Identification of Actinomyces, Arachnia, Bacterionema, Rothia, and Propionibacterium Species by Defined Immunofluorescence

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Fractionated fluorescein-isothiocyanate (FITC)-conjugated immunoglobulin G (dye-to-protein ratio <10), produced against whole cells of Actinomyces spp., Arachnia, Bacterionema, Rothia, and Propionibacterium spp., give species-specific conjugates with controlled nonspecific staining reactions when appropriately diluted on the basis of their antibody content (10 mg/ml). Using this standardization in immunofluorescence, serotype-specific conjugates are also available after dilution for all serotypes of these organisms except for Actinomyces viscosus type 2, and Propionibacterium acnes type 1. Adequately adsorbed conjugates could be used to differentiate these serotypes from A. viscosus type 1 and P. acnes type 2, respectively. A serological classification in defined immunofluorescence corresponded to species and serotype designation proposed on the basis of other serological analysis and biochemical characteristics. This includes a separation in immunofluorescence of two serotypes of Propionibacterium acnes. The detection of certain actinomycetes of the family Actinomycetaceae and Propionibacterium species by the defined immunofluorescence in direct smears prepared from clinical specimens agreed to 88% with parallel culturing when including a prereduced (PRAS) medium technique for isolation. Qualitative studies revealed that single cells of these organisms could be specifically identified by immunofluorescence when admixed with morphologically similar bacteria and a large number of other contaminants.

A rather substantial literature is developing which suggests the importance of strains of gram-positive filamentous or diphtheroidal bacteria, or both, in the pathogenesis of periodontal disease. The pathogenicity of Actinomyces spp. has recently been demonstrated in experimental animals (11, 12, 20, 31, 50). However, the etiological role of these organisms in human periodontal disease remains to be determined. This is due in part to the fact that cultivation and isolation of these fastidious organisms require specific cultural techniques and are time-consuming procedures (e.g., 19, 27, 29). Thus, the need for specific and rapid identification procedures in ecological and epidemiological work has become important.

Immunofluorescent techniques have been found to be of value for detection of Actinomyces spp. in tissue impression smears from experimentally infected mice (34), in tissue or exudate smears from human tonsillar materials (4), and in human dental calculus (46). However, new applications of immunofluorescent techniques pose special problems and require their evaluation and characterization with respect to specific staining titer, fluorescein concentration, protein concentration, fluorescein-to-protein ratio (F/P ratio), and usable diagnostic titer (cf. 3, 10, 24).

The aim of this investigation was to determine the optimal criteria for fluorescein-isothiocyanate (FITC) conjugated antisera against certain genera of the family Actinomycetaceae and certain species of Propionibacterium in direct immunofluorescence. The evaluations were made using type species and oral isolates. The accuracy of identifying these organisms by defined immunofluorescence in direct smears was determined by parallel cultures.

MATERIALS AND METHODS

Strains. Reference strains were obtained from American Type Culture Collection (ATCC), Rockville, Md., National Collection of Type Cultures...
Actinomyces kindly supplied Microbiology, West Virginia University Medical School, Morgantown, W. Va. One strain of Corynebacterium acnes group 2 (D 34) (Propionibacterium granulosum) was supplied by J. G. Voss, The Proctor & Gamble Co., Miami Valley Lab., Cincinnati, Ohio.

Immunization. Type strains (Table 1) were grown in Trypticase soy broth (Difco) for 48 to 72 h. After centrifugation the cultures were suspended in 0.15 M NaCl with 5% Formalin (vol/vol) for 1 h and washed twice in 0.15 M NaCl. One-milliliter cell suspensions containing roughly 10⁹ organisms per ml were injected intravenously in rabbits every third to fourth day for 4 weeks. The rabbits were bled 7 days after the last injection. Slide agglutination titers of the sera ranged from 1/64 to 1/128 with living cells as antigens. For each strain, two rabbits were immunized.

Preparation of IgG. Rabbit immunoglobulin G (IgG) was prepared from sera by precipitation with ammonium sulfate at 37% saturation. The precipitate was dissolved in distilled water, and the procedure was repeated twice. The precipitated and redissolved material was dialyzed against 0.0175 M Na₃HPO₄ (pH 6.3), further purified by chromatography on a diethylaminoethyl (DEAE)-cellulose column equilibrated and eluted with the dialysis buffer, and finally concentrated with an ultrafiltration cell (Diaflow model 51, Amicon Corp.) to a protein concentration of 18 to 20 mg/ml. The protein concentration was determined by measuring the optical density at 280 nm. The IgG preparations gave a single IgG line in immunoelectrophoresis against anti-rabbit plasma protein serum (Behringwerke AG, Germany) (42).

Conjugation. Rabbit IgG was conjugated with FITC (BDH Biochemicals, Poole, England). FITC was added to IgG in the proportion of 30 μg of dye per mg of protein under vigorous stirring at room temperature for 1 h (pH 9.5). Nonreactive FITC was removed by passing the solution through a Sephadex (Pharmacia AB, Sweden) G-25 column equilibrated with phosphate-buffered saline (PBS) (35).

Fractionation of conjugates. To obtain fractions giving minimal nonspecific staining (24, 51), the conjugates were chromatographed on a DEAE-cellulose column equilibrated with 0.0175 M Na₃HPO₄ (pH 6.3). After elution of a small amount of nonconjugated material with the starting buffer, a stepwise gradient was applied using 0.125 M NaCl, 0.25 M NaCl, and 0.500 M NaCl in 0.0175 M Na₃HPO₄ (pH 6.3), respectively. The fractions were concentrated by

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**Table 1. Specific staining titers for homologous strains and nonspecific staining of gingival tissue cells by fractions of FITC conjugates with different F/P ratios**

| FTIC-labeled antigens (10 mg/ml) | F/P ratio (μg/mg) | Specific staining titer* | Nonspecific staining | FTIC-labeled antigens (10 mg/ml) | F/P ratio (μg/mg) | Specific staining titer* | Nonspecific staining |
|---------------------------------|-------------------|-------------------------|---------------------|---------------------------------|-------------------|-------------------------|---------------------|
| *Rothia dentocariosa* (ATCC 17931) | 9.0               | 128                     |                     | *Arachnia propionica type 1* (ATCC 14157) | 10.0              | 16                      |                     |
|                                   | 13.0              | 32                      | ++                  |                                 | 12.0              | 8                       | +                   |
|                                   | 20.0              | 4                       | ++                  |                                 | 20.0              | 4                       | +                   |
| *Bacterionema matruchotii* (ATCC 14265) | 7.5               | 64                      | ++                  | *Arachnia propionica type 2* (WVU 346) | 5.5               | 64                      |                     |
|                                   | 16.0              | 2                       | (+)                 |                                 | 12.5              | 8                       | +                   |
|                                   | 20.0              | 2                       | ++                  |                                 | 18.0              | 1                       | +                   |
| *Actinomyces viscosus* type 1 (ATCC 15987) | 4.0               | 64                      | +                   | *Propionibacterium acnes* type 1 (NCTC 737) | 7.5               | 64                      |                     |
|                                   | 13.0              | 8                       |                     |                                 | 15.0              | 4                       | +                   |
|                                   | 21.0              | 4                       |                     |                                 | 21.0              | 4                       | +                   |
| *Actinomyces viscosus* type 2 (ATCC 19246) | 4.0               | 64                      | ++                  | *Propionibacterium acnes* type 2 (ATCC 11828) | 7.0               | 64                      | +                   |
|                                   | 12.0              | 4                       |                     |                                 | 12.0              | 4                       | +                   |
|                                   | 20.0              | 4                       |                     |                                 | 17.0              | 4                       | +                   |
| *Actinomyces naeslundii* (ATCC 12104) | 8.0               | 64                      | ++                  | *Propionibacterium avidum* (ATCC 25577) | 9.0               | 32                      | +                   |
|                                   | 15.0              | 16                      |                     |                                 | 15.0              | 4                       | +                   |
|                                   | 19.0              | 3                       |                     |                                 | 20.0              | 4                       | +                   |
| *Actinomyces israelii* type 1 (ATCC 12103) | 6.0               | 64                      | +                   | *Propionibacterium granulosum* (ATCC 25564) | 10.0              | 16                      | +                   |
|                                   | 13.5              | 2                       |                     |                                 | 13.0              | 8                       | +                   |
|                                   | 20.0              | 2                       |                     |                                 | 19.0              | 16                      | +                   |
| *Actinomyces israelii* type 2 (WVU 307) | 5.0               | 128                     |                     | *Propionibacterium jensenii* (ATCC 4867) | 10.0              | 32                      | +                   |
|                                   | 12.0              | 8                       | ++                  |                                 | 16.0              | 4                       | +                   |
|                                   | 20.0              | 2                       | ++                  |                                 | 18.0              | +                       | +                   |
| *Actinomyces odontolyticus* (ATCC 17982) | 9.0               | 32                      |                     |                                 |                   |                         |                     |
|                                   | 15.0              | 4                       |                     |                                 |                   |                         |                     |
|                                   | 20.0              | 1                       |                     |                                 |                   |                         |                     |

*Expressed as reciprocal of titer.*
ultrafiltration (Diaflo model 50, Amicon Corp.) to a final concentration of 10 mg/ml and dialyzed against PBS (pH 7.2). The protein content and F/P ratio (μg/mg) were estimated as described by Wells et al. (8). Portions of conjugates were stored at 0 to 5 C or frozen in several small portions for storage.

Preparation of FITC-labeled F(ab')2 fragments. To obtain F(ab')2 fragment of IgG from antisera to Rothia dentocariosa, pepsin digestion was performed (17, 36) using 1 mg of enzyme per 50 mg of IgG in 0.1 M sodium acetate (pH 3.0) for 7 h. The F(ab')2 fragments were conjugated with FITC as described above and fractionated on Sephadex G-25 equilibrated with 0.0175 M Na2HPO4 (pH 4.7) into fractions with different F/P ratios (17). The first peak to emerge from the column was collected, and its pH was adjusted to 7.2. Conjugated F(ab')2 fragments with high F/P ratios, which showed firmer adsorption to the gel and which therefore eluted in a second peak, were discharged. The F/P ratio was calculated according to Forsum (17).

Dilution and adsorption of conjugates. The fractionated conjugates were diluted in PBS (pH 7.2), and the highest dilution giving 3+ staining of the homologous organism was recorded as the specific titer of each fraction.

Adsorption of antisera was performed with equal volumes of conjugated antisera and packed living cells from a 48-h culture of the heterologous, cross-reacting organism at 37 C for 2 h.

Sampling of clinical materials. Five patients with severe periodontal disease with pockets (Periodontal Index [PI], according to Russel [41], 4.7 to 5.8) were selected for this study.

The subjects were instructed to refrain from tooth brushing for 24 h before collection of dental plaque and calculus materials. Supragingival specimens were collected from the buccal surfaces of the upper molars with a sterile McCall curette (Sandvikens Coromant AB, Sweden) during preoperative periodontal treatment. Subgingival dental plaque, calculus, and gingival tissue specimens were taken during surgical treatment. Specimens were immediately transferred both to tubes containing 1.5 ml of sterile FTA-hemagglutination buffer (BBL) supplemented with 0.05% cysteine-hydrochloride, and to sterile, CO2-filled, stoppered tubes, containing 1.5 ml of prereduced salt solutions according to the Anaerobe Laboratory Manual (27). All samples were ground in an all-glass tissue grinder (Grave AB, Stockholm, Sweden) and dispersed in a mixer (Vortex-Genie, Scientific Industries Inc.) for 30 s. Samples of excised gingival tissue and granulation tissue were directly refrigerated, and within 3 h after operation they were frozen to -76 C.

Preparation of smears. Smears from each clinical specimen were Gram stained, and the morphotypes present were recorded.

Smears for immunoﬂuorescent staining were made from the suspensions in FTA-hemagglutination buffer and prereduced salt solution by streaking 0.01 ml of the suspension on a slide. For immunofluorescent staining of gingival tissue and granulation tissue, frozen samples were cut in a cryostat chamber (Lab.-Tek., Sweden) maintained at about -20 C. Sections 4 μm thick were transferred to slides and fixed in acetone for 10 min. For estimation of specific staining reactions of type organisms and for identification of isolates, smears were prepared from 24- to 48-h pure cultures. Nonspecific staining of leukocytes were evaluated in smears prepared from freshly drawn human venous blood. After coagulation for 10 min at room temperature and then for 20 min at 37 C, the clot was removed by washing with 0.15 M NaCl.

Staining of smears and recording procedures. One drop of conjugate was placed on a smear and incubated for 20 min at 37 C. The smear was washed with PBS for 15 min and mounted under a cover glass with phosphate-buffered glyc erin (pH 7.2).

The preparations were read under a Zeiss fluorescence microscope equipped with an Oram HBO 200 mercury lamp, oil immersion dark-field condenser, and a 4-mm BG 12 filter as primary and a Zeiss 50 filter as secondary filter. Tissue specimens were read with a Zeiss 44 filter as secondary filter. All observations were made under ×500 magnification. The following criteria were used for recording the specific fluorescent staining of microorganisms: 3+, intensely fluorescent margins, well marked edges; 2+, faintly fluorescent margins, edges usually diffuse; 1+, barely distinguishable fluorescent margins with diffuse edges. Nonspecific staining of gingival tissue cells and leukocytes was recorded using the following criteria: 3+, brilliant nonspecific staining; 2+, clear-cut nonspecific staining; 1+, doubtful nonspecific staining.

Cultural and isolation procedures. The clinical specimens were cultured aerobically and anaerobically on conventional and prereduced media. The FTA buffer and the prereduced salt solution suspensions were diluted serially and 10-fold in cold (4 C) PBS, pH 7.2, and prereduced salt solution, respectively. Samples of the PBS dilutions 10-2, 10-4, and 10-6 were plated in duplicate on freshly prepared NAYS agar (14) and on NAYS agar plates supplemented with 25 IE/ml of polymyxin-P-sulfate (Novo Industri A/S, Copenhagen, Denmark). One plate of each medium was incubated aerobically with 10% CO2 and one in an anaerobic jar using the Gas-Pak anaerobic system (BBL). Samples from the dilutions 10-1, 10-3, and 10-4 of the prereduced salt solutions were cultured on prereduced (PRAS) PY agar medium (27) and in prereduced PY agar roll tubes (Scott Laboratories Inc.) using the VPI Anaerobic Culture System (Bellco Inc.). All plates and tubes were incubated at 37 C for 3 to 5 days before examination and were then examined at a magnification of approximately ×10 using a stereoscopic zoom microscope (Zeiss AG, Germany) with transmitted or reflected lighting, or both. On the basis of colonial morphology, a Gram-stained smear was prepared from each colonial type. Two colonies of each type identified as gram-positive diphtheroidal or filamentous organisms, or both, were selected. One colony was transferred to an aerobic NAYS-agar plate and one colony was transferred to an anaerobic, prereduced PY-agar plate. Organisms growing aerobically were subjected to the diagnostic keys for this group of organisms proposed by Holmberg and Hallander (25). Anaerobic isolates were identified by tests and media devised by the Anaerobe Laboratory Manual (27) as diagnostic keys for facultative to anaerobic Ac-
tinomyces spp., A. propionica, and Propionibacterium spp. Isolates of A. odontolyticus were identified by the diagnostic tests for this species devised by Georg (19) and Gerencser and Slack (22).

RESULTS

Characterization of conjugates. The FITC-labeled and fractionated conjugates were characterized with respect to F/P ratios, specific staining of homologous strains, and extraneous fluorescence.

Specific staining reaction. The staining titers for homologous strains obtained with three fractions of each conjugate (10 mg/ml) with various F/P ratios are presented in Table 1. The highest dilution giving 3+ fluorescent staining was recorded as the specific titer. The highest specific titers were consistently obtained with the fractionated conjugates having F/P ratios less than 10. These fractions stained 3+ at dilutions of 1:32 to 1:128. Fractions with F/P ratios around 20 usually failed to give specific staining.

Nonspecific staining reaction. The nonspecific staining of gingival tissue smears was negligible with fractions with low F/P ratios (Table 1). As expected, nonspecific staining increased in direct proportion to the F/P ratios. A marked nonspecific staining which interfered with titration and impeded accurate recording of the specific staining appeared when fractions with F/P ratios around 20 were used. In leukocytes an intense nonspecific cytoplasmatic fluorescence was observed with all fractions of the conjugates with F/P ratios above 10. Low F/P ratios reduced this fluorescence to 2+, but in no instance was it eliminated.

Cross-reactions with Staphylococcus aureus. Since S. aureus with high protein A content (1,500–3,000 ng/10⁶ bacteria) reacts with all FITC-labeled antisera at the same dilutions as do the homologous strains, giving an unwanted cell margin fluorescence due to a reaction between protein A and the Fc-part of IgG (16). FITC-labeled F(ab’)₂ fragments were prepared from sera against R. dentocariosa, known to form coccoidal cells. When smears were prepared from suspensions of pure 18-h cultures of staphylococci and R. dentocariosa and each was stained with anti-R. dentocariosa IgG and anti-R. dentocariosa F(ab’)₂ fragments of IgG, nonspecific staining of the staphylococci was obtained with IgG but not with the F(ab’)₂ fragments. Both IgG and F(ab’)₂ fragment preparations stained the type species of R. dentocariosa at their specific staining titers.

Cross-reactions. Fractions of each conjugate with optimal staining properties were tested for cross-reactions in immunofluorescence with various, presumably related, type species.

A limitation of type-specific immunofluorescence to a 3+ reaction as endpoint yielded type specificity for the individual species except for anti-Actinomyces viscosus type 2 sera, which cross-reacted with A. israelii type 1 antigens, and for some antisera to serotypes that cross-reacted with the other serotypes within the same species. Dilution of antisera abolished the cross-reactions with 3+ fluorescent intensity at low titers and provided serotype-specific conjugates for all species except for anti-A. viscosus type 2 and Propionibacterium acnes type 1—conjugates which, at high titers near the specific staining titers for these conjugates, still stained A. viscosus type 1 and P. acnes type 2 antigens with a 3+ reaction. The results are given in Table 2.

Since dilution in providing serotype-specific conjugates for A. viscosus type 2 and P. acnes type 1 was inefficient because of cross-reactions at high titers, adsorption of these conjugates with cross-reacting antigens was performed. This procedure rendered the reagents serotype specific, although it resulted in weakened conjugates.

An extension of the type-specific fluorescence to 1+ reactions gave a scattering of cross-reactions between the conjugate prepared and several species, indicating a broad spectrum of antigenic similarities (Table 3). Dilution of conjugates eliminated most cross-reactions at low titers and rendered the conjugates species specific at dilutions of 1:32 to 1:64; homologous antigens were still stained with a 2+ to 3+ reaction.

To confirm the cross-reactions found between species, another set of type-species received as the following: R. dentocariosa (Nocardia sali-vae), NCTC 10207; Leptotrichia dentium (Bac-terionema matruchotii), ATCC 14919; A. viscosus type 1, ATCC 15988; Actinomyces naeslundii, NCTC 10301; A. israelii type 1, ATCC 10048; P. acnes type 1, CDC 554; P. acnes, type 2, CDC 605; and P. avidum, CDC 9064, were tested in immunofluorescence with the conjugates prepared. No other relationships were detected with these strains as antigens, and the cross-reactions found were of the same degree as in the primary tests.

The sera were also tested with a number of oral isolates biochemically identified as R. den-tocariosa, B. matruchotii, A. viscosus, A. naes-lundii, A. israelii type 1 and 2, Actinomyces odontolyticus, Arachnia propionica, P. acnes type 1 and 2. The pattern of cross-reactivity obtained with the type species was reproduced,
### Table 2. Highest dilutions of FITC-labeled antisera against Actinomyces, Arachnia, Bacterionema, Rothia and Propionibacterium species giving 3+ fluorescence of homologous and heterologous antigens

| Antigen | Fractionated (FITC-conjugated IgG) | FITC-Conjugated | Fractionated (FITC-conjugated IgG) | FITC-Conjugated |
|---------|-----------------------------------|-----------------|-----------------------------------|-----------------|
| R. dentocariosa ATCC 17931 | 256 | 256 | B. matruchottii ATCC 14265 | 64 |
| A. vicosus type 1 ATCC 15987 | 512 | 512 | A. vicosus type 2 ATCC 19246 | 512 |
| A. naeslundii ATCC 12104 | 2 | 2 | A. naeslundii ATCC 12103 | 2 |
| A. israelii type 1 ATCC 12103 | 4 | 4 | A. israelii type 2 WVU 307 | 4 |
| A. odontolyticus ATCC 17982 | 286 | 286 | A. odontolyticus ATCC 17982 | 286 |
| A. propionica type 1 ATCC 14157 | 4 | 4 | A. propionica type 1 ATCC 14157 | 4 |
| A. propionica type 2 WVU 346 | 512 | 512 | A. propionica type 2 WVU 346 | 512 |
| P. acnes type 1 NCTC 737 | 256 | 256 | P. acnes type 1 NCTC 737 | 256 |
| P. acnes type 2 ATCC 11826 | 8 | 8 | P. acnes type 2 ATCC 11826 | 8 |
| P. avidum ATCC 25577 | 1 | 1 | P. avidum ATCC 25577 | 1 |
| P. granulosum ATCC 25564 | 2 | 2 | P. granulosum ATCC 25564 | 2 |
| P. jensenii ATCC 4867 | 64 | 64 | P. jensenii ATCC 4867 | 64 |

### Table 3. End point titers of FITC-labeled Fab', fragments of IgG.

| Antigen | Fractionated (FITC-conjugated IgG) | FITC-Conjugated | Fractionated (FITC-conjugated IgG) | FITC-Conjugated |
|---------|-----------------------------------|-----------------|-----------------------------------|-----------------|
| R. dentocariosa ATCC 17931 | 256 | 256 | B. matruchottii ATCC 14265 | 64 |
| A. vicosus type 1 ATCC 15987 | 512 | 512 | A. vicosus type 2 ATCC 19246 | 512 |
| A. naeslundii ATCC 12104 | 2 | 2 | A. naeslundii ATCC 12103 | 2 |
| A. israelii type 1 ATCC 12103 | 4 | 4 | A. israelii type 2 WVU 307 | 4 |
| A. odontolyticus ATCC 17982 | 286 | 286 | A. odontolyticus ATCC 17982 | 286 |
| A. propionica type 1 ATCC 14157 | 4 | 4 | A. propionica type 1 ATCC 14157 | 4 |
| A. propionica type 2 WVU 346 | 512 | 512 | A. propionica type 2 WVU 346 | 512 |
| P. acnes type 1 NCTC 737 | 256 | 256 | P. acnes type 1 NCTC 737 | 256 |
| P. acnes type 2 ATCC 11826 | 8 | 8 | P. acnes type 2 ATCC 11826 | 8 |
| P. avidum ATCC 25577 | 1 | 1 | P. avidum ATCC 25577 | 1 |
| P. granulosum ATCC 25564 | 2 | 2 | P. granulosum ATCC 25564 | 2 |
| P. jensenii ATCC 4867 | 64 | 64 | P. jensenii ATCC 4867 | 64 |
and dilution rendered all the sera species-specific.

No cross-reactions were observed with any of the prepared conjugates, at full concentration, and the following related organisms: Corynebacterium diptheriae, ATCC 13812; Corynebacterium pseudodiptheriticum, ATCC 10700; Corynebacterium xerosis, ATCC 7094; Corynebacterium ovis, ATCC 3450; Corynebacterium pyogenes, ATCC 5224; Bifidobacterium eriksonii, ATCC 15423; Bifidobacterium adolescentis, ATCC 15104; Leptotrichia buccalis, ATCC 19419; Lactobacillus casei, ATCC 11974; Lactobacillus acidophilus, ATCC 11974; Lactobacillus fermentii, ATCC 14932; Nocardia asteroides, ATCC 19247; Nocardia caviae, NCTC 1934; Nocardia corallina, M6 5007; Mycobacterium smegmatis, NCTC 8151; Mycobacterium phlei, NCTC 8152; Mycobacterium fortuitum, NCTC 8697; and Streptomyces azureus, ATCC 14921. To elucidate a presumed serological relationship between P. acnes strains and strain D34, antisera were prepared against the latter strain. Strain D34 was originally named Corynebacterium acnes group 2 (53), but on the basis of cell wall analyses it has been referred to the P. granulosum group (30). In immunofluorescence, strain D34 showed complete antigenic identity with the reference strain of P. granulosum, ATCC 25577. No cross-reactivity with P. acnes strains was observed, nor was D34 stained by anti-P. acnes conjugates.

Immunofluorescent staining of direct smears and parallel cultures. Twenty clinical specimens of plaque, calculus, and granulation tissue from periodontal lesions of five patients were examined by light microscopy, immunofluorescence, and culture. Gram-stained smears prepared from each specimen revealed a number of gram-positive filamentous or diphtheroidal organisms, or both, in each specimen. In smears stained with the conjugates prepared, fluorescent cells were observed, indicating the presence of one or more of the organisms under study in each specimen. Using the different cultural techniques in combination, all the cellular morphotypes observed in the Gram-stained smears were usually recovered among the isolates. Up to six different gram-positive filamentous or diphtheroidal organisms, or both, were encountered from individual specimens. Four isolates could not be identified by the diagnostic methods used. One of these isolates was later tentatively identified as Corynebacterium equi, and the other was identified as a Streptomyces species. Comparing the results of immunofluorescence with those of culture, it was found that strains of R. dentocariosa, B. matruchottii, A. viscosus type 2, A. naeslundii, A. israelii type 1, A. odontolyticus, A. propionica type 1 and 2, and P. acnes type 1 and 2 were detected both by immunofluorescence and by parallel cultures (Table 4). In 65 (41%) of the 160 smears prepared for the detection of these organisms by immunofluorescence, a single species was detected both by immunofluorescence and in parallel cultures. Sixty-seven (42%) of the smears prepared were negative, and the species was not recovered in culturing. Disagreement between smear and culture results occurred in 15 (9%) of the diagnostic pairs due to failure to identify a species observed in immunofluorescence among the isolates and in 13 (8%) due to negative smears but positive culture results. Repeated culturing from the clinical specimens, primarily negative in culture but positive in immunofluorescence for a specific species, resulted in recovery of the species in 8 of the 15 specimens. Thus, the agreement between the two methods was 88%. Although no systematic quantitative estimations were made, it was found that less than five fluorescent cells in smears of a species frequently resulted in negative cultures, due to confluent growth of the admixed bacteria, especially streptococci at dilutions lower than 10⁻⁴. The sensitivity of immunofluorescence for detecting these types of organisms was determined in a single experiment. A 48-h pure culture of A. israelii was washed and resuspended in PBS and quantitated by viable count. A dental plaque specimen, negative both in culture and in immunofluorescence for A. israelii, was mixed with PBS in several dilutions. The total concentration of bacteria in each dilution was determined by viable count. One milliliter from 10-fold dilutions of the A. israelii suspension was added to samples of the dental plaque specimen and thoroughly mixed. Smears prepared from the pure suspensions of A. israelii and from each mixture were stained with anti-A. israelii conjugates and examined for the presence of fluorescent cells. Single cells of A. israelii were detectable in smears from PBS suspensions of A. israelii containing 10⁴ cultivable cells or more. Single cells of A. israelii were detected in smears with a total admixed dental plaque population of more than 10⁴ cultivable organisms per ml.

Isolates, identified as A. viscosus, A. naeslundii, and A. israelii occurred in specimens from all the individuals studied. Strains of R. dentocariosa, B. matruchottii, A. odontolyticus, Arachnia propionica, and P. acnes were isolated from some of the subjects. No strains of Pro-
pionibacterium avidum, P. granulosum, and P. jensenii were encountered.

Strains of R. dentocariosa, B. matruchotii, A. viscosus, A. naeslundii and A. odontolyticus appeared in specimens from supragingival sites whereas A. naeslundii, A. israelii, Arachnia propionica, and P. acnes were found mostly in subgingival specimens.

### DISCUSSION

The usefulness of immunofluorescence as a rapid and simple technique for detection of bacteria in pure cultures as well as on direct smears is well documented (10, 24). Increasing attention has been paid to standardization in immunofluorescence with respect to criteria for preparation of conjugates and to a quality control of the conjugates to define their specific and nonspecific staining properties (3, 26).

This study presents standards for optimal FITC-conjugated antisera to whole cells of certain actinomycetes (Actinomyces, Bacterionema, Rothia, Arachnia) and Propionibacterium species. Earlier studies on detection of these types of organisms in direct smears have been seriously disturbed by nonspecific staining (4, 34, 40). A detailed evaluation of the degree of standardization in previous studies is, however, impossible. High specific staining titers with brilliant staining of the cell margins of homologous strains and effective reduction of nonspecific staining was obtained if fractionated conjugates with F/P ratios below 10 were used. Nonspecific staining increased in direct proportion to the F/P ratios of the fractions, which is in accordance with observations from other applications, where an F/P ratio of approximately 1 to 14 has been found optimal (e.g., 39). Another problem of unwanted fluorescence arises due to the reaction between the Fc part of IgG and protein A of S. aureus (16, 17). This may cause diagnostic difficulties if coccoidal

| Sources | Identified by immunofluorescence/culture | Number of specimens positive for: |
|---------|------------------------------------------|----------------------------------|
|         |                                          | R. dentocariosa | B. matruchotii | A. viscosus | A. naeslundii | A. israelii | A. odontolyticus | Ar. propionica | P. acnes |
| Supragingival Plaque | +/+ | 4 | 1 | 3 | 2 | 0 | 0 | 1 | 0 |
|                     | +/- | 0 | 2 | 0 | 0 | 1 | 2 | 0 | 1 |
|                     | -/+ | 0 | 0 | 2 | 1 | 0 | 0 | 1 | 0 |
|                     | +/- | 1 | 2 | 0 | 2 | 4 | 3 | 3 | 4 |
| Calculus | +/+ | 2 | 3 | 5 | 4 | 3 | 2 | 3 | 1 |
|          | +/- | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
|          | -/+ | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
|          | +/- | 2 | 1 | 0 | 1 | 2 | 2 | 1 | 3 |
| Subgingival Plaque | +/+ | 0 | 2 | 0 | 4 | 5 | 0 | 2 | 2 |
|          | +/- | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
|          | -/+ | 2 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
|          | +/- | 2 | 3 | 2 | 0 | 2 | 3 | 3 | 3 |
| Granulation tissue | +/- | 0 | 1 | 1 | 5 | 5 | 0 | 2 | 2 |
|          | +/- | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 |
|          | -/+ | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
|          | +/- | 5 | 4 | 3 | 0 | 0 | 3 | 2 | 2 |
| Total   | +/+ | 6 | 7 | 9 | 15 | 13 | 2 | 8 | 5 |
|          | +/- | 2 | 2 | 1 | 0 | 1 | 5 | 1 | 3 |
|          | -/+ | 2 | 2 | 4 | 2 | 0 | 0 | 3 | 0 |
|          | +/- | 10 | 9 | 6 | 3 | 6 | 13 | 8 | 12 |

| Sources | Identified by immunofluorescence/culture | Number of specimens positive for: |
|---------|------------------------------------------|----------------------------------|
|         |                                          | R. dentocariosa | B. matruchotii | A. viscosus | A. naeslundii | A. israelii | A. odontolyticus | Ar. propionica | P. acnes |
| Supragingival Plaque | +/+ | 4 | 1 | 3 | 2 | 0 | 0 | 1 | 0 |
|                     | +/- | 0 | 2 | 0 | 0 | 1 | 2 | 0 | 1 |
|                     | -/+ | 0 | 0 | 2 | 1 | 0 | 0 | 1 | 0 |
|                     | +/- | 1 | 2 | 0 | 2 | 4 | 3 | 3 | 4 |
| Calculus | +/+ | 2 | 3 | 5 | 4 | 3 | 2 | 3 | 1 |
|          | +/- | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
|          | -/+ | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
|          | +/- | 2 | 1 | 0 | 1 | 2 | 2 | 1 | 3 |
| Subgingival Plaque | +/+ | 0 | 2 | 0 | 4 | 5 | 0 | 2 | 2 |
|          | +/- | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
|          | -/+ | 2 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
|          | +/- | 2 | 3 | 2 | 0 | 2 | 3 | 3 | 3 |
| Granulation tissue | +/- | 0 | 1 | 1 | 5 | 5 | 0 | 2 | 2 |
|          | +/- | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 |
|          | -/+ | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
|          | +/- | 5 | 4 | 3 | 0 | 0 | 3 | 2 | 2 |
| Total   | +/+ | 6 | 7 | 9 | 15 | 13 | 2 | 8 | 5 |
|          | +/- | 2 | 2 | 1 | 0 | 1 | 5 | 1 | 3 |
|          | -/+ | 2 | 2 | 4 | 2 | 0 | 0 | 3 | 0 |
|          | +/- | 10 | 9 | 6 | 3 | 6 | 13 | 8 | 12 |
variants of *R. dentocariosa* (7) and spherical cells of *Arachnia propionica* (9, 22, 38) appear mixed with *S. aureus*. Using F(ab')₂ fragments of the IgG preparations, this non-specific immunological activity is completely abolished, but the specific immunological activity is retained. Cross-reactions due to antigenic similarities between these organisms could be foreseen from results reported in earlier papers (30, 37, 44).

The active antigenic components of *Actinomyces*, *Arachnia*, *Rothia*, and *Propionibacterium* species have been found to be polysaccharides in the cell walls or cytoplasm, which seem to possess rather narrow serological type specificity (30, 33). The data obtained should make it possible to determine the extent to which cross-reactivity may be a problem in defined immunofluorescence studies of actinomyces, *Arachnia*, and *propionibacteria*. In diagnostic work, a 3+ immunofluorescent reaction is essential for reliable species identification. Appropriate dilutions of sera on the basis of their protein content per milliliter, which effectively eliminate cross-reactions with a 3+ fluorescent intensity, can be determined. This is a major advantage since adsorption, which often results in weakened specific staining reactions, can be avoided. Defined immunofluorescence of lower intensity than 3+ is of doubtful diagnostic value in differentiating between morphologically and serologically similar organisms. Dilutions could also be used for preparation of serotype-specific antisera. Adsorption was necessary only for preparing serotype-specific sera against type 2 of *A. viscosus* and type 1 of *P. acnes*. In practice, serological identification of species in mixed specimens will be facilitated by the use of pools of conjugates containing sera against species serotypes.

Various techniques have been used for serological classification of the actinomyces and *propionibacteria*. Immunofluorescence seems to be the best approach (44), since agglutination, immunodiffusion, and complement-fixation tests have given considerable cross-reactions (21, 48).

*R. dentocariosa* has a distinct reaction in immunofluorescence, probably due to a species-specific, fructose-containing cell wall antigen which distinguishes it from other gram-positive filamentous organisms (25, 48).

The taxonomic position of *B. matruchotii* is uncertain (29, 37), but it seems to constitute a quite distinct serological group in immunofluorescence, thereby confirming the results of earlier serological analyses by agglutination and immunodiffusion tests (32, 48). Within the genus *Actinomyces*, *A. odontolyticus* occurs as a rather distinct serological entity. In the present study no isolate was recovered which could be referred to as serotype 2, cross-reacting with *V. viscosus* type 1 sera as described by Slack, Landfried, and Gerencser (46). The other *Actinomyces* species appear to be serologically complex, with overlapping of antigens. There was a scattering of low-level cross-reactions between *A. viscosus*, *A. naeslundii*, and *A. israelii* strains. The degree of relationship, however, differed between examined strains. Diluted, species-specific antisera gave serological groups of the *Actinomyces* which corresponded to the serological classification designed by Slack and Gerencser (44). A subdivision of *A. viscosus* in animal strains, referred to as serotype 1 and human strains referred to as serotype 2, with a one-way cross-reaction between serotype 1 antibodies and serotype 2 antigens suggested by Gerencser and Slack (23) and Bellack and Jordan (2) was confirmed in this study. Two types of *A. israelii* have been described (6, 28). These two types agree with cell wall antigen constituents (13). However, the antigenic homogeneity among the serotype 1 strains has been found to vary. The low-titer cross-reactivity we observed between the serotypes parallels the findings of Slack, Landfried, and Gerencser (45) but is in contrast to the one-way cross-reaction between the serotypes, with serotype 1 conjugate staining serotype 2 antigens as reported by Blank & Georg (4).

Questions have been raised about the serological relationships of *Actinomyces* to *Propionibacterium*. A relationship in immunofluorescence between *Actinomyces* species and *Corynebacterium* (*Propionibacterium*) was reported by Slack et al. (47), which induced these authors to propose that *P. acnes* be included in the genus *Actinomyces*. As in later reports of Slack and Gerencser (43, 44), *P. acnes* strains formed a rather homogenous group in immunofluorescence with the exception that *P. acnes* sera, in full concentration, cross-reacted with *A. naeslundii* and *A. israelii* strains. There were also low-level cross-reactions between the *P. acnes* strains and *Arachnia propionica*, *P. avidum*, and *P. granulosum*. This may be explained by the presence of some combination of galactose, glucose, and mannose in the cell walls of all actinomyces, *Arachnia* and *propionibacteria* strains (5, 30, 37).

Two groups of *P. acnes* strains could be defined by immunofluorescence. The reactions obtained support the group designation proposed on the basis of biochemical reactions (8), susceptibility to phage, and gel-diffusion anal-
yses of soluble antigens in whole broth cultures (53). The existence of two serotypes, 1 and 2, has recently also been confirmed by cell wall analyses and cell wall agglutination tests (30).

Arachnia propionica was recognized by immunofluorescence methods to be a homogeneous group quite distinct from other genera of the Actinomycetaceae. It is also quite distinct from the Propionibacterium species studied. Pine (37) has recently suggested that A. propionica should be transferred to the family Propionibacteriaceae on the basis of cell wall composition. However, on the basis of a study of deoxyribonucleic acid (DNA) compositions of Arachnia to Propionibacterium species, Johnson and Cummins (30) state that A. propionica shows no DNA homology with the two major groups of anaerobic coryneforms. For the present time the phylogenetic position of Arachnia is difficult to determine; however, it will still be included in the Actinomycetaceae in the new edition of Bergey’s Manual.

Serological classification of the actinomycetes and propionibacteria, by defined immunofluorescence based on whole cell antigens, allows the establishment of distinct antigenic determinants for each species and supports classifications based on morphological and biochemical criteria as well as cell wall analyses (29, 30, 37).

It is evident that defined immunofluorescence is a method for detection of species of Actinomyces, Arachnia, Rothia, Bacterionema, and Propionibacterium in direct smears as specific and sensitive as cultural techniques. The two methods agreed to 88%, an agreement comparable to that observed for other bacteria (10, 18). Cross-reactions or inadequate cultural and isolation techniques may not have contributed to the observed disagreement between the methods; such factors seriously disturbed an earlier attempt to evaluate immunofluorescence for detection of Actinomyces species and some related bacteria in direct smears (46). The cultural techniques employed appear to be optimal for isolation of these types of organisms (15, 19, 27). Failure to isolate an organism identified by immunofluorescence probably reflects the effect of the dilution procedures and confluent growth of admixed bacteria. The overwhelming majority of the negative immunofluorescence but positive cultural results can be assigned to the dilution factor inherent in preparing smears.

Several factors favor the use of immunofluorescence over that of culturing for identification of these organisms. The difficulties and the time involved in anaerobic culturing and the attendant risk of contamination (1, 30) emphasize the advantages of direct immunofluorescence. Additional information emerging from the present study pertains to the presence of Rothia, Bacterionema, Actinomyces spp., Arachnia, and Propionibacterium acnes in the resident microflora of supra- and subgingival plaques and calculus and in granulomatous tissues in severe periodontal disease. In the light of the possible etiological role of these organisms in periodontal disease (49) the defined immunofluorescence techniques described appears to be an excellent diagnostic tool for use in further ecological and epidemiological studies.

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