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Fluorescent-Antibody Studies on Selected Strains of *Bacteroides fragilis* Subspecies *fragilis*

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Antisera against seven strains of *Bacteroides fragilis* subspecies *fragilis* were produced from dense suspensions of whole cells. These sera exhibited high agglutination titers with homologous antigens. Reciprocal cross-reactions in agglutination tests with each immunizing strain yielded lower titers. Both the indirect and direct fluorescent-antibody techniques were used to evaluate these reagents in the serological identification of 24 defined strains of *B. fragilis* subspecies *fragilis*. Subspecies and even strain specificities were noted with particular antisera. A pooled antiserum and conjugate were prepared and studied. Study results showed that specific and high-titered antisera against strains within this subspecies can be produced by the methods described herein and that possibly more than one serotype exists within the seven strains studied. The development of more antibody pools will be necessary to encompass a wider antigenic coverage before the fluorescent-antibody technique can be relied upon altogether for serologically identifying isolates of *B. fragilis* subspecies *fragilis*. Test data showed that the indirect method of fluorescent-antibody staining with whole antiserum is an excellent means of identifying strains of this organism.

Members of the genus *Bacteroides* are part of the indigenous human microflora. In fact, anaerobic bacteria outnumber aerobes in the skin, mouth, and intestinal tract (19).

Analysis of routine clinical specimens in early studies by Dack (3) and Stokes (22) showed that 4 and 10% of their respective cultures contained anaerobic bacteria. However, more recent studies reported that these organisms made up 35 to 40% of all isolates screened and identified (8). Stauffer et al. (21) found that 49% of the specimens they cultured yielded anaerobes and that strains belonging to *Bacteroidaceae* were isolated from 57% of these anaerobe-positive cultures.

Strains of *Bacteroides* have been implicated as the most frequently occurring etiological agents of anaerobic bacteremia (7, 23, 24), which documents their increased incidence and importance in such infections. Of the five subspecies of *Bacteroides fragilis* (subspecies *fragilis, thetaiotaomicron, vulgatus, distasonis, ovatus*), *B. fragilis* subspecies *fragilis* is the most common subspecies isolated from clinical specimens (1, 7). Also, the mortality rate of patients with *B. fragilis* subspecies *fragilis* and *B. fragilis* subspecies *thetaiotaomicron* bacteremia approached 70% in one investigation (7). These reports suggest that some members of the *B. fragilis* group are more clinically significant than others in human infections (1), and their inclination toward and involvement in infections cannot be ignored. Since many strains of *B. fragilis* are refractory to antibiotic therapy (8, 15, 18), their early detection and rapid identification are of utmost importance in patient management and in choosing the chemotherapy to be administered.

Fluorescent-antibody (FA) techniques have been successfully used to identify members of the *Bacteroidaceae* (9, 11, 16, 21; G. L. Jones, Ph.D. thesis, University of North Carolina, Chapel Hill, 1974; G. L. Lombard, Ph.D. thesis, University of North Carolina, Chapel Hill, 1972). Lombard and Jones used the direct FA technique to differentiate *B. fragilis* from other species and from other genera within the family. The indirect FA method has also been described as useful for detecting and identifying *Bacteroides* species in clinical materials (21).

This paper describes an FA serological study of seven strains of *B. fragilis* subspecies *fragilis* and includes data from homologous, intraspecies, and heterologous FA reactions. The purpose of the study was to prepare and evaluate antisera and conjugates against the seven

strains, to study the feasibility of using a pooled serum, and to explore the antigenic coverage provided by the pool used.

MATERIALS AND METHODS

Source of cultures. The bacterial strains used in the present study were taken from reference cultures at the Center for Disease Control (CDC). These cultures were obtained predominantly from clinical specimens received by the CDC.

Characterization of *Bacteroidaceae* cultures. All reference strains of anaerobes were identified by gas-liquid chromatography and by biochemical tests as described by Dowell and Hawkins (5) and Holdeman and Moore (13). CDC reference organisms were: *B. fragilis* subspecies *fragilis* 5462, 9053, 9498, 10306, 11710, 11777, 12103, 12330, 12336, 12959, 13037, 13712, 14073, 14462 (ATCC 23745), 14787, 15796, 15802, P-77, P-80, P-83, P-85, P-86, P-89, FN-2; *B. fragilis* subspecies *ovatus* 11457, 15866; *B. fragilis* subspecies *vulgatus* 8482, 12967, 14363, 18315; *B. fragilis* subspecies *distasonis* 15436, 15756, 15975, 15988, 16075, 16573; *B. fragilis* subspecies *thetaiotaomicron* 14363, 14373, 14389, 15801, P-15; *Bacteroides melaninogenicus* 19003, 19004, 19005; *Bacteroides oralis* 17624; *Fusobacterium necrophorum* 2018, 16298, 18644; *Fusobacterium mortiferum* 15695, 15815, 17628; *Fusobacterium nucleatum* 9052, 15758, 17634; *Fusobacterium russii* 9054; and *Fusobacterium varium* 16043, 17300, P-142.

Preparation of antigens. Seven strains of *B. fragilis* subspecies *fragilis* (11710, 12103, 12330, 12336, 12959, 13712, 14787) were chosen as immunizing antigens because of their lack of serological reactivity in two previous FA investigations (Jones, Ph.D. thesis; Lombard, Ph.D. thesis). After it was identified and characterized, each strain was inoculated into thioglycolate broth (0135C, BBL) and incubated anaerobically for 48 h. Approximately 3 ml of each culture was aseptically transferred and streaked over the entire surface of a large petri dish (15 by 150 mm) containing Schaedler agar (BBL) with 0.001% vitamin K_1 added. The plates were incubated under anaerobic conditions for 48 to 72 h. Cells were carefully harvested, so as not to disrupt the agar, into 0.4% Formalin-treated saline (0.85% NaCl, pH 7.2). Cell suspensions were packed by centrifugation and suspended in 0.01 M phosphate-buffered saline (pH 7.2). Antigen preparations were checked for purity and viability by Gram stains and subcultured into aerobic and anaerobic media. The vaccines were stored at 4°C until injected.

Injection schedule and production of antisera. The density of each immunizing cell suspension was adjusted to 20% transmission at a wavelength of 540 nm as determined by a spectrophotometer (cell suspension opacity exceeded that of a McFarland #10 nephelometer standard). Serum samples from New Zealand white rabbits were collected and titrated with each immunizing strain of *B. fragilis* subspecies *fragilis* being tested. Sera without demonstrable antibodies against the test antigens were used as negative controls in subsequent tests. Pairs of rabbits were each injected intravenously into marginal ear veins with one of the seven immunizing strains. The injections were given at 4-day intervals in the following amounts: 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ml. Blood obtained by cardiac puncture 5 days after the final antigen injection was allowed to clot at room temperature for 2 h, and the serum was obtained. Merthiolate (1:10,000, final concentration) was added as a preservative, and the antiserum was divided into 5-ml aliquots and frozen at −20°C until used.

After 2 weeks of no injections, each rabbit was given a 5.0-ml booster of antigen, and 5 days later antisera were again obtained as described above. This latter procedure was carried out to determine: (i) whether there was any increase in homologous titer, (ii) whether cross-reactivity with heterologous antigens was broadened, and (iii) to obtain a greater quantity of antiserum.

Total protein concentrations of all antisera were adjusted to 50 mg/ml as determined by the biuret method (10). Agglutination titers were resolved by making twofold dilutions of the seven antisera with sterile saline. A constant amount of either the homologous (vaccine) or a heterologous antigen (#3 McFarland) was added to each tube, including a saline control. All tubes were shaken, incubated in a water bath at 45°C for 2 h, and refrigerated at 4 to 8°C overnight. The highest dilution of antiserum in which discernible clumping occurred was defined as the agglutination titer.

Indirect FA tests. Smears of antigens were made on slides either from suspensions of pure cultures (24 to 48 h old), or from the antigens used for immunization. Smears were allowed to air dry, fixed in acetone for 5 to 10 min, and overlayered with increasing twofold dilutions of homologous whole antiserum. The slides were incubated for 30 to 45 min at room temperature in a moist chamber, washed once each with phosphate-buffered saline and distilled water, and air dried. Smears were then covered with goat anti-rabbit immunoglobulin-globulin (Difco), which had been conjugated to fluorescein isothiocyanate by the methods of Spendlove (20). This reagent was used at a concentration of 1 mg of total protein per ml, as determined by preliminary staining tests. Stained slides were washed again as described above and mounted with buffered-glycerol mounting fluid (Difco). Slides were examined for fluorescence and graded as follows: − (no fluorescence), 1+ (faint fluorescence), 2+ (dim fluorescence), 3+ (bright peripheral fluorescence), or 4+ (brilliant peripheral fluorescence). FA titers were recorded as the highest dilutions of antisera yielding 4+ reactions. A pool composed of equal amounts of each of the seven sera was titrated against each strain in the same manner as the individual antiserum.

Immunoglobulin fractions used in the indirect FA tests were obtained by fractionating portions of each antiserum with ammonium sulfate as recommended by Hebert et al. (12). All immunoglobulins were adjusted to contain 10 mg of total protein per ml. A pooled immunoglobulin was prepared by combining equal volumes of the individual immunoglobulins and was titrated with each antigen.

Positive (homologous FA reaction) and negative (omitting the middle layer or using preimmune serum) controls were used in all indirect FA tests.

Direct FA tests. Portions of each fractionated immunoglobulin were conjugated to fluorescein iso-

thiocyanate (12). Each conjugate was titrated by covering smears of homologous antigen with twofold dilutions of the conjugate. The test slides were then treated as in the indirect test, and FA titers were assessed. Positive (homologous antigen-conjugate) and negative (conjugated normal serum) controls were used in titer determinations and in cross-reactions.

Microscopy and photomicrography. A Leitz microscope equipped with a cardioid dark-field condenser was used to examine all test slides. Illumination was provided by an Osram HBO 200-W mercury-vapor lamp. The filter system consisted of a Corning 5-58 excitation filter and an OG-2 barrier filter. Photographs were taken with Tri-X-Pan film (Kodak) with a 2-min exposure time.

RESULTS

Agglutination tests. Homologous agglutination titers for the seven antisera against *B. fragilis* subspecies *fragilis* ranged from 512 to 4,096 (Table 1). Results in reciprocal cross-reactions among these antisera were lower in most tests, ranging from negative to 2,048. However, some of the antigens were agglutinated in heterologous antisera at the same or even in higher dilutions than in homologous antisera (e.g., 11710 cells in 12330 antiserum and 12336 antigen in 14787 antiserum). Increased titers were observed in only a limited number of either homologous or cross-reactions after booster inoculations of antigens.

Indirect FA tests. Table 2 contains FA titers obtained by the indirect method. Initially, FA titers were determined by making dilutions of whole antisera, which were adjusted to contain 50 mg of total protein per ml. Homologous FA titers (4+) obtained with whole antisera were quite high (128 to 2,048). Subsequently the fractionated immunoglobulins were adjusted to 10 mg of total protein per ml, used as the middle layer in staining tests, and were also titrated. Individual immunoglobulins yielded 4+ reactions with homologous antigens at dilutions ranging from 1:10 to 1:320. Figures 1 through 4 show fluorescing cells in which 4+ reactions occurred. Pleomorphic and spheroplast-like cells were obvious in some preparations (Fig. 3 and 4).

Each of the seven strains used in immunizing procedures was reacted individually with the six other antisera. Heterologous titers were determined to be equal to the respective homologous titers in five such tests. FA titers of 4+ were achieved in 15 tests by using twofold dilutions that were only one dilution lower than the homologous titers. In the remaining tests, except in two instances (antigen 14787 with antisera 12336 or 12959), 4+ fluorescence was obtained with lower dilutions of the various antisera. Cells of lines 11710, 12103, 12959, and 13712 were stained 4+ with rather high dilutions of all antisera, except with anti-14787. Antigens 12330 and 12336 stained 4+ with dilutions lower than the four strains mentioned above but at higher dilutions than cells of 14787. In fact, this particular strain (14787) yielded fewer high-titered reactions than did any of the other six strains. Also, anti-14787 serum produced lower FA titers in cross-reactions than did any of the remaining antisera.

Pooled agglutinins were prepared from whole antisera and from immunoglobulins. The result-

TABLE 1. *Agglutination titers of antisera produced with selected strains of Bacteroides fragilis subspecies fragilis*

Antiserum	Antigens						
	11710	12103	12330	12336	12959	13712	14787
11710	512[a]	16	512	128	512	256	16
	(512)[b]	(32)	(512)	(64)	(512)	(256)	(16)
12103	256	2,048	64	16	64	256	128
	(256)	(2,048)	(64)	(16)	(64)	(NTP)[c]	(NTP)
12330	1,024	256	2,048	32	64	256	32
	(2,048)	(NTP)	(2,048)	(32)	(NTP)	(512)	(32)
12336	512	128	64	1,024	128	128	512
	(512)	(128)	(NTP)	(1,024)	(128)	(256)	(512)
12959	—[d]	16	8	—	2,048	256	2
	(—)	(16)	(32)	(32)	(2,048)	(1,024)	(16)
13712	64	256	—	1,024	256	1,024	64
	(128)	(256)	(8)	(NTP)	(1,024)	(2,048)	(64)
14787	64	1,024	256	2,048	512	512	2,048
	(64)	(1,024)	(256)	(2,048)	(NTP)	(512)	(4,096)

[a] Titers expressed as the reciprocal of the highest dilution giving discernible agglutination.

[b] Parenthetical numbers represent agglutination titers obtained after "booster" immunization, which consisted of an additional 5.0-ml injection of vaccine.

[c] NTP, No test performed.

[d] —, No fluorescence observed at 1:10 dilutions of these immunoglobulins.

TABLE 2. *FA staining titers of whole antisera and anti-immunoglobulins against strains of B. fragilis subspecies fragilis as determined by the indirect method of staining.*

Antigens	Antibodies						
	11710	12103	12330	12336	12959	13712	14787
11710	1,024[a] (320)[b]	1,024 (80)	512 (80)	512 (40)	1,024 (160)	1,024 (80)	128 (NTP)[c]
12103	1,024 (160)	2,048 (160)	512 (NTP)	512 (NTP)	64 (40)	1,024 (80)	64 (NTP)
12330	256 (NTP)	512 (NTP)	1,024 (80)	32 (NTP)	128 (NTP)	128 (NTP)	64 (NTP)
12336	512 (NTP)	512 (NTP)	64 (NTP)	1,024 (80)	512 (NTP)	256 (NTP)	32 (NTP)
12959	1,024 (80)	256 (160)	512 (NTP)	64 (NTP)	1,024 (320)	256 (80)	64 (NTP)
13712	1,024 (160)	1,024 (160)	128 (NTP)	256 (NTP)	512 (80)	2,048 (160)	16 (NTP)
14787	16 (—)[e]	32 (—)	64 (10)	2[d] (—)	2[d] (—)	4 (—)	128 (10)

[a] FA titers expressed as the reciprocal of the highest dilution of antiserum giving maximum fluorescence (4+).
[b] Parenthetical numbers represent FA titers obtained with immunoglobulin fraction of antiserum.
[c] NTP, No test performed.
[d] 2+ FA reactions achieved with this dilution of antiserum.
[e] —, No fluorescence observed at 1:10 dilutions of these immunoglobulins.

ant titers from these reactions are given in Table 3. Staining titers for the polyvalent antiserum ranged from 32 to 2,048, whereas those for the pooled immunoglobulins were determined to be from 20 to 320. As in individual tests, some strains were stained with higher dilutions of the pools than others. In three tests, the pooled antiserum stained the antigens at the same dilution as did the homologous antiserum, i.e., antigens 11710, 12959, and 13712. In the remainder of tests using the pooled antiserum, the titers were lower than when homologous antisera were used. Fluorescence of 4+ was obtained with the pooled immunoglobulin with each immunizing strain. These titers were lower than those for the pooled antibodies; however, the total protein content of the immunoglobulins was 10 mg/ml as compared with 50 mg/ml for the pooled antiserum.

All antisera were tested against organisms other than the *Bacteroidaceae* strains previously listed. Included were: *Propionibacterium acnes* (3 strains), *Bifidobacterium breve* and *Bifidobacterium bifidum* (1 strain each), *Clostridium sporogenes, Clostridium perfringens,* and *Clostridium sordellii* (2 strains each), *Escherichia coli* (10 strains), *Pseudomonas aeruginosa* and *Pseudomonas cepacia* (1 strain each), *Streptococcus pyogenes* and *Streptococcus faecalis* (1 strain each), *Staphylococcus aureus* and *Staphylococcus epidermidis* (2 strains each), *Actinomyces israellii* and *Actinomyces bovis* (1 strain each). These organisms were also taken from stock cultures of the Anaerobe Laboratory at the CDC. Interfering cross-reactions did not occur with any of these organisms.

Cross-reactions did occur when a pooled antiserum was reacted with three *Bacteroidaceae* strains. *F. necrophorum* 16298 stained 4+ with a 1:256 dilution but not at 1:512. This same organism stained 3+ with the immunoglobulin pool undiluted but not at a 1:20 dilution, a concentration well below the staining titer. This interference was removed by adsorbing with cells of this organism without affecting the subspecies titers.

B. fragilis subspecies *distasonis* 15988 was stained with pooled serum at 1:32 (2+) but not at higher dilutions. Smears of this strain did not react with the pooled immunoglobulin undiluted. *B. fragilis* subspecies *vulgatus* 14363 fluoresced 4+ when reacted with a 1:4 dilution of the polyvalent antiserum but did not fluoresce when the pool was diluted 1:64. Adsorption was not necessary in these two cases, since the highest dilution giving fluorescence was still lower than the FA staining titer. All other reactions with heterologous *Bacteroidaceae* were negative and, therefore, not troublesome.

Excluding the immunizing strains, 17 isolated and defined strains of *B. fragilis* subspecies *fragilis* were reacted with the pooled serum. Four of these organisms were stained 4+ with a 1:1,024 dilution. Eight cultures had 4+ FA titers with a one-lower dilution (1:512); two gave 4+ reactions at 1:256; two stained 4+ at 1:128; and one stained only 2+ with the antiserum undiluted. Similarly, the pooled immunoglobulin reagent stained nine

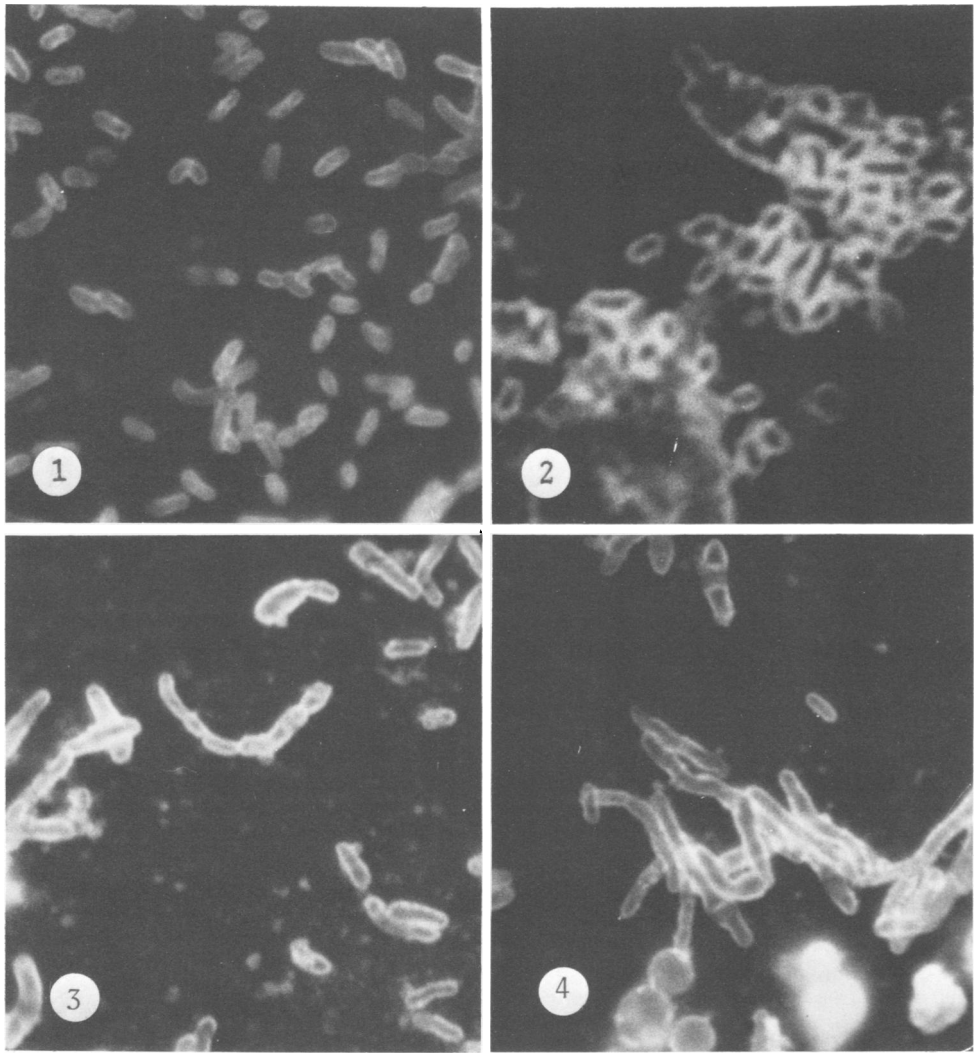

FIG. 1. *B. fragilis subspecies fragilis 12103 grown on Schaedler agar and stained by the direct FA method. ×1,700.*

FIG. 2. *B. fragilis subspecies fragilis 12103 grown in thioglycolate broth and stained by the indirect FA method. ×2,000.*

FIG. 3. *B. fragilis subspecies fragilis 11710 cultured on Schaedler agar and stained with homologous FA conjugate (×1,700) showing some pleomorphic cells.*

FIG. 4. *B. fragilis subspecies fragilis 12959 stained with pooled antiserum against the seven immunizing strains (×17,00) showing spheroplast-like cells.*

of the isolates at higher dilutions (160 or 80), whereas lower dilutions were required to stain the remaining strains. Isolate P-86 was stained with a 1:2 dilution (4+) but was only 2+ at 1:4.

Direct FA tests. The results using the seven monovalent conjugates are shown in Table 4. These labeled agglutinins were more specific than whole antisera in that cross-reactions were not observed with other subspecies or with heterologous genera. Antisera that exhibited high FA titers in the indirect tests also gave high titers in the direct tests. Conjugates 11710, 12103, 12959, and 13712 had titers ranging from 64 to 256 with homologus antigens. Titers of conjugates 12330 and 12336 were 8 and 16, respectively. Strain 14787 fluoresced weakly with its conjugated antiserum (3+ undiluted, 2+ at 1:4).

The pooled conjugate stained the seven test

TABLE 3. *B. fragilis subspecies fragilis strains tested by the indirect FA staining method*

Strain no.	Staining titers	
	Pooled sera[a]	Pooled immunoglobulins[b]
11710[c]	1,024	320
12103[c]	1,024	320
12330[c]	256	40
12336[c]	512	40
12959[c]	1,024	160
13712[c]	2,048	160
14787[c]	32	20
5462[d]	512	40
9053[d]	128	40
14462[d]	1,024	160
9498	512	160
10306	1,024	160
11777	512	80
13037	512	40
14073	256	40
15796	512	80
15802	1,024	160
FN-2	1,024	80
P-77	512	40
P-80	512	80
P-83	256	40
P-85	512	80
P-86	(undiluted)[e]	2
P-89	128	40

[a] Titers expressed as the reciprocal of the highest dilution of pooled serum (50 mg of total protein per ml) giving a 4+ FA reaction.

[b] Titers expressed as the reciprocal of the highest dilution of pooled immunoglobulins (10 mg of total protein per ml) giving a 4+ FA reaction.

[c] Strains used for preparation of antisera in present study.

[d] Strains used in previous investigation by Lombard (Ph.D. thesis) and by Jones (Ph.D thesis).

[e] Yielded only a 2+ reaction.

TABLE 4. *Staining results obtained by using the direct method with 24 strains of B. fragilis subspecies fragilis*

Strain	Direct method titers	
	Homologous conjugate[a]	Pooled conjugate[b]
11710	128	128
12103	256	256
12330	8	32
12336	16	32
12959	64	64
13712	256	128
14787	(undiluted)[c]	4
5462	NTP[d]	64
9053	NTP	64
14462	NTP	128
9498	NTP	128
10306	NTP	64
11777	NTP	64
13037	NTP	32
14073	NTP	32
15796	NTP	128
15802	NTP	256
FN-2	NTP	128
P-77	NTP	64
P-80	NTP	64
P-83	NTP	32
P-85	NTP	64
P-86	NTP	8
P-89	NTP	32

[a] Titers recorded as the reciprocal of the highest dilution of the conjugate giving 4+ FA reaction.

[b] Pooled conjugate consisted of equal volumes of each of the seven conjugates. Titer given as reciprocal of highest dilution giving 4+ fluorescence.

[c] 3+ reaction obtained.

[d] NTP, No test performed.

strains 4+ but at various dilutions. For the most part, these titers were comparable to those obtained with monovalent, homologous fluorescein isothiocyanate-labeled antibodies. Antigen 14787 was stained 4+ with a 1:4 dilution of the polyvalent conjugate but only 2+ at 1:8. FA reactions with the pooled conjugate and the 17 additional strains of *B. fragilis* subspecies *fragilis* gave rather high titers, except for strain P-86, which demonstrated the lowest titer of this group.

DISCUSSION

Both agglutination and FA test results showed that higher-titered antisera against *B. fragilis* subspecies *fragilis* were obtained in this investigation than in several earlier studies (11; Jones, Ph.D. thesis; Lombard, Ph.D. thesis). The high titers were interpreted as immunological responses to antigenic stimulation, since antibodies were not detected by preliminary agglutination or FA tests with any of the preimmune sera. These results could be attributed to differences in preparing or injecting antigens. In our case, the antigens were subcultured from thioglycolate broth onto Schaedler agar plates, whereas Stauffer (21), Lombard (Ph.D. thesis), and Jones (Ph.D. thesis) reported using broth suspensions. It is known that, in some instances, physiological manipulations of cultures can affect their antigenicity. Also, very dense suspensions and large doses of antigens were used in this study when compared to the above studies. Since antigenicity can be altered after storage or multiple transfers of a culture, we tested young (24 h) broth and agar cultures as well as older Formalin-fixed cultures that had been used for immunization and then stored in a refrigerator at 4 to 8°C for up to a year. Significant differences in either agglutination or FA titers were not detected. Some cultures taken immediately from thioglycolate broth containing

0.3% agar did not stain as brilliantly as homologous cultures taken simultaneously from agar-free broth or from agar plates. Therefore, inoculation of an agar-free broth may enhance and aid in staining clinical specimens containing strains of *B. fragilis* subspecies *fragilis*.

Staining tests in which antigens were boiled for 1 or 2 h prior to staining yielded 4+ fluorescence with homologous antisera; thus, titers were not reduced in the process. This indicated that the antigens involved with FA were heat stable. Lambe and Moroz (17) found that heated antigens of *B. fragilis* subspecies *fragilis* were also agglutinated as readily as unheated cells.

Homologous titers were not increased appreciably, nor did cross-reactions with heterologous or subspecies strains occur more frequently after a booster injection of antigen. The removal of interference by other subspecies or genera in indirect FA tests was by adsorption of the antiserum with the offending organism, allowing for a more specific reagent.

Agglutination and FA titers were comparable in six of the seven homologous tests, i.e., yielding positive reactions at high dilutions. These two types of titers cannot always be correlated, however, and similar differences with other organisms have been reported (2, 6).

Based on our agglutination and FA test results, it appears that more than one serotype of *B. fragilis* subspecies *fragilis* exists in the strains tested. These findings corroborated other reports concerning this subject (4, 21; Jones, Ph.D. thesis). Results from a limited number of absorption tests indicated that strains 12103 and 12959 shared a common antigen, whereas strain 14787 lacked an antigen common to either of these strains. More detailed work in this area is required to delineate the results from all possible cross-reactions. Kasper (14) has reported that antisera against strains of *B. fragilis* subspecies *fragilis* in his study varied in their binding capacity to cell wall polysaccharide. Perhaps then, there were quantitative differences in the antigenic materials of our strains.

Data from immunodiffusion tests (to be reported later) demonstrated that some strains contained more antigens in their cell-wall extracts than did others. Some bands formed identical arcs; others formed arcs that were similar; and still others formed arcs that were not similar. The number of bands in homologous immunodiffusion tests varied from one to four, whereas in cross-reactions bands were either absent or varied from one to two bands. These results also implied that more than one serotype exists within the seven test strains. Danielsson et al. (4) suggested the possibility of more than one serotype within this subspecies in an earlier report.

Pleomorphic and spheroplast-like cells stained in some preparations, displaying brilliant fluorescence like that of normally appearing cells. This would be helpful in identifying clinical isolates, since at times these structures are formed spontaneously or appear as a result of chemotherapy (21).

Stauffer et al. (21), in studying the detection of *Bacteroidaceae* by immunofluorescence, found antiserum against strains of *B. fragilis* subspecies *fragilis* quite specific for subspecies and even for strains within this group. Our results were similar in that other of the subspecies were stained but not to any great extent. One of our antisera stained a *F. necrophorum* strain, a problem not encountered in the aforementioned study.

Because only a limited number of strains were included and studied, some strains of *B. fragilis* subspecies *fragilis* would evidently be missed by using pooled antisera from this study. Therefore, more pools must be developed and evaluated to broaden the antigenic coverage, since accumulating evidence suggests that several serotypes exist. Adequate coverage would then warrant the use of FA tests in the presumptive identification of organisms of this subspecies in cultures and in clinical specimens. Although FA conjugates were more specific in tests, minor cross-reactions in indirect tests were eliminated without undue difficulty. Results from this study showed that the indirect method of FA with whole antiserum is an excellent method of identifying strains of *B. fragilis* subspecies *fragilis* and that the use of either fractionated immunoglobulins (indirect test) or conjugated immunoglobulins (direct test) were no more effective.

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