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NOTES

Serological Studies of Oral *Bacteroides intermedius*

F. NAKAZAWA,[1] J. J. ZAMBON,[2]* H. S. REYNOLDS,[2] AND R. J. GENCO[2]

Department of Oral Microbiology, School of Dentistry, Niigata University, Niigata 951, Japan,[1] and Departments of Oral Biology and Periodontology, School of Dental Medicine, State University of New York at Buffalo, Buffalo, New York 14214[2]

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***Bacteroides intermedius* is a gram negative, anaerobic microorganism associated with certain forms of human periodontal disease, including adult periodontitis and acute necrotizing ulcerative gingivitis. Previous studies have indicated the presence of two DNA homology groups which could be distinguished by analysis of protein patterns on polyacrylamide gel electrophoresis, as well as at least two serogroups within *B. intermedius*. The present study examined the serology of *B. intermedius* and determined the distribution of *B. intermedius* serogroups in clinical isolates and patient plaque samples. Serological reactions with unabsorbed rabbit antisera and antisera immunoabsorbed with *B. intermedius* strains demonstrated a previously unreported antigenic group within *B. intermedius*, serogroup C, in both immunodiffusion and immunofluorescence assays. Of 79 *B. intermedius* isolates from 68 subjects examined with specific antisera, 55% of the isolates and 52% of the subjects were categorized in serogroup C, 40% of the isolates and 46% of the subjects were in serogroup B, and 5% of the isolates and 6% of the subjects were in serogroup A. In 31 samples of subgingival dental plaque from adolescents known to harbor *B. intermedius*, 81% demonstrated serogroup B, 16% had serogroup A, and 3% had serogroup C.**

The black-pigmented *Bacteroides* species are gram negative, anaerobic, nonmotile bacilli which appear as brown- to black-pigmented colonies when grown on medium containing blood. The black-pigmented *Bacteroides* species of the oral cavity can cause medically important extraoral infections, including brain abscess (17), lung abscess (1), mediastinitis (20, 32), and podiatric infections in diabetics (23). In the oral cavity, black-pigmented *Bacteroides* species have been implicated in the etiology of human periodontal disease, odontogenic abscesses, and endodontic lesions (6, 33). Among the black-pigmented *Bacteroides* species, *Bacteroides intermedius* has been associated with several forms of periodontal disease, including adult periodontitis (21, 25, 30, 34, 36), acute necrotizing ulcerative gingivitis (2, 12, 15, 27), and pregnancy gingivitis (11).

B. intermedius, however, appears to represent a heterogeneous group of microorganisms with possibly differing levels of virulence. DNA-DNA hybridization studies indicate the presence of two distinct DNA homology groups within *B. intermedius* (10), while previous antigenic studies have demonstrated the presence of two serologically distinct groups within oral *B. intermedius* (19). Heterogeneity within this species is also suggested by studies of patient antibody responses to *B. intermedius* infection. Patients with acute necrotizing ulcerative gingivitis, for example, develop high levels of serum antibody to only certain representative strains of *B. intermedius* (2).

In the study presented here, the serology of *B. intermedius* was examined and the distribution of *B. intermedius* serogroups was determined for both pure isolates and patient subgingival plaque samples.

The strains of *B. intermedius* examined in this study included 49 laboratory strains and 30 fresh isolates. The laboratory strains included ATCC 25611^T (type strain) and ATCC 25261 (American Type Culture Collection, Rockville, Md.); NCTC 9336 (National Collection of Type Cultures, London, United Kingdom); 13025, D25B-1, D16B-7, 4203, D28D-12, D22B-23, 13044, D11B-5, 9042, D10A-24, 13042, D16A-32, 9849, and 13029 (courtesy of L. V. H. Moore, Virginia Polytechnic Institute, Blacksburg); M86-688, M86-449, M86-596, M86-607, M86-675, M86-678, M86-679, M86-692, M86-698, M86-699, M86-714, M86-730, M86-734, M86-735, and Ball (courtesy of J. Slots, School of Dental Medicine, University of Pennsylvania, Philadelphia); 39, 564, 333, 334, 158, 96, 85, 73, 135, and 308 (courtesy of G. Bowden, University of Manitoba, Winnipeg, Canada); 5W2, 520-2, 525-1, M22-4, MR45B-2, and PD15M-1 (courtesy of G. Bourgeau, Université Laval, Quebec, Canada); and SUNYaB20-3 (School of Dental Medicine, State University of New York at Buffalo).

The fresh isolates, SUNYaB FL8-2, SUNYaB M2D2K-1, SUNYaB 17-9K-3, SUNYaB 16-12K-1, SUNYaB 17-12K-1, SUNYaB BLX-28B, SUNYaB BLX-28BK-1, SUNYaB BLX-83B-1, SUNYaB BLX-29BK-1, SUNYaB A9A2-13, SUNYaB A6A2-3, SUNYaB A6A2-30, SUNYaB G8-9K-3, SUNYaB T19M-1. SUNYaB T8M-BM-2, SUNYaB 16-5K-8, SUNYaB (L2, -3, -5, -6, -7, and -8), SUNYaB (A9A1-4, -5, -9, and -10), and SUNYaB (A6A1-1, -3, -5, and -7), were obtained from samples of human dental plaque taken with paper points and anaerobically cultured on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 5% rabbit blood and with 5.0 μg of hemin and 0.5 μg of vitamin K_1 per ml, as previously described (35, 36). Strains of gram-negative anaerobic rods were classified as *B. intermedius* if they (i) fermented glucose, fructose, maltose, and sucrose, but not cellobiose or lactose; (ii) were indole positive and catalase negative; (iii) did not hydrolyze starch or esculin; and (iv) produced succinic, acetic, isovaleric, and sometimes

* Corresponding author.

formic, isobutyric, and propionic acids as metabolic acid end products (7, 9).

Serological studies of *B. intermedius* used rabbit polyclonal antisera produced in female New Zealand White rabbits. Bacterial cells for immunization and for sonication in the preparation of antigen extracts were anaerobically cultured in brain heart infusion broth for 36 h, harvested by centrifugation, washed, and suspended at a concentration of 10 mg (wet weight) per ml in sterile saline. Twelve 1.0-ml samples were intravenously injected via the marginal ear vein by the protocol of McCarty and Lancefield (18). Trial bleedings were obtained from the central ear artery, and the antibody titer was determined by serial dilution in immunodiffusion assays. Once a satisfactory antibody titer was achieved, the rabbits were exsanguinated by cardiac puncture. Antiserum samples were heated to 56°C for 30 min and stored in small portions at −70°C.

The reactivity and pattern of precipitin bands was examined in double-immunodiffusion assays carried out in gels containing 1.2% agarose (SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine) in 0.33 M Veronal buffer, pH 8.2, by the method of Ouchterlony (22).

Sonic extracts of 10 strains of *B. intermedius*, including ATCC 25611^T, ATCC 25261, NCTC 9336, SUNYaB F L8-2, SUNYaB G 8-9K-3, SUNYaB T 19M-1, SUNYaB T 8M-BM-2, SUNYaB 20-3, SUNYaB 16-5K-8, and SUNYaB 17-9K-3, at a protein concentration of 10 mg/ml (16) were examined by double gel diffusion against the corresponding unabsorbed rabbit antisera. Three immunoprecipitin reaction patterns were demonstrated, which suggested three serogroups: serogroup A, designated by ATCC 25611^T and SUNYaB FL8-2; serogroup B, designated by NCTC 9336, ATCC 25261, and SUNYaB 17-9K-3; and serogroup C, designated by SUNYaB G 8-9K-3, SUNYaB T 19M-1, SUNYaB T 8M-BM-2, SUNYaB 20-3, and SUNYaB 16-5K-8 (Table 1). The only cross-reactions noted were between the two serogroup A antisera and the five serogroup C sonicates.

Serogroup-specific antiserum was prepared by immunoabsorption of serum samples representative of the three groups, including ATCC 25611^T (serogroup A), NCTC 9336 (serogroup B), and SUNYaB G 8-9K-3 (serogroup C). Antiserum to *B. intermedius* ATCC 25611^T was absorbed with whole cells of strains NCTC 9336 and SUNYaB G 8-9K-3 to produce a serogroup-A-specific antiserum. Likewise, antiserum to strain NCTC 9336 was absorbed with cells of ATCC 25611^T and SUNYaB G 8-9K-3 to produce a serogroup-B-specific antiserum, and antiserum to SUNYaB G 8-9K-3 was absorbed with strain ATCC 25611^T and NCTC 9336 to produce serogroup-C-specific antiserum. Immunoabsorptions were performed by adding 100 mg (wet weight) of whole bacterial cells to 1 ml of rabbit antiserum. The mixture was placed in a shaker for 1 h at 37°C and then at 4°C for 12 h. After centrifugation at 12,000 × *g* for 60 min, the antiserum supernatant was removed, and the absorption was repeated with bacterial cells from the other heterologous serogroup. Gel filtration chromatography was then used to eliminate nonprecipitating particulates and antigen-antibody complexes. The immunoabsorbed antisera were applied to Bio-Gel A5m columns (Bio-Rad Laboratories, Richmond, Calif.) and eluted with phosphate-buffered saline (PBS) containing 0.02% sodium azide. Fractions were monitored in double-gel-diffusion assays with bacterial sonicates from representatives of each of the serogroups. Reacting fractions were pooled and concentrated to the original volume of serum using a Minicon B15 clinical sample concentrator (Amicon Division of W. R. Grace & Co., Danvers, Mass.).

The three serogroup-specific antisera were then reacted in double-immunodiffusion assays against the same 10 cell sonicates. Precipitin bands formed between the absorbed antisera and sonicates from each *B. intermedius* strain in that serogroup, but there were no bands formed against sonicates from *B. intermedius* strains in the other serogroups. In addition, the precipitin bands demonstrated reactions of nonidentity. The precipitin line formed by the reaction of the serogroup-A-specific antiserum with sonic extracts from ATCC 25611 shows a reaction of nonidentity with that formed by the reaction of the serogroup-B-specific antiserum reacting with sonic extracts from NCTC 9336 and with that formed by serogroup-C-specific antiserum reacting with sonic extracts from SUNYaB G 8-9K-3 (Fig. 1). Similarly, precipitin lines formed in the B-specific and C-specific reactions also exhibit nonidentity (Fig. 1).

To verify the serogroup specificity of the absorbed antisera and to determine the serogroup distribution of *B. intermedius* isolates and patient plaque samples, immunofluorescence assays were performed as described by Mouton et al. (19). Pure *B. intermedius* isolates were cultured on tryptic soy agar (Difco) supplemented with 5% sheep blood (Crane Laboratories, Inc., Syracuse, N.Y.) and with 5 μg of hemin and 0.5 μg of vitamin K_1 per ml. Cultures were incubated for 48 h at 37°C in an anaerobic chamber (Forma Scientific, Marietta, Ohio) containing an atmosphere of 85% N_2, 5%

TABLE 1. Immunoprecipitin reaction patterns in Ouchterlony gel diffusion assays

Antiserum[a] strain	Antigen reaction[b]									
	Serogroup A		Serogroup B			Serogroup C				
	ATCC 25611^T	SUNYaB FL8-2	NCTC 9336	ATCC 25261	SUNYaB 17-9K-3	SUNYaB G 8-9K-3	SUNYaB T19M-1	SUNYaB T8M-BM-2	SUNYaB 20-3	SUNYaB 16-5K-8
ATCC 25611^T	+	+	−	−	−	+	+	+	+	+
SUNYaB FL8-2	+	+	−	−	−	+	+	+/−	+	+/−
NCTC 9336	−	−	+	+	+	−	−	−	−	−
ATCC 25261	−	−	+	+	+	−	−	−	−	−
SUNYaB 17-9K-3	−	−	+	+	+	−	−	−	−	−
SUNYaB G8-9K-3	−	−	−	−	−	+	+	+	+	+
SUNYaB T19M-1	−	−	−	−	−	+	+	+	+	+
SUNYaB T8M-BM-2	−	−	−	−	−	+	+	+	+	+
SUNYaB 20-3	−	−	−	−	−	+	+	+	+	+
SUNYaB 16-5K-8	−	−	−	−	−	+	+	+	+	+

[a] Unabsorbed rabbit antiserum.
[b] Sonic extracts from bacterial cells at 10 mg (dry weight) per ml in PBS.

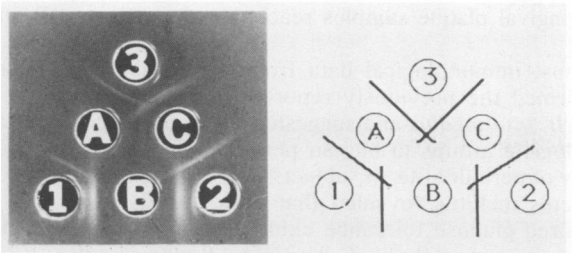

FIG. 1. Serogroup reactions demonstrated in double-immunodiffusion assays. Wells: A, serogroup-A-specific antiserum (rabbit antiserum to strain ATCC 25611^T absorbed with whole bacteria cells of strain NCTC 9336 and SUNYaB G8-9K-3); B, serogroup-B-specific antiserum (rabbit antiserum to strain NCTC 9336 absorbed with whole bacterial cells of strains ATCC 25611^T and SUNYaB G8-9K-3); C, serogroup-C-specific antiserum (rabbit antiserum to strain SUNYaB G8-9K-3 absorbed with whole bacterial cells of strains ATCC 25611^T and NCTC 9336); 1, sonicates from serogroups A (ATCC 25611^T) and B (NCTC 9336); 2, sonicates from serogroups B and C (SUNYaB G8-9K-3); 3, sonicates from serogroups C (SUNYaB G8-9K-3) and A (ATCC 25611^T). The bacterial sonic extracts were used at a concentration of 10 mg (dry weight) per ml in PBS, with equal volumes in each well.

CO_2, and 10% H_2. The bacterial cells were harvested from the surface of the agar plates with sterile cotton swabs and dispersed in sterile Ringer solution to an optical density of 0.7 at 540 nm. The serotype distribution of *B. intermedius* in subgingival plaque samples obtained from 31 adolescents shown by previous immunofluorescence assays to harbor *B. intermedius* was also determined with serogroup-specific antisera in immunofluorescence assays. Subgingival plaque was obtained from the mesial surface of the first four molar teeth with sterile paper points. Samples were then pooled in 1.0 ml of sterile Ringer solution with 2% Formalin and dispersed by a vortex mixer.

Samples (10 μl each) of bacterial suspension or patient plaque were placed on glass slides, air dried, and gently heat fixed. Working titer concentrations (the highest twofold serial dilution still giving 4+ fluorescence) of serogroup-specific antiserum and fluorescein isothiocyanate-conjugated antisera were determined by checkerboard titration. Serum (10 μl) at a working titer concentration in PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T) was applied to each bacterial smear. After 10 min, the specimens were gently rinsed with PBS-T, washed in PBS, and rinsed with distilled water. Slides were then incubated with 25 μl of affinity-purified goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (isomer I, fluorescein-to-protein ratio of 25 μg/ml; BBL Microbiology Systems, Cockeysville, Md.). After 20 min of incubation at 37°C in 100% humidity, the slides were washed as described above and glass cover slips were mounted with glycerol in PBS (2:1 [vol/vol], pH 9.0).

The stained bacterial smears were examined with a Zeiss standard 14 microscope equipped for phase-contrast illumination and for incident light fluorescence. The light source was a 100-W halogen lamp with a BP 450 to 490 interference filter in its excitation pathway and with an LP 520 barrier filter. Fluorescence was graded from 0 to 4+ according to the following scheme: 0, no fluorescence; 1+, bare fluorescence, with single cells not distinguishable; 2+, faint fluorescence, with single cells visible but no definition of cell shape; 3+, moderate fluorescence, with good cell envelope definition and a dark cell center; 4+, brilliant fluorescence, with good cell envelope definition and a dark cell center. Grades 3+ and 4+ were considered positive reaction results.

Like double-diffusion assays, immunofluorescence assays demonstrated the presence of three distinct serogroups within *B. intermedius*. Each serogroup-specific antiserum at working titer (1:512, 1:512, and 1:64 for serogroups A, B, and C, respectively) gave positive fluorescence reactions only with *B. intermedius* strains of the same serogroup.

The serogroup distribution of 78 *B. intermedius* isolates from 68 subjects was determined by indirect immunofluorescence (Table 2). The vast majority of isolates were in serogroups B and C. Serogroup A, represented by the type strain, ATCC 25611, accounted for 5% of the isolates and 6% of the subjects, while serogroup B, represented by ATCC 25261, accounted for 40% of the isolates and 46% of the subjects. Most of the *B. intermedius* isolates (55%) and subjects (52%) belonged to serogroup C.

There appeared to be a strong correlation between the *B. intermedius* serogroups and DNA homology groups as reported by Johnson and Holdeman (10). Of the 17 *B. intermedius* isolates for which DNA homology data were available, *B. intermedius* isolates in DNA homology group I were all found in either serogroup A or C, while *B. intermedius* isolates in DNA homology group II were all found in serogroup B. The close relationship between serogroups A and C is also reflected in the serological studies with unabsorbed antisera. There was cross-reaction between unabsorbed serogroup A antisera to strains 25611^T and SUNYaB FL8-2 with sonicate to the serogroup C strains in immunodiffusion assays (Table 1). This reaction was eliminated by absorption of the serogroup A antisera with bacteria from serogroup C. Neither unabsorbed nor absorbed antisera to the serogroup C strains, however, reacted with the serogroup A microorganisms in these assays.

The distribution of *B. intermedius* serogroups was also determined in subgingival plaque samples from 31 adolescent patients. Of the samples, 81% reacted with serogroup B antiserum, 16% reacted with serogroup A antiserum, and 3% reacted with serogroup C antiserum. None of the patient plaque samples reacted with more than one of the *B. intermedius*-serogroup-specific reagents.

The black-pigmented *Bacteroides* species were first described as *Bacterium melaninogenicum* by Oliver and

TABLE 2. *B. intermedius* serogroup distribution by indirect immunofluorescence assay with serogroup-specific antisera

Serogroup	Strains[a]
A	ATCC 25611 (I), SUNYaB FL8-2, M86-688, 2D2K-1
B	NTC 9336 (II), ATCC 25261 (II), SUNYaB 17-9K-3, SUNYaB 16-12K-1, SUNYaB 17-12K-1, 13025 (II), D25B-1 (II), D16B-7 (II), 4203 (II), D28D-12 (II), D22B-23 (II), SUNYaB BLX-228, SUNYaB BLX-28BK-1, SUNYaB BLX-83B-1, SUNYaB BLX-29BK-1, SUNYaB A9A2-13, SUNYaB A6A2-3, SUNYaB A6A2-30, -39, -564, -333, -334, -158, -96, -85, -73, 5W2, 520-2, 525-1, MM22-4, MR45B-2, Ball
C	SUNYaB G8-9K-3, SUNYaB T19M-1, SUNYaB T8M-BM-2, SUNYaB 16-5K-8, SUNYaB 20-3, SUNYaB (L2, -3, -5, -6, -7, and -8), 13044 (I), D11B-5 (I), 9042 (I), D10A-24 (I), 13042 (I), D16A-32 (I), 9849 (I), 13029 (I), PD15M-1, 35, 308, M86-449, M86-596, M86-607, M86-675, M86-678, M86-679, M86-692, M86-698, M86-699, M86-714, M86-730, M86-734, M86-735, SUNYaB (A9A1-4, -5, -9, and -10), SUNYaB (A6A1-1, -3, -5, and -7)

[a] DNA homology groups I and II reported by Johnson and Holdeman (10).

Wherry in 1921 (21). This species was then subdivided according to fermentation reactions into strong fermenters, weak fermenters, and nonfermenters (3, 24) as the following subspecies: *melaninogenicus*, *intermedius*, and *asaccharolyticus*, respectively (9). In view of significant differences in guanine-plus-cytosine content between groups, these subspecies were then raised to species level as *B. melaninogenicus*, *B. intermedius*, and *B. asaccharolyticus* (4). This scheme has been further refined so that the saccharolytic black-pigmenting *Bacteroides* spp. now include the following: *B. melaninogenicus*; *Bacteroides loescheii*, formerly grouped with *B. melaninogenicus* (8); *Bacteroides denticola*, formerly a DNA homology group within *B. melaninogenicus*; *Bacteroides corporis*, formerly a DNA homology group and serogroup within *B. intermedius* (10); and *B. intermedius*, which contains two DNA homology groups (10).

B. intermedius is often found in high numbers in subgingival dental plaque in patients with the adult form of periodontitis (26, 28, 29, 31, 36) and with acute necrotizing ulcerative gingivitis (15) and in periodontal lesions in patients with non-insulin-dependent diabetes mellitus (35). Progress in examining the role of *B. intermedius* in the pathogenesis of periodontal disease has been hampered, however, by the presence of major antigenic and DNA homology subgroups within this species which may, themselves, have differing pathogenic potential.

Serological studies of *B. intermedius* have also revealed that this species is a heterogeneous group. *B. intermedius* (*B. melaninogenicus* subsp. *intermedius*) was described by Lambe (13) as serologically distinct from *B. melaninogenicus* subsp. *melaninogenicus* and *B. melaninogenicus* subsp. *asaccharolyticus*, who used unabsorbed antisera in both immunofluorescence and agglutination assays (14). Lambe assigned serogroups A, B, and C to *B. melaninogenicus* subspp. *melaninogenicus*, *asaccharolyticus*, and *intermedius*, respectively. A subsequent study examining a larger number of *B. melaninogenicus* subsp. *intermedius* strains uncovered a group of strains which was unreactive to antisera directed to serogroup C. This group was designated C-1 and became *B. corporis* (10, 14).

Antigenic subgroups within *B. intermedius* have also been described by Mouton et al. (19) and by Gmür and Guggenheim (5). Mouton et al. found antigenic differences between two oral strains of *B. intermedius* in immunofluorescence assays. Gmür and Guggenheim reported the production of four monoclonal antibodies to *B. intermedius*, including two species-specific monoclonal antibodies which could categorize 28 test strains into three reactivity groups based on enzyme-linked immunosorbent assays, which were in turn proposed as *B. intermedius* serogroups. One monoclonal antibody reacted with 57% of the *B. intermedius* strains tested, including the type strain ATCC 25611. This monoclonal antibody did not react with ATCC 25261. Therefore, the present data classifying these strains into distinct serogroups support these previous findings. The serogroup represented by ATCC 25261 accounted for 25% of the total, with the remaining serogroup accounting for 17%. By contrast, the present study shows a rather different distribution of *B. intermedius* serogroups. Serogroup A, represented by ATCC 25611^T, accounted for only 5% of the 79 isolates and 6% of the 66 subjects, while serogroup B, represented by ATCC 25261, accounted for 40% of the isolates and 46% of the subjects. Most of the *B. intermedius* isolates (55%) and subjects (52%) were classified as being in the serogroup C. In adolescents, however, 81% of the *B. intermedius*-positive subgingival plaque samples reacted with serogroup B antisera.

Thus, immunological data from the present study have confirmed the previously reported antigenic heterogeneity with *B. intermedius* and suggested the importance of certain serological groups in human periodontal disease. A recent study of periodontitis in subjects with non-insulin-dependent diabetes mellitus revealed that periodontitis patients with impaired glucose tolerance exhibited significantly elevated serum immunoglobulin G levels to *B. intermedius* 25261, serogroup B. In contrast, there were no differences in serum antibody titers between periodontally healthy and periodontally diseased individuals to any of a number of other subgingival microorganisms (35). Elevated levels of serum antibodies to this same *B. intermedius* serogroup in patients with another form of periodontal disease, acute necrotizing ulcerative gingivitis, have also been reported by Chung et al. (2).

DNA homology groups I and II in *B. intermedius* have also been correlated with distinct serogroups, although the homology data have been reported for only three strains (5). The present study also finds a correlation between the DNA homology group and serogroup in a subset of 17 *B. intermedius* strains. Strains categorized as belonging to DNA homology group I (*B. intermedius* I) were members of either serogroup A or C, while those strains categorized as being DNA homology group II (*B. intermedius* II) were members of serogroup B. The type strain ATCC 25611 is therefore *B. intermedius* I, serogroup A; ATCC 25261 and NCTC 9336 are *B. intermedius* II, serogroup B; and SUNYaB G8-9K-3 is *B. intermedius* I, serogroup C.

While DNA homology has been the basis for defining bacterial species, the DNA homology groups of *B. intermedius* have not yet been raised to species level because of the lack of a readily available distinguishing phenotypic trait. Data from the present study and from previous studies make it clear that the DNA homology groups in *B. intermedius* can be phenotypically distinguished by a serogroup-defining polyclonal antiserum or monoclonal antibody in serological assays such as immunofluorescence, double immunodiffusion, or enzyme-linked immunosorbent assay. There are also data to indicate that analysis of *B. intermedius* soluble proteins by polyacrylamide gel electrophoresis can differentiate DNA homology groups. Together, these data suggest the possibility of raising *B. intermedius* DNA homology group II to species status on the basis of an antigenic phenotypic trait.

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