Slot Immunoblot Assay for Detection and Quantitation of Periodontal Disease-Associated Microorganisms in Dental Plaque

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A rapid method for qualitative and quantitative detection of specific oral microorganisms from subgingival dental plaque is described. Plaque samples were suspended in phosphate-buffered saline containing protease inhibitors and 0.5% formaldehyde, briefly sonicated to disperse bacterial aggregates, and applied to nitrocellulose membranes in a slot blot manifold. Subsequent incubations with species-specific rabbit antibody and anti-rabbit antibody–alkaline phosphatase conjugate and development with BCIP-NBT substrate resulted in an easily discernible, permanent stain being deposited at the sample application site. Comparison with known concentrations of pure, cultured microorganisms applied to the same membranes permitted qualitative or semiquantitative plaque characterization by visual inspection. Analysis of the blots by a computer-linked flatbed scanner provided quantitative data on microbial content. The reproducibility of the results (standard error of the mean, <10%) obtained with slot immunoblotting greatly exceeded that of the results obtained with immunofluorescence analysis (standard error of the mean, >57%). Because it is versatile, rapid, sensitive, reproducible, permanent, and relatively inexpensive, slot immunoblotting lends itself to use in large-scale investigations for the detection and quantitation of specific microbial species.

Numerous investigators have reported relationships between colonization by specific oral microorganisms in subgingival dental plaque and the presence and/or severity of periodontal disease (13, 15, 17). The most commonly used methods of detection and enumeration of these species have been culture and biochemical analyses, which have generally been considered the "gold standard." However, given the labor-intensive nature of and the expense associated with these procedures, a variety of immunologic methods have been developed to detect and quantitate a panel of microorganisms previously determined by culture analysis to be present in plaque from diseased subgingival sites. Primary among the immunologic techniques used is immunofluorescence, in which antibodies reactive with various species are used to detect the presence and calculate the numbers of microorganisms microscopically (2-4, 9, 11, 12, 14, 15, 16, 18, 19). The major limitations of these assays include the labor-intensive nature, the subjective aspects of interpreting the observations, and the lack of a permanent record of the assay results. Reports by Ebersole et al. have also described the potential usefulness of the enzyme-linked immunosorbent assay (ELISA) in the serologic identification of oral Bacteroides spp. and other microorganisms (5, 6).

In this report, we describe a method that is based on dot blot methodology and that provides rapid screening of dental plaque specimens for the presence of specific oral microorganisms. This method provides a permanent record of the assay, which is both quantitative and qualitative and is amenable to evaluation by low-cost laboratory instrumentation or by visual inspection.

MATERIALS AND METHODS

Subgingival plaque specimens. Subgingival plaque was obtained with a curette from diseased sites (pocket depth >5 mm) in patients exhibiting adult periodontitis as previously described by Loesche et al. (13). Samples were placed in cryotubes (Vanguard) containing 0.5 ml of phosphate-buffered saline (PBS; 0.05 M sodium phosphate, 0.15 M NaCl [pH 7.4]) with 0.5% formaldehyde, EDTA (2 mM), phenylmethylsulfonyl fluoride (1.0 mM), pepstatin A (0.1 mM), and leupeptin (0.5 mg/liter). Sample vials were stored at −20°C until used.

Pure cultures. Porphyromonas gingivalis (ATCC 33277) and Treponema denticola (ATCC 35405) were grown under anaerobic conditions (85% N₂, 10% H₂, 5% CO₂) at 37°C in 500-ml batches as previously described (1). After being harvested by centrifugation at 13,000 × g for 20 min and washed three times with PBS, the microorganisms were killed and fixed by incubation with 0.5% formaldehyde in PBS at room temperature for 18 h on a rotary shaker. The formalinized bacteria were washed two times with PBS and once with distilled water, lyophilized, and stored at −20°C.

Antibacterial antibodies. High-titer (greater than 1:40,000, as determined by ELISA) polyclonal antibodies against P. gingivalis and T. denticola were produced in rabbits. In brief, rabbits were immunized subcutaneously with 1 mg (dry weight) of the bacterial preparation suspended in 1 ml of aluminum hydroxide adjuvant gel. The rabbits were reimmunized after 1 week and boosted at approximately 5 weeks following the first immunization. Bleedings were obtained weekly after the booster immunization. All antisera were screened by the micro-ELISA procedure for titers and specificity for both related and unrelated species (1). In preliminary studies, antisera were screened against approximately 70 American Type Culture Collection strains and clinical isolates (characterized by a classical microbiologic technique) representing 10 genera and 25 species (data not shown). The antisera were found to react strongly with all isolates previously determined to be of the same species as that used for immunization. At the final titers used in the assays, these antisera were found to be monospecific for the immunizing antigens.

Indirect immunofluorescence assay. After being vortexed,
10-μL plaque samples or pure cultures were applied to wells of 12-well immunofluorescence slides (Scientific Products). Each slide contained positive (pure-culture) and negative controls. The remaining 10 wells of each slide received sample plaque suspension. The slides were air dried at 37°C, and the samples were gently heat fixed. The appropriate antibacterial antibody was diluted in PBS containing 2% bovine serum albumin, and 10.0 μL was applied to each well, except for the fluorescein isothiocyanate conjugate control. The slides were incubated in a humid chamber for 30 min at room temperature, washed twice for 5 min each time in PBS, and rinsed in distilled water. Goat anti-rabbit antibody–fluorescein isothiocyanate conjugate (Zymed Laboratories, South San Francisco, Calif.) diluted in PBS-2% bovine serum albumin (10 μL per well) was applied, and the slides were again incubated for 30 min in a humid chamber at room temperature prior to being washed and rinsed as described above. The slides were mounted with glycerol in PBS (2:1 [vol/vol]) containing paraphenylenediamine (pH 9.0) and sealed with a coverslip and nail polish prior to microscopy. Randomly selected high-power fields were examined, and the fluorescing microorganisms were counted.

Immunofluorescence was evaluated with a Leitz Dialux microscope equipped with a Ploempak 2.3 fluorescence illuminator for epifluorescence. The light source was a 200-W HBO mercury superpressure lamp. Fluorescence was graded from 0 to 4+, with grades 3+ and 4+ being considered serologically positive reactions. Staining was considered positive for the selected microorganisms if it revealed strongly fluorescent cells with well-defined outlines and dark or lightly shining centers and constituting more than 1% of the total count.

**Total particle count.** Samples were prepared and applied to 12-well immunofluorescence slides as described above. Crystal violet stain was applied for 10 min, and the slides were washed with distilled water. The total microorganism count from randomly selected high-power fields was recorded. This value was used as the 100% value when the percentage of positive fluorescent cells was calculated for each specimen.

**SIB assay.** The samples used for indirect immunofluorescence were utilized in the slot immunoblot (SIB) assay. Prior to the assay, the samples were subjected to ultrasonication (3 min at 100% power: Kontes Instruments) to disrupt aggregates of plaque particles.

Nitrocellulose sheets (BA-85; Schleicher & Schuell, Keene, N.H.) were soaked for 15 min in TBS (0.05 M NaCl, 10.0 mM Tris-HCl, 10.0 mM Tris base [pH 7.4]) prior to insertion in the slot blot manifold (Minifold II; Schleicher & Schuell). Standards (pure cultures) or undiluted plaque samples were applied (10 μL) to each well of the slot blot manifold, which was then evacuated with the gentle application of vacuum. The nitrocellulose sheets were removed from the manifold for further processing. All subsequent incubations were performed on a rocking table (Hoefer Scientific Co.) at room temperature.

**Microbial detection and quantitation.** Microbial detection and quantitation were performed after first immersing the nitrocellulose membrane in TBS containing 0.5% nonfat dried milk (BLOTTO [10]) for 30 to 60 min to block unoccupied binding sites on the nitrocellulose membrane. The appropriate antibacterial antibody, diluted in TBS-Tween 20 (TBS-T; 0.05% Tween 20, except when indicated otherwise) containing 0.5% BLOTTO, was applied and allowed to incubate for 1 h. Following three 5-min washes in TBS-T, goat anti-rabbit immunoglobulin G (heavy and light chains) conjugated to alkaline phosphatase (Bio-Rad Laboratories) diluted in TBS-T containing 0.5% BLOTTO was applied and incubated for 1 h. After all washes, BCIP-NBT substrate solution (Kirkegaard & Perry Laboratories) was applied and color development was allowed to proceed to its maximum.

The estimation of the carbohydrate content of the plaque samples was performed on another nitrocellulose membrane to which plaque samples had been applied. After removal from the manifold, the membrane was incubated for 30 min with TBS-T (2.0% Tween 20) (no BLOTTO). Following a wash with TBS-T, the membrane was immersed in TBS-T containing biotinylated concanavalin A (Vector Laboratories, Burlingame, Calif.) and incubated at room temperature for 1 h. Following three 5-min washes with TBS-T, the membrane was incubated for 30 min at room temperature in TBS-T containing streptavidin-alkaline phosphatase conjugate (Zymed Laboratories). After a final wash in TBS-T, the membrane was immersed in BCIP-NBT substrate solution, incubated until full color development occurred, and dried.

The protein content of the plaque samples on the nitrocellulose membrane was estimated by the method of Hancock and Tsang (8). In brief, after the application of the standards and plaque samples, the membrane was incubated overnight in a solution of India ink (Pelikan Fount) diluted 1:1,000 in TBS-T. The membrane was rinsed for 5 min in distilled water prior to being dried.

Because of its ready availability and suitability for use as both a protein standard and a carbohydrate standard, nonfat powdered milk was used as the standard for protein and carbohydrate determinations in the immunobinding assays. The protein content of the powdered milk was determined by the bicinchoninic acid assay (Pierce Chemical Co.). The total hexose content was established by the anthrone reaction. Once calibrated, a single lot of powdered milk was used for all assays. These two preliminary assays established the reference levels from which the subsequent dose-response intensity of the titrated standard could be measured.

Quantitative data were obtained from the nitrocellulose membranes with a flatbed optical scanner (Hewlett-Packard ScanJet Plus) linked to a Macintosh II computer. Scans were analyzed with densitometry software (Biosoft, Milltown, N.J.), which calculated peak height and area for each slot on the membrane. Peak height was used in all calculations.

Dose-response curves for each microorganism were calculated from the pure-culture titrations on each membrane. Semiquantitative data were obtained from the nitrocellulose membranes by visual scoring of the color intensity of each slot. The data were scored on a scale from 0 to 3, where 1 represented the faintest line detectable, 3 represented the maximum intensity, and 2 represented the intermediate intensity. Since this scoring was performed on the pure cultures as well as the plaque samples, the scale could be related to relative concentrations of pure microorganisms.

**RESULTS**

**Detection and quantitation of P. gingivalis and T. denticola.**

(i) **Standard titrations by and sensitivity of SIB.** To establish the sensitivity of the assay, we applied graded doses of pure cultures of either organism to the nitrocellulose filters in the slot blot manifold and processed them with the appropriate antibacterial antibody. Figure 1 shows two typical scans of these experiments. For both antigens, there was a dose-dependent relationship when approximately 2 × 10⁶ to 3 × 10⁷ particles were applied to the membranes. For subsequent studies, the lowest concentration detectable visually was...
FIG. 1. Titrations of pure cultures. The computer-generated graph illustrates typical linear portions of titrated pure-culture microorganisms detected by SIB on nitrocellulose.

assigned a value of 1. Thus, a score of 1 represented a concentration of at least $2 \times 10^4$ particles. In the same manner, the highest intensity was assigned a value of 3, representing a concentration of at least $3 \times 10^5$ particles. All intermediate levels of intensity were assigned a value of 2. Thus, when the appropriate bacterial standards were run with unknown samples, each slot could be scored visually (0 to 3) without the need for a spectrophotometer or scanner.

(ii) Mixture effects in SIB. In practice, the value of this assay is in the rapid screening of plaque samples, rather than pure cultures. Thus, we next determined whether the presence of other microorganisms or substances in plaque compromised the ability of this assay to detect specific microorganisms. Dental plaque samples found previously to be deficient in a specific test microorganism were divided into aliquots; to separate aliquots were added increasing concentrations of specific pure cultures of oral microorganisms. These mixtures were added to individual wells of the slot blot manifold. To separate wells were added the same graded doses of pure cultures. The membranes were processed with the appropriate antibodies and scanned. The wells containing the mixtures of plaque and pure culture were compared with the wells containing only pure culture. There was no discernible difference between the titrations, indicating that no confounding factors interfered with the detection of specific microorganisms in the plaque samples (Fig. 2).

(iii) Effect of blood on SIB results. Since plaque samples obtained from diseased sites are often contaminated with significant amounts of blood, we assessed the potential interference of whole blood, neutrophils, and mononuclear leukocytes in SIB (data not shown). In no case did the presence of blood products interfere with the detection or quantification of plaque microorganisms. In addition, no residual enzymatic activity that could affect the development of the BCIP-NBT substrate was associated with these blood products.

(iv) Relationship between indirect immunofluorescence and SIB. Forty-five plaque samples obtained from diseased and nondiseased subgingival sites were analyzed by indirect immunofluorescence and SIB. The blots were visually scored (0 to 3) as described above, and the relationship between the two assays was compared by regression analysis. There were moderate correlations (P. gingivalis, $r = 0.64$; T. denticola, $r = 0.72$) between the results of the two assays (Fig. 3).

(v) Comparison of the reproducibilities of indirect immuno-

FIG. 2. Titration of T. denticola (pure culture) in a plaque sample. A pure culture of T. denticola was added to a patient plaque sample previously determined to be devoid of the organism and then titrated. A pure culture of T. denticola without plaque was titrated on the same sheet of nitrocellulose.

FIG. 3. Comparison of immunofluorescence assay counts with SIB scores. Identical samples ($n = 45$) were evaluated by immunofluorescence and SIB. Mean values for each immunofluorescence assay count ($n = 3$) were plotted versus each SIB score.
fluorescence and SIB. To evaluate reasons for the "looseness" of the correlations, we performed studies to examine the reproducibilities of indirect immunofluorescence and SIB measurements, since significant variability in obtained measurements could account for the moderate correlations. Dental plaque samples were vortexed for 30 s, and nine 10-μl aliquots of the same sample were applied to separate immunofluorescence slides, stained with the appropriate antibacterial antibody as described above, and examined. We found extensive variability in the counts from a single sample. Typically, the standard error for all of the wells in a single trial exceeded 57% of the mean for those wells. The primary contributing cause to the excessive variability appeared to be the inability to uniformly disperse the plaque; random selection of microscopic fields for counting was secondary. Blotting was assessed in a similar fashion, with the exception that the sample was sonicated for 3 to 5 s prior to analysis and then identical aliquots were applied to six wells. In these studies, the measurements for each well were quite consistent, resulting in a standard error of less than 10% of the mean. The high reproducibility was probably due to the sonication, which resulted in uniform dispersal of the sample.

Estimation of carbohydrate and protein contents of plaque samples. A major difficulty in screening a large number of plaque samples lies in the inability to determine whether the sample is sufficient (or whether the sample exists at all), since plaque collection is by no means a quantitative procedure. While microscopic counts may be a solution, they are time-consuming and impractical for mass screening. To address the question of sample amount, we attempted to assess the gross carbohydrate content of the plaque sample by using SIB. Carbohydrate could be detected by the binding of labeled concanavalin A, and protein could be detected by the binding of India ink. A typical titration of a pooled dental plaque sample for carbohydrate content is shown in Fig. 4. This relationship between concanavalin A or India ink binding and the plaque sample is for the determination of mass only and provides no indication of the bacterial content.

DISCUSSION

Rapid, specific identification of microbial species is required for the detection and semiquantitation of plaque microorganisms in the processing of large numbers of specimens. In this report, we describe a method having significant advantages over previously used methods of dental plaque characterization. This method provides the major benefits of rapid processing; semiquantitation of microbial constituents with either inexpensive computer-assisted scanning or visual inspection; specificity and reproducibility; permanent record; and low cost of reagents and supplies.

In comparison with the indirect immunofluorescence assay, the SIB assay presents numerous benefits. Unlike the transient fluorescence of the former, which must be read immediately, the SIB assay provides a permanent record. The SIB assay is not dependent on subjective interpretation, since the scoring is related only to the intensity of the color development and is highly reproducible. As shown in this report, indirect immunofluorescence assay results, while very good for the detection of a specific microbial species, do not provide the quantitative reproducibility offered by SIB assay results. This difference is presumably based on the fact that dental plaque specimens are not uniform suspensions, but rather often appear as large clumps which make quantification difficult. Since the retention of morphologic integrity is not important in the SIB assay, the samples may be exposed to ultrasonic disruption prior to analysis, permitting the formation of a uniform suspension.

As we have shown, SIB may be evaluated visually or with a scanner. In practice in our laboratory, computer scanning is rarely used, since visual scoring (0 to 3) provides information on the approximate concentration of the microorganism present in the plaque sample, which is usually all that is necessary, since the actual collection of the plaque sample is not quantitative. Scanning of SIB has one limitation as far as quantification is concerned. Since the assay results in the formation of an insoluble dye precipitate, the linear range that can be measured by reflectance or in comparison with a gray scale is limited. Thus, if one desires to generate absolute quantitative data, the assay must be repeated at a higher dilution of the plaque sample to ensure that the endpoint falls within the linear range of the measurement system.

One difficulty in the immunologic assays that are used for microbial detection is associated with small samples. In rapid screening methods, one is always confronted with the dilemma of discriminating between a negative finding due to the lack of the sought-after species in the plaque versus the possibility that the plaque sample itself is too small to allow the detection of a specific species. To solve this problem, we use India ink and a biotinylated concanavalin A probe to estimate the amounts of protein and carbohydrate present. Obviously, these methods do not provide information on the total count of bacteria present (which does not correlate with the number of bacteria present), nor do they truly indicate the actual amount of protein or carbohydrate present, but they do provide an estimate of the "bulk" of the sample collected. Thus, laboratory guidelines may be established to characterize samples with low concanavalin A reactivity as not detectable. It should be emphasized that while powdered milk was used in these studies, any suitable standards for protein and carbohydrate that will adhere to the nitrocellulose membrane may be used.

Earlier studies described the use of SIB to detect plaque microorganisms (7); however, we have extended its useful-
ness as a qualitative resource to one that is also quantitative. In addition, by the addition of carbohydrate and protein detection, we have ensured that there is a mechanism by which the reporting laboratory may determine that insufficient sample was collected and that a negative result is therefore meaningless. This assay has been recently compared with standard culture methods, DNA probes, and indirect immunofluorescence in a study of microbial detection and quantification (unpublished data). In an examination of plaque samples from 70 patients for the presence of *P. gingivalis*, *T. denticola*, *Actinobacillus actinomycetemcomitans*, and *Bacteroides forsythus*, DNA probes and SIB were found generally to produce very comparable results, with much greater sensitivity than the classical methodology. These results call into question the use of culture methods for the detection and quantification of specific microbial species.

This report addresses the utility of SIB for the detection of known species of microorganisms. One should keep in mind the significant limitations of any assay dependent on the use of a specific probe, such as an antibody or a DNA probe. That is, one is limited to detecting only those species reactive with that probe. In addition, the specificity of the detection system is dependent on the specificity of the probe used. Thus, when using this type of assay, one must critically characterize the probe being used and be aware that one is limited to detecting specific species. This type of assay is suitable in clinical studies only when one is interested in detecting specific microbial agents and is not useful when one is investigating a disease of unknown etiology.

In conclusion, SIB is a rapid, semiquantifiable assay which generates a permanent record. Since it can be performed with relatively inexpensive equipment and supplies, it is amenable to large-scale epidemiologic studies which seek to detect and quantify specific microbial species. The utility of this assay is also reflected in the large numbers of determinations that can be made from a single plaque specimen. Typically, in our studies, a single subgingival plaque sample obtained with a curette is placed in 0.5 ml of a formaldehyde cocktail. Since only 10 μl of this sample is needed for a single analysis, as many as 50 different species determinations may be made with this sample.

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REFERENCES


