Evidence of Host-Specific Subgroups among “*Histophilus ovis*” Isolates

C. KIRKHAM,* E. L. BIBERSTEIN, AND R. B. LEFEBVRE

Department of Veterinary Microbiology and Immunology, School of Veterinary Medicine,
University of California, Davis, California 95616

An investigation of 20 bovine and 24 ovine isolates of organisms variously described as “*Histophilus ovis*,” “*Haemophilus agni*,” and “*Haemophilus somnus*” revealed no consistent host-related differences in 47 biochemical characteristics; however, agarose gel electrophoresis of predominantly plasmid-derived deoxyribonucleic acid demonstrated the presence of variable numbers of plasmids of divergent size in each of the ovine strains but in none of the bovine isolates. Restriction enzyme analysis of deoxyribonucleic acids from an ovine culture and a bovine culture also suggested chromosomal differences. Our observations support the concept of one species, *Histophilus ovis*, comprising ovine and bovine isolates, but point to the existence of subspecific divisions related to host predilection.

Between 1956 and 1960 several fastidious gram-negative organisms were named as causes of disease in ruminants. One, “*Histophilus ovis*” (23), was associated with suppurative mastitis in sheep; another, “*Haemophilus agni*,” was associated with septicemic disease of lambs (13). A third, now commonly called “*Haemophilus somnus*” (12; W. E. Bailie, Ph.D. thesis, Kansas State University, Manhattan, 1969), was the agent of bovine septicemia and meningoencephalitis. The use of these three names, based largely on strain origin and local preferences, has persisted despite the demonstration of the close phenotypic resemblance of these agents to one another and the results of deoxyribonucleic acid (DNA)-DNA homology tests which showed them to be, by current standards (27), members of one species. By the principle of priority this species should be named “*Histophilus ovis*,” once the genus “*Histophilus*” has received a workable phenotypic definition (1, 20, 25, 26).

There remain suggestions of subgroups within the species, related to host derivation. Although a serological study, in which an enzyme-linked immunosorbent assay was used, confirmed cross-reactivity between “*Haemophilus agni*” and “*Haemophilus somnus*,” it also revealed significant differences in titers between homologous and heterologous reactions (3), and the homology percentages in hybridization tests between “*Haemophilus somnus*” DNA and “*Histophilus ovis*” or “*Haemophilus agni*” DNA were significantly lower than the homology percentages among DNA preparations obtained from “*Haemophilus somnus*” strains (26).

In this study we searched for other subspecific distinctions between bovine and ovine isolates belonging to the genus “*Histophilus*.”

MATERIALS AND METHODS

Bacterial cultures. The geographic, zoological, and anatomic-pathological origins of the strains used are shown in Tables 1 and 2. While they were under active study, the agents were maintained on chocolate agar plates (G-C base, bovine hemoglobin; Remel, Lenexa Kans.). Incubation was carried out under 5% carbon dioxide in a CO₂ incubator (Napco, Portland, Oreg.).

The species designations under which the cultures were

received were accepted as long as the cultures consisted of gram-negative pleomorphic coccobacilli that were approximately 0.3 to 1 by 0.5 to 1.2 µm and grew optimally on sheep blood or chocolate agar under 5 to 10% carbon dioxide to a colonial size of 1 to 2 mm in 48 h. The colonies were convex, moist, smooth, glistening, butyrous, and, on chocolate agar, distinctly yellow.

Differential tests. Acid production from carbohydrates was determined in phenol red broth (Difco Laboratories, Detroit, Mich.) containing substrate at a concentration of 1%. Incubation was at 37°C for 1 week in air.

Oxidase activity was tested on filter paper impregnated with tetramethyl-phenylenediamine (Ceptiseal; Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.) according to the directions of the supplier.

The catalase test was performed on slides by suspension of a bacterial colony in 3% hydrogen peroxide (Mallinckrodt, Inc., St. Louis, Mo.) and observation of the preparation for frothing.

Tests for arginine dihydrolase and lysine and ornithine decarboxylase were carried out in Møller medium (Difco), and tests for urease were carried out by the micromethod of Lautrop (15).

Indole production was determined by adding Kovacs reagent (9) to a 48-h mycoplasma broth culture (PPLO medium; Difco).

Reduction of nitrate to nitrite was determined by addition of sulfanilic acid and *N,N*-dimethyl-naphthylamine to a 48- to 72-h culture in 0.1% nitrate broth (Difco) (9).

Esculin broth containing ferric chloride indicator (6) was inoculated and incubated to determine esculin hydrolysis.

Enzyme profiles. Enzyme profiles of five strains of “*Histophilus ovis*,” five strains of “*Haemophilus agni*,” and five strains of “*Haemophilus somnus*” were prepared by use of an API-ZYM set in accordance with the directions of the supplier (Analytab Products, Plainview, N.Y.).

Plasmid demonstration. Strains were propagated on chocolate agar for 48 to 72 h, and plasmids were isolated from the cells by the colony lysis method of Kado and Liu (11). The purified plasmids were characterized by agarose gel electrophoresis (11). Following electrophoresis the gel was stained with ethidium bromide, illuminated by ultraviolet irradiation, and photographed. A plasmid control (*Pasteurella multocida* 1059-1) and a plasmid molecular weight standard (molecular weights, 1.8 × 10⁶ to 65 × 10⁶) were included.

* Corresponding author.

TABLE 1. Geographic origins of the isolates studied

Name as received	Location	No. of strains
" <i>Haemophilus somnus</i> "	California	9
	Colorado	6
	Oklahoma	2
	Switzerland	2
	New Zealand	1
" <i>Histophilus ovis</i> "	California	4
	Idaho	6
	Alberta, Canada	2
	Quebec, Canada	2
	Australia	1
" <i>Haemophilus agni</i> "	California	3
	Alberta, Canada	6

For demonstration of plasmid DNA in a cesium chloride-ethidium bromide gradient (8), cells were propagated for 24 h at 37°C in 100 ml of mycoplasma broth (PPLO broth; Difco) and harvested by centrifugation at $10,000 \times g$ for 20 min at 4°C. Subsequent preparatory steps followed the procedure of Guerry et al. (8). Centrifugation for separation of plasmid DNA from chromosomal DNA was done at $100,000 \times g$ for 48 h at 15°C. Gradients were scanned under an ultraviolet lamp.

Restriction enzyme analysis. Cells were grown in 100 ml of mycoplasma broth (PPLO broth; Difco) to a density of 10^7 to 10^8 cells per ml (late log phase) by overnight incubation at 37°C, and whole-cell DNA was isolated from 20 ml of this culture by using the method of LeFebvre et al. (16). Plasmid-enriched fractions were isolated by using the method of Birnboim (2). The purified DNA was digested with restriction endonucleases *EcoRI* and *HindIII*, which were purchased from Bethesda Research Laboratories, Inc., Bethesda Md., and were used according to the specifications of the manufacturer. The resulting DNA fragments were fractionated by gel electrophoresis in a 0.7% agarose gel (24.5 by 20 cm; Bethesda Research Laboratories) at 60 V for 15.5 h. The gel was stained with ethidium bromide, illuminated by ultraviolet irradiation, and photographed.

RESULTS

The 20 "*Haemophilus somnus*," 9 "*Haemophilus agni*," and 15 "*Histophilus ovis*" cultures which we studied were oxidase positive and catalase negative, decarboxylated ornithine, and reduced nitrates to nitrites. They were arginine dihydrolase, lysine decarboxylase, and urease negative and

TABLE 2. Anatomic origins of the isolates studied

Name as received	Anatomical area	No. of strains
" <i>Haemophilus somnus</i> "	Bovine lung	7
	Bovine brain	3
	Bovine trachea	2
	Bovine uterus	2
	Bovine vagina	5
	Bovine prepuce	1
	Unknown	1
" <i>Histophilus ovis</i> "	Ovine vagina	2
	Ovine prepuce	4
	Ovine epididymis	8
	Unknown	1
" <i>Haemophilus agni</i> "	Ovine brain	4
	Ovine lung	1
	Ovine spleen	2
	Ovine septicemia	4

FIG. 1. Agarose gel electropherogram of DNAs from representative strains of the "*Histophilus ovis*" group. Lane A, "*Haemophilus somnus*" M608; lane B, "*Haemophilus somnus*" 805; lane C, "*Histophilus ovis*" A; lane D, "*Histophilus ovis*" 642A; lane E, "*Histophilus ovis*" 3384Y; lane F, "*Haemophilus agni*" 6; lane G, "*Haemophilus agni*" 902; lane H, molecular mass standards. All bacterial lanes (lanes A through G) have a diffuse chromosomal zone (arrow). DNAs from strains of ovine origin (lanes C through G) in addition have one or more plasmid bands in various locations. There are no plasmid bands in the preparations derived from bovine cultures (lanes A and B). MDa, Megadalton.

did not hydrolyze esculin; 18 of 20 "*Haemophilus somnus*" strains and all 15 "*Histophilus ovis*" isolates produced indole, but only two of nine "*Haemophilus agni*" strains produced indole ($P < 0.01$; Fisher test).

All strains produced acid from fructose, glucose, mannose, sorbitol, and xylose and no change in adonitol, arabinose, cellobiose, dextrin, dulcitol, lactose, melibiose, raffinose, rhamnose, salicin, and trehalose. Inositol was acidified by only 2 of 20 "*Haemophilus somnus*" cultures, while maltose was fermented by 3 "*Haemophilus somnus*" strains and 2 of 15 "*Histophilus ovis*" isolates. Mannitol was fermented by all but three isolates (all "*Haemophilus somnus*").

With respect to the enzyme profiles, the five test strains belonging to each host species gave positive reactions for alkaline phosphatase, esterase (C_4), esterase-lipase (C_8), leucine aminopeptidase, acid phosphatase, phosphoamidase (except one "*Histophilus ovis*" culture), and beta-glucuronidase. None was positive for lipase (C_{14}), cystine aminopeptidase, trypsin, chymotrypsin, alpha- and beta-galactosidases, alpha- and beta-glucosidases, beta-glucosaminidase, alpha-mannosidase, or alpha-fucosidase. One "*Haemophilus somnus*" culture and one "*Histophilus ovis*" culture were feebly positive for valine aminopeptidase.

No plasmids were found in any bovine isolate ($n = 20$), while from one to six plasmids ranging in molecular weight from 2×10^4 to 185×10^4 were demonstrable in all cultures of ovine origin ($n = 24$) (Fig. 1). No single plasmid (as judged by molecular mass) was present in all ovine strains. Interestingly, an ovine strain reaching us under the label "*Haemophilus somnus*" contained plasmids.

FIG. 2. Chromosomal and plasmid DNAs from "*Haemophilus somnus*" and "*Haemophilus agni*" digested with either *Hind*III or *Hind*III and *Eco*RI, fractionated on a 0.7% agarose gel, stained with ethidium bromide, illuminated with ultraviolet irradiation, and photographed. Lane A, *Hind*III digest of "*Haemophilus somnus*" chromosomal DNA; lane B, *Hind*III digest of "*Haemophilus agni*" chromosomal and plasmid DNAs; lane C, *Hind*III digest of "*Haemophilus agni*" plasmid DNA; lane D, *Hind*III-*Eco*RI digest of "*Haemophilus somnus*" chromosomal DNA; lane E, *Hind*III-*Eco*RI digest of "*Haemophilus agni*" chromosomal and plasmid DNAs; lane F, *Hind*III-*Eco*RI digest of "*Haemophilus agni*" plasmid DNA.

Comparative cesium chloride gradients of a bovine strain (strain 805) and an ovine strain (strain M642) revealed a satellite band suggestive of plasmid DNA in the ovine preparation but not in the bovine preparation.

Figure 2 shows the results of restriction enzyme analysis of DNAs from a bovine strain (strain 805) and an ovine strain (strain 902). The plasmid bands are readily visible in the genomic lanes of both the *Hind*III and *Eco*RI-*Hind*III digests of the ovine-derived DNA. This figure shows a high degree

of similarity between the banding patterns of "*Haemophilus somnus*" and "*Haemophilus agni*" DNAs for these digests, although differences were detectable in the higher-molecular-weight range. Similar results were observed with *Pst*I and *Hha*I preparations (data not shown).

DISCUSSION

Our observations on biochemical characteristics reveal no differentiating patterns among the three groups of cultures reaching us under the labels "*Haemophilus somnus*," "*Haemophilus agni*," and "*Histophilus ovis*." The apparent lack of indole production among "*Haemophilus agni*" cultures (two of nine strains) may be due to the origin of three isolates (13) from one source and five isolates from another (18), so that the nine cultures of "*Haemophilus agni*" may represent perhaps no more than three strains.

Among the nearly 500 strains which have been characterized by differential tests and reported in the literature (1, 4, 5, 7, 10, 14, 17, 18, 21-25, 28), variability has been noted in practically all of the key biochemical and cultural features that have been described. These include oxidase (28) and catalase tests (1, 5), raised carbon dioxide requirement (1, 26), hemolytic activity (5, 22), indole production (1, 5, 14, 18, 20, 25), decarboxylase tests (7, 21), reduction of nitrate (5), esculin hydrolysis (21, 22), and acid production from carbohydrates (24). These variations often appear to be related to strain origin by host species. However, differences between laboratory methodologies and in interpretive criteria are likely contributing factors. The question of host-related differences deserves further critical study.

While restriction enzyme analysis of chromosomal DNA revealed a general overall pattern similarity (19), differences between a strain of bovine origin and a strain of ovine origin in fragment distribution were seen at the higher-molecular-weight range. The relationship of these differences to host specificity requires confirmation with additional strains, but the presence of plasmids in ovine isolates appears to constitute a uniform mark of distinction between the two groups of strains. The consistent finding of extrachromosomal DNA in ovine strains from widely separated areas on three continents further supports the distinctness of the two populations ($P < 0.001$; Yates chi-square test), which was also previously suggested by the apparent host-specific pathogenicity of the organisms (12), as well as by serological and DNA-DNA hybridization data (20, 26). Beyond that, there are puzzling questions regarding the function and significance of the plasmids, particularly in view of their diversity in size and number. Future studies, involving attempts at curing ovine strains and cloning of plasmids, may shed some light on the significance of these plasmids and their selective occurrence.

Meanwhile, our studies have confirmed and extended previous observations leading to the conclusion that "*Histophilus ovis*," "*Haemophilus agni*," and "*Haemophilus somnus*" are members of one species, *Histophilus ovis*, although with apparently host-specific subdivisions, which may eventually merit subspecific recognition.

ACKNOWLEDGMENTS

We gratefully acknowledge the kind cooperation of the following individuals, who contributed cultures to this study: R. E. Corstvet, P. F. Frelser, R. Higgins, A. B. Hoerlein, A. Julian, P. B. Little, J. Nicolet, S. Ostrowski, and R. L. Walker. We are indebted to Lori Hansen for much helpful advice in connection with the plasmid studies and their interpretation.

LITERATURE CITED

1. Biberstein, E. L. 1981. *Haemophilus somnus* and *Haemophilus agni*, p. 125-132. In M. Kilian, W. Frederiksen, and E. L. Biberstein (ed.), *Haemophilus, Pasteurella, Actinobacillus*. Academic Press, Inc. (London), Ltd., London.
2. Birnboim, H. C. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.* **100**:243-255.
3. Canto, J., E. L. Biberstein, T. A. Schulte, and D. Behymer. 1983. Cross-reactivity of *Haemophilus somnus* antibody in agglutination and complement fixation tests and in the enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **17**:500-506.
4. Claxton, P. D., and R. E. Everett. 1966. Recovery of an organism resembling *Histophilus ovis* in a ram. *Aust. Vet. J.* **42**:457-458.
5. Corboz, L., and J. Nicolet. 1975. Infektionen mit sogenanntem *Haemophilus somnus* beim Rind: Isolierung und Charakterisierung von Stämmen aus Respirations- und Geschlechtsorganen. *Schweiz. Arch. Tierheilkd.* **117**:493-503.
6. Cowan, S. T., and K. J. Steel. 1965. Manual for the identification of medical bacteria, p. 112. University Press, Cambridge.
7. Garcia-Delgado, G. A., P. B. Little, and D. A. Barnum. 1977. A comparison of various *Haemophilus somnus* strains. *Can. J. Comp. Med.* **41**:380-388.
8. Guerry, P., J. VanEmbsden, and S. Falkow. 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. *J. Bacteriol.* **117**:619-630.
9. Hendrickson, D. A. 1985. Reagents and stains, p. 1093. In E. H. Lennette, A. Balows, W. J. Hausser, and H. J. Shadomy (ed.), *Manual of clinical microbiology*, 4th ed. American Society for Microbiology, Washington, D.C.
10. Higgins, R., F. Godbout-DeLasalle, S. Messier, Y. Couture, and P. Lamothe. 1981. Isolation of *Histophilus ovis* from vaginal discharges in ewes. *Can. Vet. J.* **22**:395-396.
11. Kado, C. I., and S.-T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **145**:1365-1373.
12. Kennedy, P. C., E. L. Biberstein, J. A. Howarth, L. M. Frazier, and D. L. Dungworth. 1960. Infectious meningoencephalitis in cattle, caused by a *Haemophilus*-like organism. *Am. J. Vet. Res.* **21**:403-409.
13. Kennedy, P. C., L. M. Frazier, G. H. Theilen, and E. L. Biberstein. 1958. A septicemic disease of lambs caused by *Haemophilus agni* (new species). *Am. J. Vet. Res.* **19**:645-654.
14. Kiupel, H., and I. Prehn. 1986. Zur *Haemophilus somnus*-Infektion des Rindes—Bakteriologische Untersuchungsergebnisse unter besonderer Berücksichtigung von Abortussubstraten. *Arch. Exp. Veterinaermed.* **40**:164-169.
15. Lautrop, H. 1960. Laboratory diagnosis of whooping-cough or *Bordetella* infections. *Bull. W. H. O.* **23**:15-31.
16. LeFebvre, R. B., J. W. Foley, and A. B. Thiermann. 1985. Rapid and simplified protocol for isolation and characterization of leptospiral chromosomal DNA for taxonomy and diagnosis. *J. Clin. Microbiol.* **22**:606-608.
17. Linneweber, B., R. Weiss, and T. Schliesser. 1986. Bakteriologische Untersuchungen über Vorkommen, Biochemie, und Antibiotika-Resistenz von *Haemophilus somnus* (spec. incertae sed) bei Rindern. *Berl. Muench. Tierarztl. Wochenschr.* **99**:221-225.
18. Lundberg, M. S. 1986. Isolation of *Haemophilus agni* from six Alberta ram lambs with septicemia. *Can. Vet. J.* **27**:501-503.
19. McGillivray, D. J., J. J. Weber, and H. F. Dean. 1987. Characterization of *Histophilus ovis* and related organisms by restriction endonuclease analysis. *Aust. Vet. J.* **63**:389-393.
20. Piechulla, K., R. Mutters, S. Burbach, R. Klussmeier, S. Pohl, and W. Mannheim. 1986. Deoxyribonucleic acid relationships of "*Histophilus ovis*/*Haemophilus somnus*," *Haemophilus haemoglobinophilus*, and *Actinobacillus seminis*. *Int. J. Syst. Bacteriol.* **36**:1-17.
21. Rahaley, R. S., and W. E. White. 1977. *Histophilus ovis* infection in sheep in Western Victoria. *Aust. Vet. J.* **53**:124-127.
22. Rauth, S., W. Bisping, and G. Kirpal. 1986. Bakteriologische Untersuchungen über die Verbreitung von *Haemophilus somnus* im Genitaltrakt von Rindern. *Berl. Muench. Tierarztl. Wochenschr.* **99**:405-408.
23. Roberts, D. S. 1956. A new pathogen from a ewe with mastitis. *Aust. Vet. J.* **32**:330-332.
24. Shigidi, M. A., and A. B. Hoerlein. 1970. Characterization of the *Haemophilus*-like organism of thromboembolic meningoencephalitis of cattle. *Am. J. Vet. Res.* **31**:1017-1022.
25. Stephens, L. R., J. D. Humphrey, P. B. Little, and D. A. Barnum. 1983. Morphological, biochemical, antigenic, and cytochemical relationships among *Haemophilus somnus*, *Haemophilus agni*, *Haemophilus haemoglobinophilus*, *Histophilus ovis*, and *Actinobacillus seminis*. *J. Clin. Microbiol.* **17**:728-737.
26. Walker, R. L., E. L. Biberstein, R. F. Pritchett, and C. Kirkham. 1985. Deoxyribonucleic acid relatedness among "*Haemophilus somnus*," "*Haemophilus agni*," "*Histophilus ovis*," "*Actinobacillus seminis*," and *Haemophilus influenzae*. *Int. J. Syst. Bacteriol.* **35**:46-49.
27. Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int. J. Syst. Bacteriol.* **37**:463-464.
28. Webb, R. F. 1983. Bacteriological characteristics of *Histophilus ovis* and its relationship to similar bacteria. *Res. Vet. Sci.* **35**:25-29.