

Morphological, Biochemical, Antigenic, and Cytochemical Relationships Among *Haemophilus somnus*, *Haemophilus agni*, *Haemophilus haemoglobinophilus*, *Histophilus ovis*, and *Actinobacillus seminis*

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Morphology, biochemical reactions, pigmentation, antigens, and cell envelope proteins were examined in 12 strains of *Haemophilus somnus*, *Haemophilus agni*, *Histophilus ovis*, and *Actinobacillus seminis*. All of the strains except *A. seminis* are related and are considered as a single *Haemophilus-Histophilus* (HH) group. In immunodiffusion tests, HH group bacteria had at least two antigens common to all members of the group, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that they have similar cell envelope protein profiles. A quantitatively variable yellow pigment with absorption maxima of 430 to 435 nm was present in strains of *H. somnus* and *H. agni*. The HH group did not produce catalase and grew only in air containing 10% CO₂. Of 10 HH group bacteria, 9 required thiamine monophosphate for growth. *A. seminis* was distinguished from the HH group by its lack of yellow pigment, production of catalase, growth in air, lack of a thiamine monophosphate requirement, and different cell envelope protein profile. In gel immunodiffusion tests, *A. seminis* antigens produced two lines of partial identity with the HH group when antiserum against *H. somnus* was used. Reference strains of *Haemophilus influenzae*, *Actinobacillus lignieresii*, and *Haemophilus haemoglobinophilus* were compared with the test strains. In immunodiffusion tests, a single antigen was found to be common to *H. haemoglobinophilus*, *A. seminis*, and the HH group. No similarities between any of the test strains and *H. influenzae* or *A. lignieresii* were noted. The close relationship of *H. somnus*, *H. agni*, and *Histophilus ovis* suggests that these unofficially named bacteria may belong to a single taxon.

Many bacteria that have not been officially named are significant pathogens of production animals. *Haemophilus somnus*, *Haemophilus agni*, *Actinobacillus seminis*, and *Histophilus ovis* are each associated with specific diseases of ruminants, but none is recognized as a species in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (6) or the List of Approved Bacterial Names (38). These bacteria are gram negative and hemophilic. They are difficult to identify owing to poor growth in routinely used biochemical test media, and isolation from a typical lesion is usually a primary determinant in their identification. However, as the number of studies conducted on these bacteria increases, they are being isolated with increasing

frequency from animals that are normal or have atypical diseases. Therefore, it is important to characterize these pathogens to allow reliable identification, official recognition, and establishment of type strains.

H. somnus was identified as the causative agent of bovine infectious thromboembolic meningoencephalitis in 1960 (22) and was named in 1969 (W. E. Bailie, Ph.D. thesis, Kansas State University, Manhattan, 1969). In a recent Canadian survey, thromboembolic meningoencephalitis was found to be the second most common cause of mortality in feedlot cattle (29). *H. somnus* is also a significant pathogen of cattle in Europe and has been implicated as a cause of pneumonia, abortion, vaginitis, and endometritis (41). An isolate is also recorded from ovine pneumonia (7). *H. somnus* also occurs commensally in the prepuces of cattle and is common in bovine semen (10, 17-19). The cultural and

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biochemical features of *H. somnus* have been described (13, 22, 37), and its DNA base composition is similar to that of *Haemophilus influenzae* (3). Mannheim et al. (28) concluded that *H. somnus* belonged in the *Actinobacillus-Haemophilus-Pasteurella* group after examining the DNA base compositions, respiratory quinones, and biochemical reactions of two strains.

In 1958, Kennedy et al. described a bacterium isolated from sheep with septicemia and named it *H. agni* (23). The biochemical reactions of *H. somnus* and *H. agni* were very similar, and complement fixation testing of hyperimmune serum samples showed a close serological relationship between the two organisms. Subsequent isolations of *H. agni* have not been reported. However, in Australia and New Zealand a similar bacterium, named *Histophilus ovis* by Roberts (35), has been frequently isolated in cases of ovine septicemia and synovitis (21, 34), ovine epididymitis (8, 12), and from ovine vaginas (32). Organisms resembling *Histophilus ovis* have also been described in South Africa (43). Recently, *Histophilus ovis* was isolated in Canada from the vaginal discharges of ewes (16).

A. seminis was first described in Australia by Baynes and Simmons (4), who isolated it from ovine epididymitis. It is a recognized cause of ovine epididymitis in the United States (27), New Zealand (15), South Africa (43, 47), and Australia. *A. seminis* has also been associated with ovine polyarthritis and posthitis (45) and has been reported in bovine semen (15, 44). A partial relationship between *A. seminis* and *Histophilus ovis* has been suggested on the basis of serological and biochemical reactions (33, 43).

Actinobacillus actinoides, originally isolated from bovine pneumonia (39), has been associated with bovine seminal vesiculitis (20). *H. somnus* was likened to *A. actinoides* by Bailie et al. (2). Another organism, *Haemophilus citreus*, was isolated from bovine coital exanthema (11). Both *A. actinoides* and *H. citreus* have cultural characteristics similar to those of *H. somnus*, and both are listed as species incertae sedis in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (6). Reported isolations of these bacteria are rare.

All of the above-mentioned bacteria produce glistening clear colonies <1 mm in diameter on blood agar after 24 h of incubation. They are gram negative and extremely pleomorphic, varying from coccobacillary to filamentous forms. The oxidase reaction is positive, and they fail to grow in Hugh-Leifson OF medium or MacConkey agar. When serum is added to media, the bacteria are slowly fermentative, but there is no response to factor X (hemin) or factor V (NAD⁺). Increased CO₂ tension during incubation is essential for growth of all the bacteria

mentioned except *A. seminis*. There is no clear basis for differentiation among all of the above-mentioned bacteria because few comparative studies have been conducted.

While investigating bovine *H. somnus* infections, we have observed an organism apparently identical to *H. somnus* in cases of ovine abortion and meningitis. Also, the pathologies of *H. somnus* infections in cattle (22), *H. agni* infections in sheep (23), and *Histophilus ovis* infections in sheep (32, 34) are remarkably similar, with basic lesions of necrotizing vasculitis, thrombosis, and septic infarction. Therefore, we undertook to examine the relationships between *H. somnus*, *H. agni*, *Histophilus ovis*, *A. seminis*, and an organism presumptively identified as *A. actinoides*. Cultures of *H. citreus* were not available. In addition, these bacteria were compared with reference strains of *H. influenzae*, *Actinobacillus lignieresii*, and *Haemophilus haemoglobinophilus*. The last organism was included in the study as an example of a *Haemophilus* species found in the canine reproductive tract (40).

MATERIALS AND METHODS

Bacteria. As some of the bacteria studied were not available from reference collections, cultures were requested from investigators who had previously reported isolating them. Table 1 lists the bacteria studied, giving the names under which they were received and their origin. Our own isolates were typical of previously described *H. somnus* (13). One of these, *H. somnus* 43826, has been used to induce thromboembolic meningoencephalitis in cattle experimentally (42). On receipt, all cultures were inoculated into the yolk sacs of 7-day-old embryonating eggs. After 24 h of incubation, the yolks were harvested and stored in -70°C in 1-ml portions.

Media. Plain blood agar supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) and chocolate blood agar were prepared by adding 7% bovine blood to brain heart infusion (BHI) agar (Difco Laboratories). Blood agar was used for day-to-day maintenance of all strains except *H. influenzae* and *H. haemoglobinophilus*, which were maintained in chocolate agar. Bacteria were subcultured daily for 5 days, after which fresh bacteria were grown from frozen storage. All tests were inoculated with bacteria grown on blood agar.

Biochemical reactions were tested in two blood-free media (L. R. Stephens, Ph.D. thesis, University of Guelph, Guelph, Ontario, Canada, 1981). BHI-thiamine-aspartate-starch (BHITAS) broth or agar was prepared containing (in grams per liter): BHI, 32, or BHI agar, 57; sodium L-aspartate (Sigma Chemical & Co., St. Louis, Mo.), 0.5; soluble potato starch (Fisher Scientific Ltd., Toronto, Ontario), 1.0; and Tris (Fisher Scientific Ltd.), 1.0. After the media had been autoclaved, a filter-sterilized aqueous solution of 1 mg of thiamine monophosphate (TMP; Sigma Chemical Co.) per ml was added to a final concentration of 1 µg/ml (1). Proteose peptone (PP) broth was prepared containing (in grams per liter): Difco Proteose Peptone

TABLE 1. Sources of isolates used in the study

Organism		Source		Country of origin	Reference
Species	Strain	Animal	Organ		
<i>H. somnus</i>	43826	Bovine	Brain	Canada ^a	42
	26-16	Bovine	Prepuce	Canada ^a	18
	45468	Bovine	Lung	Canada ^a	
	2426	Bovine	Brain	U.S.A. ^b	5
	111	Bovine	Lung	U.K. ^c	31
Strain UQV 179	UQV 179	Bovine	Lung	Australia ^d	
<i>Histophilus ovis</i>	43803	Ovine	Brain	Canada ^a	
	H989	Ovine	Blood ^e	Australia ^f	33
	9L	Ovine	Vagina	Canada ^g	16
<i>H. agni</i>	1344	Ovine	Septicemia	U.S.A. ^b	23
<i>A. seminis</i>		Ovine	Epididymis	Australia ^h	
	ATCC 15768	Ovine	Semen	U.S.A. ⁱ	4
<i>H. haemoglobinophilus</i>	ATCC 19416	Canine	Prepuce	U.S.A.	
<i>H. influenzae</i>	ATCC 19418	Human		U.S.A.	
<i>A. lignieresii</i>	ATCC 19393	Bovine		U.S.A.	

^a Isolated by the authors.

^b E. L. Biberstein, Department of Veterinary Microbiology, University of California, Davis.

^c D. G. Pritchard, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, United Kingdom.

^d Animal Pathogen Culture Collection, Department of Veterinary Pathology and Public Health, University of Queensland, Australia; courtesy of M. D. Mutimer.

^e Isolated from a case of septicemia.

^f G. Riffkin, Department of Agriculture, Regional Veterinary Laboratory, Hamilton, Australia.

^g R. Higgins, Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada.

^h P. Headlam, Department of Agriculture, Attwood Veterinary Research Laboratory, Westmeadows, Victoria, Australia.

ⁱ American Type Culture Collection, Rockville, Md.

(Difco Laboratories), 10; sodium L-aspartate, 0.5; soluble potato starch, 1.0; Tris 1.0; Na₂HPO₄ (anhydrous), 2.0; NaCl, 5.0; and dextrose, 2.0. After the broth had been autoclaved, TMP was added as described above. Also, an aqueous solution of 50 mg of L-cysteine hydrochloride (Sigma Chemical Co.) per ml, neutralized with NaOH and filter sterilized, was added to a final concentration of 0.5 g/liter. When *H. influenzae* or *H. haemoglobinophilus* was grown in these media, factor X and V strips (Becton, Dickinson Co., Mississauga, Ontario, Canada) were added.

Sugar fermentation tests were performed in PP broth in which the dextrose was replaced with appropriate sugars and phenol red (0.005 g/liter) was added. After 24 h of incubation, tests were examined for growth and acid production.

Hugh and Leifson OF medium, MacConkey agar, Simmons citrate agar, SIM agar, triple sugar iron agar, and Christensen urea agar (Difco Laboratories) were all prepared as directed by the manufacturers, except that TMP (1 µg/ml) was added after autoclaving.

Biochemical tests. Standard techniques were used (30). Indole production was detected by the addition of Kovacs reagent to a 24-h culture in BHITAS broth. Nitrate production was tested in BHITAS broth con-

taining 0.1 or 0.01% (vt/vol) KNO₃. Production of H₂S during growth in BHITAS or PP broth was detected with lead acetate-impregnated paper strips. The ability to synthesize porphyrins from delta-aminolevulinic acid was used to ascertain dependence on factor X; this method is more accurate than the use of factor X strips placed on agar (25).

Colonial and microscopic morphology. Colonial morphology on BHITAS and blood agar was observed after 24 and 48 h of incubation. Gram stain reaction and motility tests were performed on 18-h BHITAS broth cultures.

Incubation conditions. All cultures were routinely incubated at 37°C in an atmosphere of 10% CO₂ and 90% air. Growth on blood agar in this atmosphere was also tested at 22°C. Aerobic growth on blood agar at 37 and 22°C was also tested.

Response to growth factors. Absolute dependence on TMP for growth was determined by visually comparing growth in PP broth and in BHITAS agar with growth in the same media prepared without the addition of TMP. Response to TMP was also assessed by a disk diffusion method. For this purpose, BHITAS agar plates prepared without TMP were inoculated with bacteria, and a paper disk 5 mm in diameter soaked in

an aqueous TMP solution (2 µg/ml [wt/vol]) was placed on the surface. Plates were observed for satellitism (increased growth) around the disk after 24 h of incubation. Response to factor X and V paper strips was assessed in the same manner. Satellitism around *Staphylococcus aureus* was also tested on BHITAS agar prepared without TMP.

Pigment production. Semi-quantitative determinations of pigment production by each strain of bacteria were made by spectrophotometric examination of lysed suspensions of the organisms. Strains of all bacteria other than *H. influenzae* and *H. haemoglobinophilus* were cultured on blood agar for 36 to 48 h. *H. influenzae* was cultured for a similar time on blood agar, adjacent to *S. aureus* streaks, and *H. haemoglobinophilus* was cultured on blood agar for 72 h. The growth from four or five plates was harvested and suspended in phosphate-buffered saline, pH 7.4, and placed in a preweighed microcentrifuge tube (Micro test tubes, Eppendorf; Johns Scientific, Toronto, Ontario, Canada). Cells were pelleted by centrifugation, the supernatant was discarded, excess fluid droplets were removed by inversion over filter paper, and the mass of the pellet was determined. A suspension of bacteria containing 20 mg (wet weight) per ml was prepared in 1.0 M Tris buffer, pH 6.8 (Fisher Scientific Ltd.), and to 3-mm samples were added 0.3 ml of 10% aqueous sodium dodecyl sulfate (SDS; Bio-Rad Laboratories, Richmond, Calif.). The suspension was gently shaken, incubated at room temperature for 5 min, and warmed until lysis was complete as indicated by clearing of the suspension.

The mean optical absorbance of three independently prepared lysates was determined over the visible spectrum by using circular optical cuvettes with a light path of 1 cm in a spectrophotometer. The mean of the three determinations was plotted against wavelength to determine the wavelength of maximum absorption (absorption maximum), and the optical absorbance of strains of similar absorption maxima were compared to determine quantitative differences in the concentrations of chromophores present (46).

Serological testing. Double immunodiffusion was performed on microscope slides in 1% agar prepared in phosphate-buffered saline, pH 7.4. Antiserum against intact *H. somnus* 43826 was prepared by hyperimmunizing a steer with whole cells of *H. somnus* in Freund complete adjuvant. Serum was collected before and after immunization. Soluble antigens of each bacterial strain to be tested were prepared by ultrasonic disruption of 0.5 ml of packed cells in 2.0 ml of phosphate-buffered saline.

Analysis of cell envelope proteins. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare cell envelope (CE) protein profiles. To prepare CE, 48-h cultures of each bacterium on BHITAS agar were harvested into 0.1 M NaCl and were rinsed twice by centrifugation. The bacteria were lysed with a Sorvall Ribi cell fractionator (I. & B. Maynard Co. Ltd., Toronto, Ontario, Canada) at 30,000 lb/in², and CE were isolated by differential centrifugation as described for *Escherichia coli* (3). The CE were stored at -70°C, and all subsequent manipulations were performed at 4°C. Immediately before electrophoresis, all CE preparations were diluted with 0.1 M NaCl to contain 10 mg of protein per ml. To denature proteins, 50 µl of CE was added to 50 µl of Tris-hydrochloride

buffer (pH 6.8)—1% (wt/vol) SDS—40 mM dithiothreitol and then held in a water bath at 100°C for 5 min. Electrophoresis was performed as described by Laemmli (26) in 0.8-mm thick slabs with separating gels at pH 8.6 containing 12% (wt/vol) acrylamide and an acrylamide-bisacrylamide ratio of 40:1. The stacking gel had a pH of 6.8 and contained 4% acrylamide. After electrophoresis, gels were fixed and stained simultaneously in methanolacetic acid-water (5:1:5) overnight. Molecular weight standards (Bio-Rad Laboratories) were included in each run. Molecular weights of unknowns were calculated from a standard curve prepared by plotting the relative migrations of the standards against the logs of their molecular weights.

RESULTS

Colonial and microscopic morphology. All strains were gram negative, nonmotile, pleomorphic bacilli ranging from coccoid to filamentous forms. *H. somnus*, *Histophilus ovis*, *H. agni*, and strain UQV 179 had identical colonial morphology. After 24 h of incubation at 37°C in 10% CO₂-90% air, colonies on blood agar were round, pinpoint, glistening, and transparent. After 48 h, the colonies were 1 to 2 mm in diameter, translucent, and umbonate. When massed on a loop, colonies were distinctly yellow and butyrous. Colonies on BHITAS agar were larger after 24 h, and yellowness was evident in individual colonies. *H. somnus* 45468 and strain UQV 179 produced a 2-mm zone of clear hemolysis around colonies on blood agar.

After 24 h of incubation at 37°C in 10% CO₂-90% air, colonies of *A. seminis* on BHITAS or blood agar were 1 mm in diameter, round, grey, and translucent. After 48 h, colonies were 2 to 3 mm in diameter, opaque, grey-white, and not umbonate. *A. lignieresii* colonies were larger than those of *A. seminis* but were otherwise similar. *H. influenzae* and *H. haemoglobinophilus* grew poorly on BHITAS agar unless appropriate growth factor strips were added.

Incubation conditions. Strains of *H. somnus*, *Histophilus ovis*, and *H. agni* grew well only in 10% CO₂-90% air. *H. somnus* 2426, *H. agni*, and strain UQV 179 produced pinpoint colonies in air at 37°C and in 10% CO₂-90% air at 22°C. Both strains of *A. seminis* grew in either atmosphere at 37°C only. *A. lignieresii* and *H. haemoglobinophilus* grew well under all conditions, whereas *H. influenzae* grew under all conditions except air at 22°C.

Biochemical reactions. Results of biochemical testing are shown in Table 2. Except for *A. lignieresii*, no strains grew on TMP-supplemented MacConkey, citrate, urea, triple sugar iron, or SIM media. Growth of no isolate except *A. lignieresii* was detectable in OF medium until the third day of incubation, when slight acidification was seen. Hydrogen sulfide was not produced in BHITAS broth except by *A. lignieresii*

TABLE 2. Biochemical reactions and growth requirements of all isolates

Organism		Oxidative (O) or fermentative (F)	Oxidase	Catalase	Indole	Nitrate	H ₂ S	ALA test ^a	Growth stimulated by TMP	Satellitism around <i>S. aureus</i>
Species	Strain									
<i>H. somnus</i>	43826	F	+	-	+	+	+	+	+	+
	26-16	F	+	-	+	+	+	+	+	-
	45468	F	+	-	+	+	+	+	+	+
	2426	F	+	-	+	+	+	+	-	-
	111	F	+	-	-	+	+	+	+	+
Strain UQV 179	UQV 179	F	+	-	+	+	-	+	+	+
<i>Histophilus ovis</i>	43803	F	+	-	+	+	+	+	+	-
	H989	F	+	-	-	+	+	+	+	-
	9L	F	+	-	-	+	+	+	+	+
<i>H. agni</i>	1344	F	+	-	-	+	+	+	+	+
<i>A. seminis</i>	ATCC	F	+	+	+	+	+	+	-	-
		F	+	+	-	+	+	+	-	-
<i>H. haemoglobinophilus</i>	ATCC	F	+	+	+	+	+	-	-	-
<i>H. influenzae</i>	ATCC	F	+	+	+	+	+	-	-	+
<i>A. lignieresii</i>	ATCC	F	+	-	-	+	+	+	-	-

^a ALA test, delta-aminolevulinic acid test for porphyrin synthesis.

and *H. haemoglobinophilus*, but most strains produced H₂S in PP broth. All strains reduced 0.01% nitrate, but only *A. lignieresii* grew in the presence of 0.1% nitrate. All strains fermented dextrose, but in many instances, growth did not occur in PP broth when dextrose was replaced by another sugar. Fermentation patterns that would assist in further characterization of the test strains were not noted. Therefore, sugar fermentation results are not detailed.

Response to growth factors. Significant stimulation of growth by TMP could only be demonstrated in PP broth. Of the 12 test strains, 9 were not seen to grow in PP broth unless TMP was added. *H. somnus* 2426 and the two *A. seminis* strains grew in PP broth with or without added TMP (Table 2). The disk method was more convenient for demonstrating a growth response to TMP, and the results agreed with those obtained from PP broth (Fig. 1). However, absolute dependence on TMP could not be determined from the disk method because most strains showed slight growth on BHITAS agar without added TMP. This is seen in Fig. 1, with luxuriant growth around the disk containing TMP and poor growth at the periphery of the plate.

None of the test strains showed a growth response to factor V. Satellitism around a factor X strip was shown by *Histophilus ovis* 43803

only, and it and all other test strains had positive porphyrin tests, indicating that factor X was not essential for growth. Of the 12 test isolates, 6 showed satellitism around *S. aureus* colonies (Table 2).

Pigment production. Lysates of all strains of *H. somnus*, *H. agni*, and strain UQV 179 were clear, sparkling, and bright lemon yellow. Lysates of *Histophilus ovis* and *H. influenzae* were clear and pale yellow, whereas those of *A. lignieresii*, *A. seminis*, and *H. haemoglobinophilus* were clear and colorless.

Absorption maxima occurred between 430 and 435 nm for *H. somnus* (all strains), *H. agni*, and strain UQV 179 (Table 3). Strains of *Histophilus ovis*, *H. influenzae*, *H. haemoglobinophilus*, *A. lignieresii*, and *A. seminis* showed progressive declines over the visible spectrum with maximum absorption in the near-UV range at 380 nm. A slight peak was present between 425 and 430 nm with *Histophilus ovis* 9L, and slight peaks occurred at 410 nm with *H. influenzae* and *H. haemoglobinophilus*. The peak optical absorbance of the strains *H. somnus*, *H. agni*, and strain UQV 179 varied between 0.239 (*H. agni*) and 0.677 (UQV 179).

Serological testing. Results of immunodiffusion testing are shown in Fig. 2. Reaction of *H. somnus* 43826 with its homologous antiserum yielded three precipitin lines. With the same

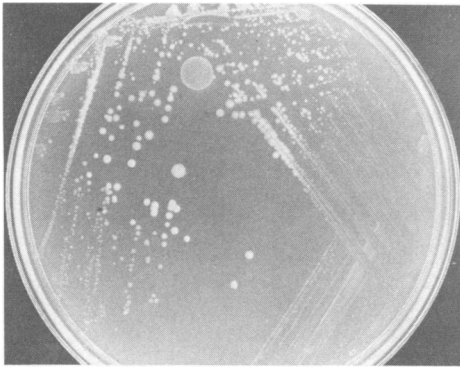


FIG. 1. Growth of *H. somnus* 43836 on BHITAS agar showing satellitism around a paper disk containing TMP.

antiserum, antigens from the other isolates of *H. somnus* and *Histophilus ovis* as well as *H. agni* and strain UQV 179 produced two to four precipitin lines (Fig. 2A and B). Dense lines of identity and weak spurs indicating partial identity can be seen. In Fig. 2C, two lines of partial identity between *H. somnus* 43826 and the two *A. seminis* isolates are seen. Also, a single strong line of identity can be seen among *A. seminis*, *H. somnus*, and *H. haemoglobinophilus*. A very faint line is apparent with *A. lignieresii*, and there was no reaction with *H. influenzae*. No precipitin lines occurred with any antigen when prevaccination serum was substituted for immune serum.

CE protein profiles. When tested by SDS-PAGE, all isolates of *H. somnus*, *Histophilus ovis*, *H. agni*, and strain UQV 179 showed similar, although not identical, CE protein profiles (Fig. 3). Each of these strains possessed major proteins with molecular weights of 75,000 to 95,000, 34,000, 28,000, and 13,000. Numerous minor proteins were also common to all of these strains, although some strains had additional high- and low-molecular-weight minor proteins of their own. The two *A. seminis* strains shared a common CE protein profile that was different from the profiles of the previous group. The protein profiles of *H. haemoglobinophilus*, *H. influenzae*, and *A. lignieresii* were different from one another and from those of all the other isolates.

The above results show considerable similarity between *H. somnus*, *H. agni*, and *Histophilus ovis*, based on colonial morphology, catalase reaction, poor growth in air, requirement for TMP, immunodiffusion reaction, CE protein profile, and pigment production. These same characters serve to distinguish the *Haemophilus-Histophilus* (HH) group from *A. seminis*. *H. influenzae* and *H. haemoglobinophilus* were readily distinguished by their requirements for X

TABLE 3. Wavelengths of absorption maxima and relative optical absorbance of all isolates examined in the study

Organism		Mean peak absorbance	Absorption maximum (nm)
Species	Strain		
<i>H. somnus</i>	43826	0.415	435
	26-16	0.365	435
	45468	0.359	430
	2426	0.355	435
	111	0.346	430-435
Strain UQV 179	UQV 179	0.677	435
<i>Histophilus ovis</i>	43803	0.140	380
	H989	0.155	380
	9L	0.208	380
<i>H. agni</i>	1344	0.239	435
<i>A. seminis</i>		0.136	380
	ATCC 15768	0.104	380
<i>H. haemoglobinophilus</i>	ATCC 19416	0.085	380
<i>H. influenzae</i>	ATCC 19418	0.257	380
<i>A. lignieresii</i>	ATCC 19393	0.107	380

and V factors, and *A. lignieresii* likewise distinguished by its less fastidious growth requirements. Strain UQV 179, submitted as representing *A. actinoides*, was included in the HH group.

DISCUSSION

The results of this study demonstrate a close phenotypic relationship between organisms currently identified as *H. somnus*, *H. agni*, and *Histophilus ovis*. Comparisons with the type species *H. influenzae* and *A. lignieresii* and with the reference species *A. seminis* and *H. haemoglobinophilus* provide further justification for inclusion of the HH group as a separate taxon in the *Actinobacillus-Haemophilus-Pasteurella* group of bacteria. However, we are unable to suggest any means by which bacteria of the HH group can be unequivocally identified. The absence of accepted criteria meant that we were unable to confirm fully the identity of bacteria sent to us as *Histophilus ovis*, *H. somnus*, or *H. agni*. Definitive identification of HH group bacteria will remain problematic until new tests are found and type species are designated. Meanwhile, we prefer to refer to these bacteria collectively rather than attempt to separate them into three species. The HH group can be presumptively identified as yellow-pigmented, gram-neg-

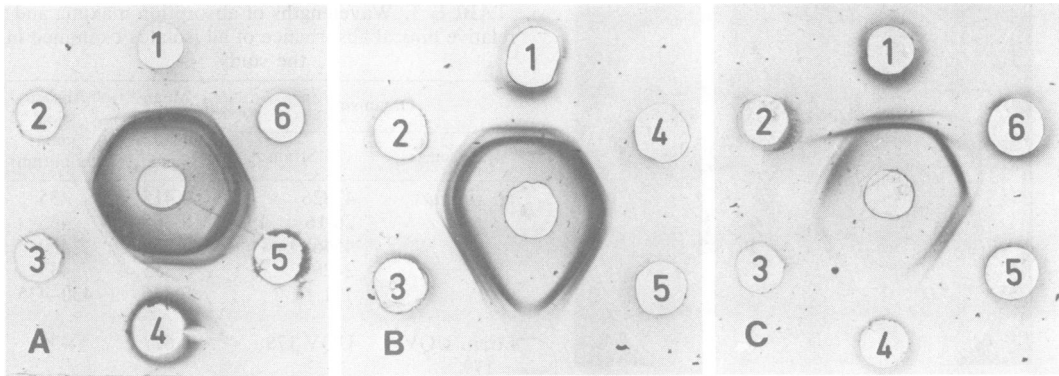


FIG. 2. Gel immunodiffusion reactions of all bacteria (peripheral wells) with antiserum prepared against *H. somnus* 43826 (central wells). (A) *H. somnus* 43826 (well 1), 26-16 (well 2), 45468 (well 3), 111 (well 4), and 2426 (well 6); and strain UQV 179 (well 5). (B) *H. somnus* 43826 (well 1); *H. agni* 1344 (well 2); and *Histophilus ovis* 9L (well 3), 43803 (well 4), and H989 (well 5). (C) *H. somnus* 43826 (well 1); *A. seminis* ATCC 15768 (well 2); *A. lignieresii* ATCC (well 3); *H. influenzae* ATCC (well 4); *A. seminis* (well 5); and *H. haemoglobinophilus* ATCC (well 6).

ative pleomorphic bacilli that ferment glucose, are oxidase positive and catalase negative, and reduce nitrate. Most strains grow well only in media containing blood, tissue extracts, or TMP, and they grow only in an atmosphere of increased CO₂ tension.

The demonstration of multiple antigens common to all bacteria in the HH group together with similar but not identical antigens, characterized in some cases by immunodiffusion lines of partial identity, indicates that, although bacteria of the HH group possess common antigens, a degree of antigenic variability occurs among strains. Common and heterologous antigens have been described for *H. somnus* (7, 9, 10), with antigenic diversity related to geographical distribution and to colonial variants. In the absence of other features, antigenic typing might be one means of characterizing organisms within the HH group. Antiserum against only one bacterium of the HH group was used in immunodiffusion tests in the present study. Further cross-absorption studies are needed to determine the degree of serological similarity among members of the HH group. Immunodiffusion tests also detected an antigen common to *H. haemoglobinophilus*, *A. seminis*, and the HH group, indicating that these bacteria may be distantly related. Support for this suggestion comes from the observation that *H. haemoglobinophilus*, *A. seminis*, *Histophilus ovis*, and *H. somnus* all inhabit the reproductive tract. A cross-absorption study has shown that *A. seminis* and *H. ovis* share a portion of their antigens (33); this agrees with the present study. Antigenic cross-reactivity among *H. haemoglobinophilus*, *A. seminis*, and bacteria of the HH group has not been previously reported.

The uniformity of the bacteria in the HH

group was further demonstrated by the similarity of their CE protein profiles in SDS-PAGE. The use of this test was based on the observation that SDS-PAGE produces numerous protein bands in common in bacteria of the same species (24). Corboz and Wild (10) examined soluble proteins of European isolates of *H. somnus* by SDS-PAGE and suggested that an antigenic protein with a molecular weight of 25,600 was specific for *H. somnus*. Allowing for technical variation, this protein is probably analogous to the matrix protein with a molecular weight of 28,000 (Fig. 3). The distinct difference between the CE protein profiles of *A. seminis* and the HH group further emphasizes the difference between these bacteria.

The ability of *H. somnus* to produce indole has been used to differentiate it from *H. agni* and *Histophilus ovis* (5), but in that report *Histophilus ovis* (35) was incorrectly described as indole negative. In the present study, 6 of the 10 bacteria of the HH group produced indole, with 5 of the 6 bovine isolates and 1 of 4 ovine isolates being indole positive. Garcia-Delgado et al. (13) observed only 2 of 68 bovine isolates of *H. somnus* to be indole negative, but Corboz and Wild (10) reported approximately one-third of 125 bovine isolates of *H. somnus* to be indole negative. Rahaley and White (34) found 9 of 10 ovine strains of *Histophilus ovis* to be indole positive. In the future, the indole reaction may be used to subdivide the HH group, but at present we can see no justification for this.

Pigmentation was bright lemon yellow in all the strains of *H. somnus* and *H. agni* and pale cream-yellow in the strains of *Histophilus ovis*. *H. somnus* and *H. agni* had different amounts of pigment. The quantitative differences found in this study confirm reports of pigment variation

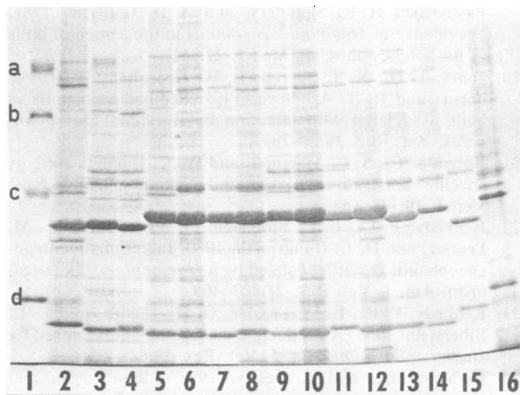


FIG. 3. Comparison of CE protein profiles of all strains by SDS-PAGE. Lane 1, Molecular weight standards: (a) Phosphorylase *b*, 92,500; (b) bovine serum albumin, 66,000; (c) carbonic anhydrase, 31,000; (d) soybean trypsin inhibitor, 21,000. Lane 2, *H. haemoglobinophilus* ATCC 19416; lane 3, *A. seminis* ATCC 15768; lane 4, *A. seminis*; lane 5, *H. agni* 1344; lanes 6 through 8, *Histophilus ovis* 9L, H989, and 43803, respectively; lane 9, strain UQV 179; lanes 10 through 14, *H. somnus* 111, 2426, 45468, 26-16, and 43826, respectively; lane 15, *H. influenzae* ATCC 19418; lane 16, *A. lignieresii* ATCC 19393.

in bovine strains in *H. somnus* in Europe (9) and suggest another means of differentiating among bacteria in the HH group. The pale yellow of *Histophilus ovis* could be readily differentiated from the white-grey pigmentation of *A. seminis*.

Growth response to the addition of TMP to broth or solid media was obvious in all bacteria of the HH group except *H. somnus* 2426. In their original description, Asmussen and Baugh (1) noted a response to TMP in all 10 isolates of *H. somnus* studied. However, strain 2426 was forwarded to us as an *H. somnus*-like strain with less stringent growth requirements. It has been referred to as a "fastidious *Pasteurella*" (5). Our finding that strain 2426 can grow in the absence of TMP helps to explain its less fastidious growth requirements. Strain 2426 was identical in all other respects to the other bacteria of the HH group. Thus, before TMP dependence can be of any value in identifying the HH group, the proportion of the population that does not possess this trait must be determined.

The rapid growth induced by TMP allowed reading of the nitrate, indole, and H₂S tests after overnight incubation. Previously, biochemical testing of *H. somnus* (13, 37) and *Histophilus ovis* (34) has been performed in media containing serum, with incubation periods of 3 to 24 days. Generally, the results of our nitrate, indole, and H₂S tests agreed with reactions reported by earlier investigators (13, 34, 37). Attempts to use PP broth as a basal medium for fermentation studies were not successful because some

strains were unable to grow in the absence of glucose. Further work is needed to develop a defined growth medium in which the fermentation reactions of bacteria of the HH group can be studied.

Previous investigations have shown that *H. somnus* exhibits satellitism around staphylococcal colonies on blood-free media (13, 22, 37). Satellitism was only observed in 6 of our 10 HH group strains, although the basal medium used may have supplied enough growth factor for some strains. The porphyrin test showed that the required growth factor was not hemin, even though *Histophilus ovis* 43803 did show a growth response to hemin. In addition, none of the test isolates responded to factor V. Therefore, the staphylococci probably provide thiamine derivatives, but it remains to be determined whether other growth factors are also supplied.

A significant finding of this investigation was that the bacterium known as *H. somnus* in North America and Europe is similar if not identical to the bacterium referred to as *Histophilus ovis* in Australia and New Zealand. Also, a bacterium identified as *A. actinoides*, isolated from bovine pneumonia in Australia, is identical to *H. somnus*. In view of the recent findings of Gourlay et al. (14), *A. actinoides* and *H. somnus* must be considered as different species. As far as the authors are aware, bovine thromboembolic meningoencephalitis has not been reported from Australia or New Zealand, and ovine *Histophilus ovis* and *H. agni* infections are rarely recorded in North America. Further investigation of the differences between ovine and bovine isolates of HH group bacteria may provide important clues about host susceptibility to these bacteria and bacterial virulence determinants.

The bacteria placed in the HH group have a sufficiently high degree of phenotypic similarity to suggest they are all one species. Marked biotypic differences in virulence between *H. somnus* 43826 and 26-16 have been described (17), and the existence of biotypes may explain differences in antigenicity, indole production, and pigmentation. Confirmation of these suggestions and the establishment of the relationship between the HH group and *A. seminis* requires a study of the genotypes of these bacteria. The taxonomic position of the bacteria of the HH group remains unclear, but they do not qualify for inclusion in the genus *Haemophilus* as presently defined (48) because of their independence of X and V factors. The bacteria of the HH group may best be accommodated in a new genus.

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