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Use of an Immunoperoxidase Method for Identification of *Bacteroides fragilis*

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An indirect immunoperoxidase (IP) slide test was evaluated for the laboratory identification of *Bacteroides fragilis*. Antigen-antibody complexes were detected with goat anti-rabbit immunoglobulin G-peroxidase conjugate with 3-amino-9-ethyl-carbazole as the peroxidase substrate. Ninety-one percent of 44 *B. fragilis* strains tested were IP positive (3+ to 4+ reactions) with ≥1:160 dilutions of rabbit antiserum produced against whole cells of *B. fragilis* ATCC 23745. The antiserum was species specific. No cross-reactions were observed with 35 *Bacteroides* strains of other species or with a variety of facultative or aerobic gram-negative bacilli. Four *B. fragilis* strains were IP negative. One of these (VPI 2393) was the deoxyribonucleic acid (DNA) homology group II reference strain. The other three were clinical isolates. IP-negative and representative IP-positive strains were tested for DNA homology with the type strains for DNA homology groups I and II (VPI 2553 and VPI 2393). Two of the three clinical isolates were classified as DNA homology group II, and the remaining strain was classified as a group I. Capsular material known to be unique to *B. fragilis* was common to both DNA homology groups as indicated by reactions with purified anticapsular antiserum. The IP technique provides a suitable alternative to fluorescent microscopy for the rapid immunological identification of *B. fragilis*.

Bacteroides fragilis is a significant participant in many anaerobic and mixed bacterial infections. This species is the most frequent anaerobic bacterium isolated from blood cultures (23). *B. fragilis* is also an important etiological agent in intra-abdominal abscesses and perirectal and pelvic septic processes (21, 22). When *B. fragilis* is suspected in an infection, antibiotic therapy should include an agent with specific activity against this species; however, many strains of *B. fragilis* are resistant to penicillin and other antibiotics that are active against most anaerobes. The drugs of choice, chloramphenicol and clindamycin, are known to exert potentially serious side effects (4, 7, 8, 24). Antibiotic therapy for mixed infections in seriously ill patients must be initiated empirically before culture and susceptibility results are available. Thus, the rapid laboratory identification of *B. fragilis* is vital to the management of such patients when considering appropriate therapy and provides clinical justification for the use of the more toxic preferred antibiotics.

The objective of this study was to evaluate an immunoperoxidase (IP) test for the rapid identification of *B. fragilis*. The basic technique has been successfully used in viral diagnosis (2) and was readily adapted for use with this microorganism.

MATERIALS AND METHODS

Bacteria. *B. fragilis* ATCC 23745, *B. distasonsis* ATCC 8503, *B. ovatus* ATCC 8483, *B. thetaiotaomicron* ATCC 12290, and *B. vulgatus* ATCC 8482 were obtained from the American Type Culture Collection (Rockville, Md.). These *Bacteroides* species were formerly accorded subspecies rank (3). Two strains of *B. fragilis* (VPI 2553 and VPI 2393) that represented deoxyribonucleic acid (DNA) homology groups I and II (11, 12) were provided by J. L. Johnson, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg. CDC strains (14787, 13712, 14462, 11710, 12103, 12330, 12336, 12959, 5462, 9053) were from G. L. Lombard (Center for Disease Control, Atlanta, Ga.) (1). Other microorganisms used throughout the study were clinical isolates obtained from the Microbiology Laboratories at University Hospital and Harborview Medical Center (Seattle, Wash.). These isolates included 42 facultative and aerobic gram-negative rods (11 *Escherichia coli*, 12 *Enterobacter* species, 4 *Proteus* species, 7 *Serratia* species, 2 *Klebsiella pneumoniae*, and 3 *Pseudomonas aeruginosa*). Eighty-two *Bacteroides* isolates of several species were tested. Each culture was restreaked three times for single colony isolation on Brucella blood agar (Pfizer Inc., New York, N.Y.) supplemented with vi-

tamin K (0.5 mg/ml) and hemin (5 μg/ml) for anaerobes or Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) for facultative bacteria. Stock cultures were maintained frozen at −70°C.

Antisera. Antisera to whole cells of *B. fragilis* ATCC 23745 and *B. vulgatus* ATCC 8482 were produced in rabbits by intravenous injection according to the method of Kasper (13). Blood was collected by cardiac puncture before immunization for control normal rabbit serum. The animals were exsanguinated 1 week after the last injection of *B. fragilis*. Rabbit serum was heat inactivated for 30 min at 56°C and absorbed with mouse liver powder to remove nonspecific cross-reacting material (2). Additionally, normal rabbit serum was absorbed with cells of *B. vulgatus* and *B. fragilis*. Small samples of serum were stored frozen at −70°C until use. Rabbit antiserum produced with purified capsular material from *B. fragilis* strain ATCC 23745 was provided by D. L. Kasper (Channing Laboratory, Boston, Mass.). Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was purchased from Miles Laboratories, Inc. (Elkhart, Ind.).

IP method. Slides were prepared by placing drops of glycerine in four rows of eight wells on standard microscopic slides. The slides were then sprayed with fluoroglide (Chemplast, Inc., Wayne, N.J.), allowed to dry and then rinsed with water and air dried.

Anaerobic cultures on Brucella blood agar were incubated in GasPak jars (BBL) for approximately 48 h. Facultative or aerobic bacteria were harvested from overnight cultures on Trypticase soy agar. Bacterial cells were suspended in phosphate-buffered saline to a turbidity matching a 0.5 McFarland standard (19). One drop (ca. 5 μl of 10^6 cells) was applied to each well. The slides were air dried, fixed in cold acetone, and then fixed in 3% HCl in ethanol.

Twofold dilutions of each rabbit antiserum, from 1:40 to 1:2,560, were tested with all ATCC, VPI, and CDC strains. Three dilutions of antisera, 1:80, 1:160, and 1:320, were routinely tested on all other clinical isolates. A 1:320 dilution of normal rabbit serum was included for a negative control on each slide. Positive controls were *B. fragilis* ATCC 23745 and *B. vulgatus* ATCC 8482 and their homologous antiserum. All tests were performed in duplicate.

A drop of antiserum or normal rabbit serum was placed in the appropriate well, and the slide was incubated in a humidified chamber for 1 h at 37°C. The slide was rinsed gently in distilled water and then washed in phosphate-buffered saline with constant agitation for 20 min. After washing, the slides were blotted dry. Goat anti-rabbit immunoglobulin G-peroxidase conjugate (diluted 1:50 to 1:60) was added to each well, and the slide was reincubated for 1 h at 37°C. The washing procedure was repeated. Next, peroxidase substrate was added to the wells and the slides were incubated for 20 to 30 min at room temperature. The peroxidase substrate was a solution of 3-amino-9-ethyl-carbazole (2 mg) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) dissolved in 0.5 ml of *N,N*-dimethyl formamide (Sigma Chemical Co., St. Louis, Mo.) buffered by the addition of 9.5 ml of 0.1 M sodium acetate buffer (pH 5.0). One drop of 3% H_2O_2 was added immediately before use. The slides were rinsed with distilled water and air dried. The preparations were counterstained with 1% methyl green for contrast, washed again with distilled water, and air dried.

Reactions were graded from 0 (colorless) to 4+ (red), macroscopically, depending on the color intensity seen in the wells. Reactions of 3+ to 4+ at a ≥1:160 dilution were considered positive; any reaction of 2+ or less was considered negative. In general, no color development (0+) or a pale pink color (1+) was observed in negative tests. Positive and negative controls were included on every slide for comparison. Microscopically, the positive specimens showed red granules at 10× and 40× magnifications.

DNA hybridization. Eleven strains of *B. fragilis* were selected for analysis of relatedness by DNA-DNA hybridization. These strains included three reference strains (ATCC 23745, VPI 2553, and 2393), three CDC strains, and five isolates from anaerobic infections obtained from the University Hospital laboratory (designated U). *B. fragilis* strains were chosen on the basis of previous positive or negative reactions in the IP test with whole cell antiserum. Broth cultures were incubated for 24 h in prereduced and anaerobically sterilized brain heart infusion broth prepared according to the VPI *Anaerobic Laboratory Manual* (10). Cells from 800 ml of culture were harvested by centrifugation, washed with 0.05 M tris(hydroxymethyl)aminomethane, 0.05 M ethylenediaminetetraacetic acid, and 0.1 M NaCl (pH 8.0) and resuspended in the same buffer containing 25% (wt/vol) sucrose. DNA was isolated as described by Marmur (18), but the method was modified by the addition of 0.5 mg of pronase (Calbiochem, San Diego, Calif.) per ml during incubation with sodium lauryl sulfate. As suggested by Marmur (18), several isopropanol precipitation steps to remove polysaccharide before incubation with ribonuclease were included. Purified DNA was dissolved in distilled water, and a small sample, containing not more than 100 μg of DNA, was frozen for in vitro radioisotopic labeling. DNA preparations were dialyzed against distilled water and sheared by ultrasonic treatment to a molecular weight of ca. 2.5×10^5.

[^{3}H]thymidine-labeled DNA was prepared from samples of pure DNA isolated from reference strains VPI 2553 and VPI 2393 by the in vitro "nick translation" procedure of Maniatis et al. (17). One microgram of unsheared DNA was allowed to react for 1 h at 15°C with 50×10^{-3} ng of deoxyribonuclease I (Worthington Biochemicals Corp., Freehold, N.J.) and 20 U of DNA polymerase I (Worthington DPFF) in the presence of $1{,}800 \times 10^{-12}$ mol of deoxyguanosine 5′-triphosphate, 360×10^{-12} mol each of deoxyadenosine 5′-triphosphate, deoxycytidine 5′-triphosphate, and tritium-labeled deoxythymidine 5′-triphosphate (40 to 60 Ci/mmol; New England Nuclear Corp., Boston, Mass.) in a reaction volume of 100 μl. ^{3}H radioactivity of these preparations was 2×10^6 to 4×10^6 cpm per μg of DNA.

The formation of DNA-DNA duplexes was assayed by the single-strand specific S1 endonuclease method of Crosa et al. (5). DNA preparations were denatured by incubation at 100°C for 10 min in 0.42 M NaCl. DNA-DNA duplex formation in solution (0.42 M NaCl) was tested by incubating 150 μg of unlabeled

DNA with ^{3}H-labeled reference DNA (containing 4,000 to 5,000 cpm) for 16 h at 62°C. The ratio of labeled to unlabeled DNA was in excess of 1:10,000. For controls in each test set, labeled reference DNA was incubated with unlabeled homologous DNA and with unlabeled heterologous DNA isolated from an *E. coli* K-12 strain. After endonuclease treatment, double-stranded DNA segments were precipitated with cold 20% trichloroacetic acid and deposited on membrane filters (type HA, Millipore Corp., Watertown, Mass.). After drying at 70°C for 10 min, the filters were immersed in vials containing toluene-Omnifluor (New England Nuclear Corp., Boston, Mass.), and the radioactivity was measured in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Reported results were the average of a minimum of two determinations.

RESULTS

Specificity of antisera. Antisera produced in rabbits against whole cells of *B. fragilis* ATCC 23745 and *B. vulgatus* ATCC 8482 were titrated against *B. fragilis* ATCC 23745, *B. vulgatus* ATCC 8482, *B. thetaiotamicron* ATCC 12290, *B. ovatus* ATCC 8483, and *B. distasonis* ATCC 8503. Antisera were specific for the homologous species, i.e., *B. fragilis* or *B. vulgatus*. No cross-reactions were observed with the other reference strains tested at a 1:40 dilution. Antisera from two rabbits, one immunized with *B. fragilis* (titer 1:1,280) and one with *B. vulgatus* (titer 1:1,280), were used throughout the study.

Eighty-two isolates of various species of *Bacteroides*, including the reference strains, were tested with antisera produced against *B. fragilis* ATCC 23745 and *B. vulgatus* ATCC 8482. *B. vulgatus* antiserum gave positive reactions with *B. vulgatus* strains only. Forty of the 44 (91%) *B. fragilis* strains gave positive reactions (3+ to 4+) with ≥1:160 dilutions of the *B. fragilis* ATCC 23745 antiserum (Table 1). Three isolates of *B. thetaiotaomicron* reacted (1+ to 2+) with low dilutions (1:40 and 1:80) of the *B. fragilis* whole cell antiserum, but were nonreactive at a 1:40 dilution in subsequent tests after subculture. No cross-reactions with other *Bacteroides* species were observed. One strain of *Veillonella parvula*, a gram-negative coccus, was tested; this microorganism was IP negative.

Forty-two facultative or aerobic bacteria belonging to six genera and 10 species were screened for cross-reactions. These strains were all negative in the IP test.

DNA-DNA homology. IP tests were negative (≤2+ reactions at 1:40) with four strains identified biochemically as *B. fragilis*. One of these strains, VPI 2393, was the reference strain for DNA homology group II as characterized by Johnson (11, 12). The other three strains were U 979, U 500, and CDC 13712. We performed DNA-DNA homology studies to determine whether these IP-negative strains belonged to DNA homology group II. Seven IP-positive (at ≥1:320) strains were also tested for comparison (Table 2); these included the reference strain for DNA homology group I (VPI 2553) and *B. fragilis* ATCC 23745 that was used to produce the whole cell antiserum. Two IP-negative strains, U 500 and CDC 13712, were found to be closely related to VPI 2393 (group II) with 97 and 99% of their DNA sequences in common. IP-negative strain U 979, however, was more closely related to VPI 2553 (group I), with 75% homologous sequences, than to the group II reference strain. A culture of U 979 was regrown from frozen stock and tested for morphological and biochemical homogeneity, for identity by IP, and for DNA-DNA homology with the two reference strains. Similar results were obtained, indicating that the discrepancy was not a result of either experimental procedure. The six other strains tested were homologous with VPI 2553 (group I) at 75 to 96% levels.

TABLE 1. *Specificity of B. fragilis antiserum[a] in the IP test*

Bacteria	No. tested	% Positive[b]
B. fragilis	44	91
B. thetaiotaomicron	15	0
B. vulgatus	4	0
B. ovatus	1	0
B. distasonsis	3	0
B. bivus	9	0
B. melaninogenicus	2	0
B. ochraceus	1	0
B. rumincola subsp. *brevis*	1	0
Bacteriodes species	2	0

[a] Antiserum was produced in rabbits immunized with whole cells of *B. fragilis* ATCC 23745.

[b] The IP test was considered positive for *B. fragilis* with reactions judged 3+ to 4+ with antiserum dilutions ≥1:160.

TABLE 2. *DNA-DNA homology of B. fragilis strains*

Strain	% Hybridization with DNA from:	
	VPI 2553[a]	VPI 2393[a]
VPI 2553	100	57
ATCC 23745	96	58
CDC 9053	91	60
CDC 12959	88	63
U 622	85	56
U 253	80	57
U 645	76	50
U 979	75	51
VPI 2393	51	100
CDC 13712	49	99
U 500	44	97

[a] *B. fragilis* VPI 2553 and VPI 2393 are the reference strains for DNA homology groups I and II (11).

Purified capsular antiserum. Strains of *B. fragilis* that were IP negative with whole cell *B. fragilis* antiserum were retested with an antiserum specific for capsular material possessed by this anaerobic species (13). IP-positive *B. fragilis* ATCC 23745 and VPI 2553 were included for comparison (Table 3). With strains VPI 2393, CDC 13712, U 500, and U 979, titers with the whole cell antiserum were <40. In contrast, titers with the antiserum prepared with purified capsular material were 320 and 640 for these four strains. Similar results with either antiserum were obtained for VPI 2553. With the homologous strain, *B. fragilis* ATCC 23745, the anticapsular titer was ≥2,560 compared to a titer of 1,280 for the whole cell antiserum.

DISCUSSION

Antiserum against whole cells of a single strain of *B. fragilis* ATCC 23745 was successfully used in an IP assay to identify 91% of 44 clinical and reference isolates. The antiserum was species specific, consistent with results reported by others (9, 20). Antiserum against a single strain of *B. fragilis* may also be specific for strains within the group, indicating that more than one serotype exists (1, 6, 16, 20). Abshire et al. (1) investigated seven strains of *B. fragilis* that were not reactive in previous fluorescent antibody studies. Antisera against these strains exhibited high titers in both agglutination and fluorescent antibody tests with homologous strains, but the results of cross-reactions with heterologous strains were generally lower, or negative in some cases. In our hands, six of these same seven (CDC) strains reacted with whole cell antiserum against *B. fragilis* ATCC 23745 at titers of ≥160. These results appear to reflect the greater sensitivity of the IP method for detecting shared antigenic components.

TABLE 3. *Reactivity of whole cell and purified capsular antisera with selected strains of B. fragilis*

Bacteroides strains	Antisera titers[a]	
	Whole cell[b]	Purified capsular[c]
ATCC 23745	1280	≥2560
VPI 2553	640	640
VPI 2393	<40	320
CDC 13712	<40	640
U 500	<40	320
U 979	<40	320

[a] The titer listed was the reciprocal of the antiserum dilution judged 3+ to 4+ reactive in the IP test system.

[b] Antiserum was produced in rabbits against whole cells of *B. fragilis* ATCC 23745.

[c] Antiserum produced against purified capsular material from *B. fragilis* ATCC 23745 was supplied by D. L. Kasper (14).

Although *B. fragilis* strains do exhibit serological diversity, common antigenic components have been characterized by Kasper and his colleagues. Both outer membrane protein antigens (15) and a capsular polysaccharide antigen (13, 14) are common to most strains of *B. fragilis*, but are rarely encountered in other *Bacteroides* species. Four of the strains we tested failed to react with antiserum against whole cells of *B. fragilis* ATCC 23745, but these strains did react with antiserum specific for purified capsular polysaccharide when tested by the same method. Kasper et al. (14) previously used this antiserum to develop a specific, indirect fluorescent antibody method for the laboratory identification of *B. fragilis*. All of the strains they tested reacted with antiserum against the capsular polysaccharide obtained from strain ATCC 23745.

Kasper (13) found that antisera against several strains of *B. fragilis* varied in their ability to bind purified capsular polysaccharide. He suggested that these results indicated quantitative rather than qualitative differences in capsular surface antigens because of the sensitivity of the radioactive assay method used and because the antigen was well characterized. Our results with the antiserum specific for capsular polysaccharide may reflect quantitative differences in this cell surface antigen among the *B. fragilis* strains tested. As mentioned above, four strains did not react with whole cell antiserum but did react with anticapsular antiserum; however, these results do not rule out the possibility of antigenic heterogeneity.

Diversity at the genetic level among microorganisms classified phenotypically as *B. fragilis* was demonstrated by the DNA-DNA homology studies of Johnson (11, 12). Two DNA homology groups were delineated with VPI 2553 (group I) and VPI 2593 (group II) used as the reference strains. The intergroup homology results obtained in this study were somewhat lower than those reported by Johnson (11, 12), 49 to 63% versus 61 to 78%, but different assay techniques were used. Nevertheless, our results confirmed the split of clinical isolates into two DNA homology groups. Randomly selected IP-positive strains (tested with whole cell antiserum) were all homologous with the group I reference strain, while three of the four IP-negative strains were homologous for the group II *B. fragilis* reference strain. Further, our results demonstrated that the capsular polysaccharide antigen described by Kasper (13, 14) could be detected in *B. fragilis* strains of both DNA homology groups.

As pointed out by Benjamin (2), IP tests have

several advantages over fluorescent antibody methods for routine use in the clinical laboratory. The reactions can be read macroscopically or rapidly with a light microscope at low power rather than with a fluorescent microscope; the preparation is permanent, and there are fewer nonspecific reactions. IP assays are more sensitive than fluorescent antibody methods because of the enzyme-amplified detection system for antigen-antibody complexes. In our experience as few as 10^4 cells of *B. fragilis* were detectable macroscopically. These results indicate that an IP assay may be applicable to the detection of this organism directly in clinical specimens as well as in the identification of *B. fragilis* from cultures.

We propose the use of an IP method as a suitable alternative to immunofluorescent microscopy for the rapid, specific laboratory identification of *B. fragilis*. However, considering the limitations of antiserum against whole cells of a single strain, further studies evaluating the relative merits of pooled antisera and antiserum against purified capsular polysaccharide in an IP assay are indicated.

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