Determination of *Bacteroides melaninogenicus* Serogroups by Fluorescent Antibody Staining

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Fluorescein isothiocyanate-labeled antibody reagents (conjugates) were prepared to one strain of each of the three subspecies of *Bacteroides melaninogenicus*: *B. melaninogenicus* subsp. *melaninogenicus*, *B. melaninogenicus* subsp. *asaccharolyticus*, and *B. melaninogenicus* subsp. *intermedius*. These three conjugates were specific; thus, they provided a new serological classification of *B. melaninogenicus*. The three serogroups were designated A, B, and C. Most test strains (98%) isolated from human clinical specimens were assigned to a specific serogroup by immunofluorescence, and the serogroup of these test strains corroborated the biochemical characterization of the three subspecies of *B. melaninogenicus*. The conjugates failed to cross-react with other anaerobes or aerobes tested. This fluorescent antibody technique provided a more rapid classification of the three subspecies of *B. melaninogenicus* than did conventional biochemical methods.

*Bacteroides melaninogenicus* is an anaerobic, gram-negative, nonsporulating bacillus that produces a brown- or black-pigmented colony. Sawyer et al. (19) reported that although biochemical differences existed among various strains of the organism, none of the strain differences were related; thus they thought that *B. melaninogenicus* should remain a single species. Later, Moore and Holdeman (12) divided the strains of this organism into the following three subspecies based upon characteristic fermentation patterns and volatile fatty acids produced: *B. melaninogenicus* subsp. *melaninogenicus*, *B. melaninogenicus* subsp. *intermedius*, and *B. melaninogenicus* subsp. *asaccharolyticus*. The clinical and epidemiological significance of this subspecies differentiation has not yet been determined.

*B. melaninogenicus* is normally found in the human intestine (2), on male and female external genitalia (2), in the throat (2, 15), and in the gingival crevices of man (2, 7, 24). It has been isolated in association with other bacteria from various types of clinical infections: tooth abscess (2, 10), soft-tissue infections (14), liver abscess (18), lung abscess (2, 3, 4, 22), an infected surgical wound (15), urine from a suspected infected kidney (15), appendicitis peritonitis (1), various types of surgical infections (26), and the uterus and blood in patients with puerperal infection (21). The organism is intimately associated with periodontal disease (11, 25) and various other types of infections (22, 23; S. M. Finegold, A. B. Miller, and D. J. Posnick, Bacteriol. Proc., p. 72, 1964).

The occurrence of *B. melaninogenicus* in clinical specimens was described as early as 1921, but the organism is still often undetected in clinical specimens for many reasons, including improper collection of the specimen, the lengthy delay between specimen collection and culture, failure to use the proper culture medium, and failure to incubate the cultures for sufficient time to permit growth and pigment production by the organism. It has been our experience, and that of others (3, 6), that some strains are difficult if not impossible to isolate in pure culture. Before 1969, this organism had never been isolated from clinical specimens submitted to this laboratory. With improved culture methods that were initiated in 1969 and are still in use, over 250 strains of *B. melaninogenicus* have been isolated from clinical specimens submitted to our laboratory. During the period from 1969 to 1973, there was no increase in the total number of patient beds in our hospital, although there was an increase in the total number of specimens cultured in the laboratory. Obviously, *B. melaninogenicus* is not as uncommon in clinical material, especially in wounds and abscesses, as we previously presumed. Since the organism is frequently found in clinical material and is difficult to culture, and since numerous biochemical tests are required for subspeciation, a rapid method of identification would be useful in two ways: it
would provide a more rapid report to the clinician, and it would reduce the cost and time of laboratory identification. Therefore, the purpose of this study was to develop fluorescent antibody (FA) reagents that would possess subspecies specificity and therefore would aid in the rapid identification of *B. melaninogenicus*.

**MATERIALS AND METHODS**

**Strains.** Isolation of *B. melaninogenicus* was enhanced by the use of brain heart infusion agar supplemented with 5% laked sheep blood, 0.5% yeast extract, hemin (0.0005%), and menadione (0.00005%). (This medium is subsequently referred to as laked blood agar plates.) These plates were used within 2 h after preparation or stored in an anaerobic atmosphere and used within 8 h. The inoculated plates were examined for brown or black colonies for at least 7 days.

The variety of clinical sources from which strains of *B. melaninogenicus* were isolated included: chest pus, mouth ulcer, decubitus ulcer, muscle at autopsy, extradural mass, gastrointestinal tract, vaginal discharge (lumbarspine, chest, umbilical), subsp. *frailis* (ATCC 73), subsp. *praevalens* (ATCC 25847) (Emory no. 209-74), *B. melaninogenicus* subsp. *asacharolyticus* (ATCC 25280) (Emory no. 208-74), *B. melaninogenicus* subsp. *intermedius* (NCTC 9338) (Emory no. 206-74), and *B. fragilis* subsp. *ovatus* (B6-V) (Emory no. 259-73).

**Identification.** Identification schemes described by Holdeman and Moore (9) were used to characterize anaerobic bacteria. Biochemical media were preduced, anaerobically sterilized media (Scott Laboratories). The biochemical tests used for identification of the anaerobic gram-negative bacilli that were studied serologically included: arabinose, cellobiose, fructose, glucose, lactose, maltose, mannitol, mannose, raffinose, rhamnose, starch, sucrose, trehalose, and xylose for acid production; esculin and starch for hydrolysis; gelatin, milk, and meat for digestion; chopped meat broth for indole production; nitrate (indole-nitrate broth; BBL) for reduction; and bile broth for stimulation or inhibition of growth. Colonies on anaerobic blood agar plates were examined for black pigmentation. The ability to convert lactate to propionate and threonine to propionate was also utilized for identification of the *Fusobacterium* strain. Gas chromatographic analyses were performed according to the methods of Holdeman and Moore (9).

**Antiserum production.** One test strain of each of the three subspecies of *B. melaninogenicus* was selected at random from clinical specimens for production of antisera. Reference strains were not used because of the possibility that the strain might have changed biochemically or serologically with prolonged storage and/or with multiple transfers. Specific strains have been used by others for the production of reference antisera (17).

**Immunization of rabbits.** Strains used for preparation of antisera were grown for 48 to 72 h on laked blood agar plates and then harvested in 0.85% sterile saline (PSS) that had been Seitz filtered and autoclaved. Formalin was added to the cell suspension to give a final concentration of 3% formalin, the mixture was incubated at room temperature (RT) for 60 min, and then the suspension was centrifuged at 3,500 rpm for 20 min in an International centrifuge (model CNS). The superantigen fluid was discarded, and the cells were washed three times with sterile PSS and then resuspended in PSS to a density of a McFarland no. 7 standard. Merthiolate was added to a final concentration of 1:5,000 to prevent contamination of the vaccine. Purity of the vaccines and killing efficacy of the formalin were determined by subculture on anaerobe and aerobe plates. The vaccines were stored in serum bottles at 4°C.

Young, albino, male rabbits weighing approximately 2 to 3 kg each were bled before injection; these preimmune sera were used ultimately as controls to establish the absence of antibody against test strains. The blood was left at RT overnight for clotting and then centrifuged; the antiserum was stored at −65°C.

The stored vaccine contained approximately 2.1 × 10⁶ cells per ml of saline. A 0.5-ml portion of the vaccine was mixed with 0.5 ml of Freund incomplete adjuvant. This mixture (1.0 ml) was injected subcutaneously into each hind and each front quadrant of the animal. After 7 days, intravenous injections of vaccine were given twice weekly. The first dose was 0.25 ml, the second dose was 0.5 ml, the third dose was 1.0 ml, the fourth dose was 1.5 ml, and the last four doses were 2.0 ml each. The animals were bled 1 week after the last injection.

Animals were bled from the heart. Merthiolate was added to each antiserum in a final concentration of 1:5,000.

**Preparation of FA reagents.** The globulin was precipitated from rabbit antiserum with 50% ammonium sulfate. The precipitate was dissolved in distilled water, and the entire procedure was repeated until the precipitate was white. The precipitate was resuspended in distilled water and dialyzed against 0.066 M K₂HPO₄-buffered saline (PBS), pH 7.2, at 4°C until the ammonium sulfate was no longer detected in the dialysate. The total protein of the immunoglobulin was determined by the biuret method. Fluorescein isothiocyanate (FITC) dye was added to the globulin in the proportion of 1 mg of dye per 20 mg of protein; this mixture was incubated at
RT for 2 h. The sample was dialyzed against 0.066 M PBS at 4 C with frequent changes for a period of 3 to 4 days until no FITC was detected in the dialyze. The FITC-labeled antibody (conjugate) was stored at -65 C with a 1:5,000 final concentration of merthiolate. Small portions were stored at 5 C during test procedures.

The three *B. melaninogenicus* conjugates were tested with each of the homologous strains to determine the optimal dilution of conjugate for FA staining of test strains (Table 1). The highest titer with a 3 to 4+ fluorescence was considered the specific staining titer. For use in staining bacteria, conjugate 138-71 was diluted 1:20, conjugate 207-72C was undiluted, and conjugate 275-70A was diluted 1:10.

**Direct FA staining.** Pure cultures of strains to be tested were inoculated onto laked blood agar plates that were incubated at 35 C for 48 h. Sufficient growth from these plates was suspended in PSS to produce a turbidity equivalent to a 1:10 dilution of a McFarland no. 5 standard as determined visually. This cell suspension (1 drop) was dispersed inside an etched circle on a microscope slide, dried, and heat fixed. The slides were stored at -65 C. Slides were removed from the freezer, dried, and heat fixed before use.

One drop of conjugate was placed on each smear. The slides were placed in a moist chamber at RT for 60 min, rinsed with PBS for 15 min, rinsed in distilled water, and air dried. The smear was mounted under a cover glass with phosphate-buffered glycerol (FA mounting fluid; Difco), pH 7.23. Smears were examined with a monocular Leitz fluorescence microscope equipped with a reflecting dark-field condenser and an Osram HBO 200 mercury lamp. A BG12 primary filter was used in combination with an OGI secondary filter. All observations were made with oil immersion. Fluorescence was graded as: 4+, brilliant fluorescence with a well-defined peripherally stained edge; 3+, moderate fluorescence with a well-defined peripherally stained edge; 2+, faint fluorescence, no well-defined edge; 1+, bare fluorescence, no well-defined edge; and 0, doubtful staining. A 3+ fluorescence or greater was considered significant.

**FA inhibition method.** Sera collected from rabbits before immunization were examined by the FA inhibition procedure (13). Heat-fixed smears of each test strain were prepared as described above for the direct FA method. One drop of rabbit preimmune serum was added to each smear. The slides were incubated in a moist chamber at RT for 30 min, rinsed with PBS for 15 min, and air dried. One drop of conjugate was added to a smear; the smear was incubated in a moist chamber at RT for 30 min, rinsed with PBS for 15 min, rinsed with distilled water, and air dried. The amount of fluorescence was recorded as above.

**Agglutination.** Strains were cultured for 48 to 72 h on laked blood agar plates. The cells were killed in 3% formalin, washed three times in PSS, and resuspended in PSS. Merthiolate in a final concentration of 1:5,000 was used as a preservative. The antigen suspension for agglutination tests was equal to the turbidity of a McFarland no. 7 standard diluted 1:2 (1+1).

| FITC-labeled antiserum (strain no.) | Specific staining titer |
|-----------------------------------|-------------------------|
| *Bacteroides melaninogenicus* subsp. *melaninogenicus* (138-71) | 1:60 |
| *B. melaninogenicus* subsp. *asaccharolyticus* (207-72C) | 1:2 |
| *B. melaninogenicus* subsp. *intermedius* (275-70A) | 1:20 |

Rabbit antisera were inactivated at 56 C for 30 min and then serially diluted twofold in PSS starting at a dilution of 1:10. An equal volume of antigen was added to 0.5 ml of the diluted antiserum. Tubes were incubated in a 45-C water bath, and the reactions were read after 18 h. Reactions were recorded as: 4+, heavy clumping; 3+, moderate clumping; 2+, light clumping; 1+, questionable clumping; and negative, no reaction. The highest serum dilution that produced a discernible agglutination was considered the agglutination titer.

**RESULTS**

**Strain identification.** All strains of *B. melaninogenicus* were anaerobic, gram-negative bacilli that produced a brown- to black-pigmented colony on the isolation medium.

*B. melaninogenicus* subsp. *asaccharolyticus* strains were asaccharolytic in all carbohydrates tested; gelatin was digested, but digestion of milk was variable. Indole and nitrate production was variable; growth was inhibited by bile; and acetic, propionic, isobutyric, butyric, and isovaleric acids were produced. Esculin and starch were not hydrolyzed.

Strains of *B. melaninogenicus* subsp. *intermedius* did not produce acid from celllobiose, mannotol, rhamnose, lactose, or trehalose. Acid was produced from glucose and starch; acid production from arabinose, fructose, maltose, mannotol, raffinose, sucrose, and xylose was variable. Gelatin and milk digestion was variable. Indole production was variable and nitrate was not reduced. Growth in bile was either inhibited or light. Acetic, isobutyric, and isovaleric acids were produced by the organism from glucose. Esculin was not hydrolyzed; starch hydrolysis was variable.

Strains of *B. melaninogenicus* subsp. *melaninogenicus* produced acid from fructose, glucose, lactose, maltose, mannotol, raffinose, starch, and sucrose. Acid production from arabinose, cellobiose, rhamnose, and xylose was variable. No acid was produced from mannotol or trehalose. Gelatin digestion was variable, but milk was not digested. Indole was not produced,
and nitrate was not reduced. Growth was usually inhibited in bile. Acetic only, or acetic, isobutryic, and isovaleric acids, were produced.

Biochemical reactions of *B. fragilis*, *B. corrodens*, and *Fusobacterium nucleatum* were characteristic of those reported by Holdeman and Moore (9).

**Direct FA staining with three *B. melaninogenicus* conjugates.** The three rabbit preimmune control sera showed no detectable antibodies to *B. melaninogenicus* test strains by the indirect FA method.

Smears of test strains that were stained by one of the three conjugates were coded and read by at least one, and sometimes two, observers who had no knowledge of the organism or the conjugate being examined.

*B. melaninogenicus* subsp. *melaninogenicus* (seven strains) gave a 3 to 4+ staining with the homologous conjugate only (Table 2). These strains either failed to stain or stained with no more than 2+ fluorescence with the heterologous conjugates (subsp. *asaccharolyticus* or *intermedius*). If 3 to 4+ staining is accepted as significant fluorescence, then *B. melaninogenicus* subsp. *melaninogenicus* represented a distinct serogroup, which I designated serogroup A. However, certain strains of serogroup A showed minor fluorescence with conjugates 207-72C and 275-70A (serogroups B and C). A 3+ staining control, as well as a negative staining control, was examined with each group of slides, which minimized the danger of misinterpreting 2+ and 3+ staining reactions.

*B. melaninogenicus* subsp. *asaccharolyticus* (30 strains) produced a 3 to 4+ fluorescent staining with the homologous conjugate only. This serogroup showed the most remarkable specificity of the three serogroups—28 strains stained specifically with the homologous conjugate only; only 2 of 30 strains showed even minor staining with the other conjugates. I have designated these strains as serogroup B.

*B. melaninogenicus* subsp. *intermedius* (12 strains) formed the third serogroup, which I have designated serogroup C. Most of the strains stained 3 to 4+ with the homologous conjugate only. There were two problem strains within this subspecies. Strain 532-70A originally stained 3+ with serogroup A conjugate and stained 1+ with serogroup B and C conjugates. Retesting of five single colonies of this strain showed a 1+ staining with serogroup A but no staining with serogroup B and C conjugates. Therefore, this strain did not stain significantly with any of the three conjugates. Strain 206-74 originally stained 2+ with serogroup A conjugate and 3+ with conjugates of serogroups B and C. FA stains were then performed on five

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**Table 2. Organisms tested by the direct fluorescent antibody staining method with conjugates to three subspecies of *Bacteroides melaninogenicus***

| Organism and strain no. | B. melaninogenicus subsp. melaninogenicus | B. melaninogenicus subsp. asaccharolyticus | B. melaninogenicus subsp. intermedius |
|------------------------|------------------------------------------|------------------------------------------|-------------------------------------|
|                        | subsp. melaninogenicus (anti-135-71)     | subsp. asaccharolyticus (anti-207-72C)   | subsp. intermedius (anti-275-70A)   |
| 138-71                 | 4+                                       | 1+                                       | 1-2+                                |
| 58-71A                 | 4+                                       | 1+                                       | 1-2+                                |
| 221-72E                | 3+                                       |                                  |                                     |
| 334-71H                | 4+                                       | 1+                                       | 1+                                  |
| 411-71D                | 4+                                       | 1-2+                                     |                                     |
| 427-70A                | 4+                                       | 1+                                       |                                     |
| 209-74                 | 4+                                       | 1+                                       |                                     |
|                        | **B. melaninogenicus subsp. asaccharolyticus** (13 strains) | | |
| 207-72C, 509-71A       | 4+                                       | 1+                                       |                                     |
| 417-71G, 407-71I       | 400-71G, 265-71F                        | 284-71I, 1510-72D                      |                                     |
| 1199-72C, 107-70D      | 208-74, 583-73E                         | 794-73D                                 |                                     |
| (15 strains)           | 735-70A, 343-73F                        | 560-73A, 924-73A                       |                                     |
|                        | 1103-73F, 722-73E                       | 424-70E, 269-73E                       |                                     |
|                        | 299-73B, 732-73E                       | 278-73D, 1122-73I                      |                                     |
|                        | 1264-73C, 506-73F                      | 256-73E                                 |                                     |
|                        | 538-72D                                 | 1-2+                                     |                                     |
|                        | 1077-73E                                | 4+                                       |                                     |
|                        | **B. melaninogenicus subsp. intermedius** | | |
| 275-70A                | 1-2+                                     | 3+                                       | 1+                                  |
| 1134-70H               | 1+                                       | 4+                                       |                                     |
| 694-71C                | 1+                                       | 4+                                       |                                     |
| 195-72A                | 1+                                       | 4+                                       |                                     |
| 906-72D                | 1-2+                                     | 3+                                       |                                     |
| 854-70E                | 1+                                       | 3+                                       |                                     |
| 679-70F                | 1+                                       | 3+                                       |                                     |
| 532-70A                | 1+                                       | 3+                                       |                                     |
| 206-74                 | 1-2+                                     | 3+                                       |                                     |
| 798-70I                | 1+                                       | 3+                                       |                                     |
| 1135-70C               | 1+                                       | 3+                                       |                                     |
| 1140-70F               | 1-2+                                     | 3+                                       |                                     |

*Conjugates were FITC-labeled rabbit antisera.*

*0 to ± fluorescence.

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single colonies; these colonies stained 1 to 2+ with serogroup A conjugate, failed to stain with serogroup B conjugate, and stained 3+ with
serogroup C conjugate. One possible explanation for these differences in staining is that either of these two strains may originally have been a mixture of more than one subspecies of B. melaninogenicus, but this was not verified biochemically.

Most of the minor cross-reactions (1 to 2+ staining) occurred with strains in serogroups A (B. melaninogenicus subsp. melaninogenicus) and C (B. melaninogenicus subsp. intermedius), the two subspecies that are most closely related biochemically. Absorption to remove these minor 1 to 2+ staining reactions was not performed. We have noted that certain strains of B. melaninogenicus subsp. intermedius are especially difficult to properly identify by biochemical reactions.

Of the three serogroups A, B, and C, serogroup B was the most common serogroup isolated in our laboratory, serogroup C was the next most common, and serogroup A occurred rarely.

The three FA conjugates were stored at -65°C for 1 year and then tested again with representative strains, as well as with recently isolated strains; the conjugates showed the same specificity as noted previously.

Each of the three conjugates failed to give more than a 1+ fluorescent staining reaction with nine strains of B. fragilis subsp. fragilis, two strains of B. fragilis subsp. thetaiotaomicron, one strain of B. fragilis subsp. distasonis, one strain of B. fragilis subsp. vulgatus, two strains of B. fragilis subsp. ovatus, two strains of B. corrodens, one strain of Bacteroides sp., one strain of F. nucleatum, two strains of Escherichia coli, two strains of Proteus mirabilis, two strains of Enterobacter aerogenes, and two strains of Serratia marcescens.

Agglutination. Sera obtained before injection of rabbits did not contain agglutinins to the immunizing antigens. The agglutination titer for each of the three homologous strains of B. melaninogenicus varied from 1:320 to 1:640 (Table 3). Titters with heterologous strains were no greater than 1:20. Absorption to remove these minor reactions was not performed. Additional strains of B. melaninogenicus were not examined by agglutination. The agglutination results of the three homologous strains corroborated the existence of the three serogroups described by FA testing.

Each of the three B. melaninogenicus antisera showed an agglutination titer of <1:10, or occasionally 1:10, with 26 strains of the five subspecies of B. fragilis tested, and a titer of 1:10 or 1:20 with two strains of B. corrodens.

### Table 3. Organisms tested by the agglutination method with antisera to three subspecies of Bacteroides melaninogenicus

| Organism and strain | Antiserum | subsp. melaninogenicus (anti-138-71) | subsp. asaccharolyticus (anti-207-72) | subsp. intermedius (anti-275-70A) |
|---------------------|-----------|-----------------------------------|-----------------------------------|----------------------------------|
| B. melaninogenicus subsp. melaninogenicus (138-71) | 640<sup>a</sup> | 20 | 20 |
| B. melaninogenicus subsp. asaccharolyticus (207-72) | 20 | 640 | 20 |
| B. melaninogenicus subsp. intermedius (275-70A) | 20 | 20 | 320 |

<sup>a</sup> Figures are the reciprocal of the highest antiserum dilution at which at least 3+ agglutination occurred.

Thus, there was no major cross-reaction with these two bacterial species.

### DISCUSSION

Immunofluorescence has only recently been applied to identification of anaerobic bacteria. The results reported here show that strains of B. melaninogenicus isolated from human sources can be divided into three specific serogroups by FA staining: serogroups A, B, and C, with serogroup B being the most frequently encountered. This division corroborates the three existing biochemical subspecies of Moore and Holdeman (12).

The serological classification of only one of 49 strains failed to correspond to the biochemical subspecies classification. Only one of 49 strains failed to react serologically; therefore, 98% of the strains tested could be assigned to one of three specific serogroups. There was no significant cross-reaction of the three specific conjugates with any additional anaerobes or aerobes tested.

In preliminary serological investigations with B. melaninogenicus, Griffin (8) prepared FA conjugates to two strains of B. melaninogenicus and noted no cross-reaction between the two strains. One test strain biochemically identified as B. melaninogenicus failed to react with the conjugates, and only one of two specimens that yielded B. melaninogenicus in culture stained with one of the two conjugates. She suggested that there were several serotypes of B. melaninogenicus.
The proposal of serological homogeneity of B. melaninogenicus by some workers, or heterogeneity by others, has been based on the results of agglutination tests (5, 16, 22, 27); however, the only results that closely corroborated our division of 49 B. melaninogenicus strains into three specific serogroups by FA was reported by Pulverer (16), who divided 29 strains of B. melaninogenicus into four serogroups (groups I, II, III, and IV) by the agglutination test. He noted the relationship between serogroups and biochemical groups, and the four groups were confirmed by the precipitation test. The strains belonging to the first three groups were isolated from humans and sheep, whereas group IV comprised cattle strains only. Since all of Pulverer's strains were saccharolytic, strains designated serogroup II, III, and IV were not included in Pulverer's four serogroups. Others have used the precipitin test to study B. melaninogenicus, but well-delineated groups were not described (20, 25). Werner and Sebald (27) reported that four strains of B. melaninogenicus represented three serotypes that could not be differentiated biochemically since the four strains had the same biochemical characteristics. Consequently, their results differed from our FA serogrouping and those of Pulverer with the agglutination test, since both our serogroups and those of Pulverer were related to biochemical characteristics.

The establishment of specific serogroups of B. melaninogenicus has important ramifications, i.e., a more rapid identification of B. melaninogenicus in the clinical laboratory. There is a definite need for reliable techniques that provide a rapid identification of anaerobes in the clinical laboratory, since present methods of biochemical identification usually require at least 5 days and often much longer. A rapid report makes it possible for the clinician to select proper antibiotic therapy for his patient as well as to know what organisms are present in the infection. This is important especially in life-threatening situations such as liver and lung abscess or septicemia in which B. melaninogenicus has been implicated. Differences in pathogenicity between the three subspecies also need to be evaluated. These B. melaninogenicus conjugates may be used for identification of colonies on original plates, and an FA report could be made the same day the culture is examined.

The use of specific conjugates permits to a considerable extent serogroup classification and, by extension, identification of the subspecies in a high percentage of the strains, since in many but not all of the strains used, the serogroup corresponded to the subspecies. Furthermore, proper subspeciation could aid in elucidating the importance of the frequency of these organisms in clinical infections. The use of specific conjugates in conjunction with definitive biochemical identification will aid in determining whether more than three serogroups exist.

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