STUDIES ON THE GRAM-NEGATIVE PLEOMORPHIC BACTERIA ASSOCIATED WITH EPIDIDYMITIS IN RAMS

by

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INTRODUCTION

The nonenteric, Gram-negative, facultatively anaerobic bacteria have been referred to as the "step-children of medical bacteriology" (Kilian et al., 1981). Implied in that terminology is a certain degree of neglect, and the result in some cases is a confusing nomenclature based more on circumstance than valid taxonomic criteria.

Included among these "step-children" are the organisms of interest in this thesis: Actinobacillus seminis (Baynes and Simmons, 1960), Histophilus ovis (Roberts, 1956), Haemophilus somnus (Bailie, 1969), and Haemophilus agni (Kennedy et al., 1958). These nomenspecies, referred to here as the AHH group, are significant because all are associated with ovine genital disease (Zeki et al., 1981; Bulgin and Anderson, 1983; Walker et al., 1986), and all exhibit confusing bacteriological and serological similarities (Webb, 1983a; Stephens et al., 1983; Erasmus, 1983). Indeed, it is generally accepted that they do not belong in their assigned genera (Kilian and Biberstein, 1984; Phillips, 1984), and their similarities may warrant inclusion in a common taxonomic group (Webb, 1983a; Stephens et al., 1983; Walker et al., 1985; Piechulla et al., 1986).

Aside from the obvious need for a consistent and systematic taxonomic method, precise characterization of bacterial pathogens is the logical prerequisite to developing reliable diagnostic protocols and

effective control measures. These objectives will remain elusive until the relationships existing among the AHH group bacteria are clarified. Such clarification was the purpose of this study. Specifically, the objectives were to:

1) Define the bacteriological characteristics of available strains of the AHH group bacteria;

2) Examine their taxonomic relationships by comparison of protein profiles obtained by electrophoretic fractionation of soluble cell extracts and;

3) Examine their serological relationships by Western immunoblotting of electrophoretically separated fractions.

I. REVIEW OF THE LITERATURE

THE AHH GROUP BACTERIA

This work originated with an interest in the etiology and diagnosis of ovine epididymitis (Rahaley et al., 1983). Brucella ovis is the primary etiologic agent (Burgess, 1982), but many other organisms have been implicated (Table 1). Most, however, are not considered specific causative agents (Bulgin and Anderson, 1983), and it has been suggested that "three species or groups of organisms (Br. ovis, gram-negative pleomorph, and Actinobacillus spp.) appear to be associated predominantly with ram epididymitis" (Ekdahl et al., 1968).

The significance of the non-Brucella species is well established (Burgess, 1982). In fact, an association between etiological agent and reproductive status suggests ovine epididymitis should be considered two diseases (Bulgin and Anderson, 1983; Bagley et al., 1985). The classic form caused by B. ovis occurs as an enzootic infection in mature breeding rams, is most common in range flocks where a large number of rams are continually maintained, and is associated with clinically normal carriers. In contrast, the second form occurs most commonly in ram lambs, and is caused by one or more "gram-negative pleomorphic rods" (GNPR's) that appear to be a transient part of the ovine genital flora (Walker and LeaMaster, 1986). Disease occurs as an ascending infection of the urogenital tract, and may be related to stress or hormonal status (Jansen, 1980; Walker et al., 1986). This second form has been called lamb epididymitis to distinguish it from the disease in mature rams (Bagley et al., 1985).

Despite numerous reports in the literature, the identity of GNPR isolates remains uncertain. Among the more common are Actinobacillus seminis (Baynes and Simmons, 1960; Van Tonder, 1979a,b; Walker et al., 1986) and Histophilus ovis (Dodd and Hartley, 1955; Claxton and Everett, 1966; Rahaley, 1977; Walker et al., 1986), but these species are not well-defined, and differentiation can be difficult (Hughes et al., 1971; Dennis, 1974; Rahaley and White, 1977). In general, Hist. ovis is more active biochemically than A. seminis (Rahaley, 1977), but the distinction is unclear because both are relatively inactive in standard bacteriological tests, and the degree of similarity is based largely on negative characteristics (Hughes et al., 1971). These difficulties are compounded by serological cross-reactivity (Rahaley, 1978; Webb, 1983a; Stephens et al., 1983), and have led to the suggestion that A. seminis and Hist. ovis are biochemical variants of the same species (Bruere et al., 1977; Webb, 1983a).

Phenotypic similarities have also been noted between Hist. ovis, Haemophilus somnus, and Haemophilus agni (Higgins et al., 1981; Webber et al., 1983; Webb, 1983a; Low and Graham, 1985). Although most commonly associated with other diseases (Humphrey and Stephens, 1983), H. somnus and H. agni have been isolated from rams with epididymitis (Zeki et al., 1981; Bulgin and Anderson, 1983), and Stephens et al. (1983) stated they "prefer to refer to these bacteria collectively rather than attempt to separate them into three species". Thus, they defined the "HH group", consisting of the "species" Hist. ovis, H. somnus, and H. agni, as "yellow-pigmented, gram-negative pleomorphic

bacilli that ferment glucose, are oxidase positive and catalase negative, and reduce nitrates". They also noted that most strains grow well only on enriched media in an atmosphere of increased carbon dioxide tension.

Actinobacillus seminis was excluded from the "HH group" by its production of catalase, lack of yellow pigment, less exacting growth requirements, and different cell envelope protein profile (Stephens et al., 1983). However, A. seminis isolates have been identified that were catalase negative (Sponenberg et al., 1983; Van Tonder, 1979c), yellow pigmented (Phillips, 1984), and dependent on serum or blood enrichment for optimal growth (Van Tonder, 1979c). The issue is furthur confused by serological cross-reactivity between A. seminis and species of the "HH group" (Rahaley, 1978; Stephens et al., 1983; Webb, 1983a).

The taxonomic relationships among these organisms have yet to be resolved. The questions that remain are difficult to answer in the absence of type strains for the "HH group", however, some strains of Hist. ovis, H. somnus, and H. agni have DNA homology levels high enough to belong to a single species (Walker et al., 1985). Although the ATCC[1] strain of A. seminis (#15768) has little DNA homology with most "HH group" strains (Walker et al., 1985; Piechulla et al., 1986), significant levels have been reported between it and one strain of H. agni (Piechulla et al., 1986).

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[1]American Type Culture Collection, Rockville, MD

Regardless of their relationships with each other, none of the AHH group bacteria belong in their assigned genera. Bergey's Manual of Systematic Bacteriology (1984) states that, while the correct taxonomic placement of A. seminis is unknown, it does not belong in the genus Actinobacillus (Phillips,1984). Likewise, both H. somnus and H. agni are currently listed as "species incertae sedis" in the genus Haemophilus, but it is noted that "by current standards", they do not qualify for inclusion (Kilian and Biberstein, 1984). Their similarity to Hist. ovis is noted, but at present the genus Histophilus has received no official recognition.

Despite these statements, the names Actinobacillus seminis, Histophilus ovis, Haemophilus somnus, and Haemophilus agni have persisted in the literature. For that reason, the remainder of this review will consider each as a distinct species.

TABLE 1. Bacterial isolates associated with genital disease in rams

Isolate	Reference
Acinetobacter sp.	Jansen, 1980
Acinetobacter lwoffi	Bulgin and Anderson, 1983
Actinobacillus sp.	Ekdahl *et al.*, 1968
Actinobacillus actinomycetemcomitans	DeLong *et al.*, 1979
Actinobacillus lignieresii	Laws and Elder, 1969
"*Actinobacillus ovis*"	Bulgin and Anderson, 1983
Actinobacillus seminis	Baynes and Simmons, 1960
Alcaligenes sp.	Jansen, 1980
Alcaligenes faecalis	Bulgin and Anderson, 1983
Bacteroides sp.	Ekdahl *et al.*, 1968
Bacillus sp.	Bagley *et al.*, 1985
Brucella abortus	Ekdahl *et al.*, 1968
Brucella ovis	Burgess, 1982
Corynebacterium ovis	Krishna *et al.*, 1977
Corynebacterium pseudotuberculosis	DeLong *et al.*, 1979
Corynebacterium pyogenes	Watt, 1978
Erysipelothrix rhusiopathiae	Bulgin and Anderson, 1983
Escherichia coli	Mason *et al.*, 1982
Flavobacterium sp.	Jansen, 1980
Francisella sp.	Jansen, 1980
"Gram-negative pleomorph"	Bruere *et al.*, 1977
Haemophius agni	Zeki *et al.*, 1981
Haemophilus parahaemolyticus	Bulgin and Anderson, 1983
Haemophilus parasuis	Bulgin and Anderson, 1983
Haemophilus somnus	Bulgin and Anderson, 1983
Haemophilus suis	Bulgin and Anderson, 1983
Histophilus ovis	Walker *et al.*, 1986

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TABLE 1. continued

Isolate	Reference
Lactobacillus sp.	Jansen, 1980
Micrococcus luteus	Jansen, 1980
Micrococcus varians	Jansen, 1980
Moraxella osloensis	Bulgin and Anderson, 1983
Moraxella nonliquefaciens	Bulgin and Anderson, 1983
Moraxella phenylpyruvica	Bulgin and Anderson, 1983
Pasteurella haemolytica	Jansen, 1980
Pasteurella multocida	Ekdahl *et al.*, 1968
Pseudomonas sp.	Jansen, 1980
Pseudomonas maltophilia	DeLong *et al.*, 1979
Pseudomonas pseudomallei	Bulgin and Anderson, 1983
Salmonella arizonae	Bulgin and Anderson, 1983
Staphylococcus sp.	Bagley *et al.*, 1985
Staphylococcus aureus	Walker *et al.*, 1986
Staphylococcus epidermidis	Jansen, 1980
Streptobacillus sp.	Jansen, 1980
Streptococcus sp.	Walker *et al.*, 1986
Streptococcus bovis	Jansen, 1980

ACTINOBACILLUS SEMINIS

Recognition of the Species. Actinobacillus seminis was first isolated in Australia from the semen of rams with epididymal lesions (Baynes and Simmons, 1960). Subsequent isolations in Australia (Simmons et al., 1966a,b), the United States (Livingston and Hardy, 1964) and South Africa (Worthington and Bosman, 1968; Van Tonder and Bolton, 1968) confirmed its etiologic role in the disease; it could be used experimentally to cause epididymitis (Baynes and Simmons, 1960; Van Tonder and Bolton, 1968), and a rise in antibody titer could be demonstrated to both autologous antigen and antigen derived from the original isolate (Livingston and Hardy, 1964; Worthington and Bosman, 1968).

Etiology. Actinobacillus seminis is one of the most common causes of ovine epididymitis, especially in immature rams (Burgess, 1982; Bulgin and Anderson, 1983; Healey et al., 1985; Walker et al., 1986). Isolates have been reported in Australia (Baynes and Simmons, 1968), New Zealand (Ekdahl et al., 1968; Bruere et al., 1977), South Africa (Van Tonder, 1973; Van Tonder, 1979a,b; Erasmus, 1983; Swanepoel, 1984) and the United States (Bulgin and Anderson, 1983; Rahaley et al., 1983; Sponenberg et al., 1983; Bagley et al., 1985; Walker et al., 1986).

Its pathogenesis and predominance in ram lambs is unclear. Walker and LeaMaster (1986) isolated A. seminis from the preputial cavity of

34% of rams and the vagina of 88% of ewes under 6 months old. It was isolated only infrequently from mature sheep. It was suggested A. seminis was a transitory part of the ovine genital flora in both rams and ewes, and that the transitory nature of infection might account for the prevalence of A. seminis-associated epididymitis in immature rams. The correlation between isolation of A. seminis from the preputial cavity of ram lambs and development of epididymal lesions suggests rams develop ascending infection of the urogenital tract (Walker and LeaMaster, 1986).

Actinobacillus seminis has also been associated with perinatal deaths (Hughes et al., 1971; Dennis, 1974), synovitis and septicemia (Rahaley and White, 1977) and polyarthritis (Watt et al., 1970) of lambs. Infections in ewes are usually subclinical (Simmons et al., 1966a; Walker and LeaMaster, 1986), but it has been isolated from aborting ewes and goat does (Van Tonder, 1973), and used experimentally to cause abortion (Baynes and Simmons, 1966; Smith and Hughes, 1974) and mastitis (Watt et al., 1970).

Isolations from species other than sheep are rare; it has been isolated from the semen of bulls with and without testicular lesions (Van Tonder and Bolton, 1970; Van Tonder, 1973; Dixon et al., 1983) and from the uterine discharge and fetal membranes of cows aborting or delivering stillborn calves (Van Tonder, 1979b; Dixon et al., 1983).

Description of the Organism. Actinobacillus seminis is difficult to identify, partly because it is relatively inactive in standard bacteriological tests (Hughes et al., 1971), and partly because of its fastidious nature and similarity to other Gram-negative pleomorphs (Rahaley, 1978; Bruere et al., 1977; Webb, 1983a). As a result, there are reports referring to a "Gram-negative pleomorph" without assigning a specific classification (Hughes et al., 1971; Dennis, 1974; Bruere et al., 1977). Others refer to isolates as "Actinobacillus-like" (Ekdahl et al., 1968) or "A. seminis-like" (Van Tonder, 1979a). Still others have identified isolates as A. actinomycetemcomitans (DeLong et al., 1979; Bulgin and Anderson, 1983; Bagley et al., 1985), however, the latter were unlike the type culture of that organism (Bulgin and Anderson, 1983), and were probably A. seminis (Bagley et al., 1985; Healey et al., 1985).

Biotypes of A. seminis have also been reported, but some isolates may have been misidentified strains of Histophilus ovis (Van Tonder, 1979c). Erasmus (1983) compared strains of A. seminis, Hist. ovis, and A. actinomycetemcomitans and suggested they could all be included in the same genus but constituted at least four species. It was also suggested that most A. seminis strains were more properly classified as A. actinomycetemcomitans or Hist. ovis. Genotypic studies do not support that conclusion (Walker et al., 1986; Piechulla et al., 1986). It remains unclear whether the Actinobacillus sp. associated with lamb epididymitis constitute more than one species or more than one biotype

of the same species (Walker et al., 1986; Cardenas and Maki, 1986).

Actinobacillus seminis is a small, Gram-negative pleomorph ranging from coccobacillary to bacillary with occasional filamentous forms (Baynes and Simmons, 1960; Livingston and Hardy, 1964). It occurs singly, in pairs, short chains, or palisade formation (Van Tonder, 1979c). It is non-motile, non-acid fast, and does not form capsules or spores (Worthington and Bosman, 1968; Van Tonder, 1979c).

Growth occurs aerobically (Stephens et al., 1983), but is enhanced by increased carbon dioxide (Van Tonder, 1979c; Webb, 1983a). Anaerobic growth is sparse (Webb, 1983a). Slight growth may occur on ordinary nutrient media (Baynes and Simmons, 1960), but serum or blood enrichment is required for optimal growth, especially on primary isolation (Van Tonder, 1979c). Colonies are pin-point at 24 hours, increasing to 3-5 mm in 3-4 days (Baynes and Simmons, 1960; Livingston and Hardy, 1964; Van Tonder, 1979c). They are non-hemolytic, round, convex, entire, and gray-white (Baynes and Simmons, 1960). Larger colonies may become umbonate with a transparent periphery and undulate edge (Van Tonder, 1979c). Colonial variants that grow slower, are more dependent on media enrichment, and express a yellow pigment have been reported (Van Tonder, 1979c). Although these closely resembled Hist. ovis (Van Tonder, 1979c), Bergey's Manual of Systematic Bacteriology states that some strains of A. seminis are yellow pigmented (Phillips, 1984). It does not grow on MacConkey agar (Baynes and Simmons, 1960).

The reported biochemical characteristics of A. seminis are variable (Table 2). In general, it is catalase and oxidase positive (Stephens et al., 1983; Walker et al., 1986; Piechulla et al., 1986), although negative reactions have been reported (Van Tonder, 1979c; Dixon et al., 1983; Sponenberg et al., 1983; Webb, 1983a). Despite numerous reports to the contrary (Table 2), Bergey's Manual of Systematic Bacteriology states that most strains do not reduce nitrate (Phillips, 1984). Swanepoel (1984) found nitrate reduction was variable based on methodology. It does not decompose urea or produce indole (Baynes and Simmons, 1960); again, exceptions have been reported (Stephens et al., 1983; Erasmus, 1983). Slight production of H_2S has also been noted (Van Tonder, 1979c; Stephens et al., 1983). Actinobacillus seminis is consistently positive for ornithine decarboxylase and, with few exceptions (Mannheim et al., 1980), negative for lysine decarboxylase and arginine dihydrolase. Both positive (Piechulla et al., 1986) and negative (Walker et al., 1986) reactions have been reported for the production of beta-galactosidase. The Voges-Proskauer reaction is usually negative (Webb, 1983a; Phillips, 1984), but positive reactions have been reported for some strains (Erasmus, 1983).

Carbohydrate metabolism is fermentative (Stephens et al., 1983), but most reactions are weak or delayed (Baynes and Simmons, 1960; Livingston and Hardy, 1964). Some authors reported that A. seminis failed to ferment any of a number of carbohydrates (Worthington and Bosman, 1968; Van Tonder, 1979c), while others observed more active

metabolism under specific growth conditions (Mannheim et al., 1980; Webb, 1983a). Positive reactions have been reported for glucose, fructose, xylose, arabinose, galactose, maltose, mannitol, mannose, trehalose and sorbitol. The 8th edition of Bergey's Manual of Determinative Bacteriology (1974) excluded A. seminis from the genus Actinobacillus based on its inability to ferment carbohydrates. That criterion has since been removed (Phillips, 1984), and Walker et al. (1986) stated that "the fermentation of certain carbohydrates should not be used as a criterion to exclude the identification of an isolate as A. seminis".

Van Tonder (1973) reported serotypes among A. seminis strains, however, some strains may have been misidentified isolates of Hist. ovis (Van Tonder, 1979c). Antigenic similarities have been observed between A. seminis and Hist. ovis (Rahaley, 1978; Webb, 1983a). Cross-reactions have also been demonstrated with B. ovis (Rahaley, 1978), and H. somnus (Stephens et al., 1983).

Healey et al. (1985) produced a series of monoclonal antibodies to an "Actinobacillus-sp." isolated from rams with epididymal lesions; three were later shown to be specific for 10, 33, and 43 kilodalton proteins on the outer membrane (Healey et al., 1986). Cross-reactivity with other Actinobacillus isolates from ovine genital disease and with A. actinomycetemcomitans was reported.

The phenotypic confusion surrounding A. seminis is reflected in the few genomic studies reported. Gumbrell and Smith (1974) found

the mol % guanine + cytosine (mol % G + C) varied from 37.8 to 48.8. The neotype strain (Baynes and Simmons, 1960) had a mol % G + C of 48.8. In contrast, Mannheim et al. (1980) reported a value of 43.7% for the ATCC strain (#15768).

While the taxonomic position of A. seminis remains in doubt, it does not belong in the genus Actinobacillus (Phillips, 1984). Its exclusion is supported by genotypic data; Piechulla et al. (1986) found only 7% DNA homology between A. seminis and A. lignieresii (the type species of the genus).

Despite its phenotypic similarity to other members of the family Pasteurellaceae (Pohl, 1981; Mannheim, 1981), A. seminis has not been shown to be genotypically related (Hollander et al., 1981). Pohl (1981) found that A. seminis had only 20% DNA homology with other species of the Haemophilus-Pasteurella-Actinobacillus group; other members of the group were linked at no less than 30%. Piechulla et al. (1986) found less than 13% DNA homology between A. seminis and any type species of the family, but suggested it might be linked by as yet unrecognized intermediates. It has also been suggested that A. seminis constitutes a distinct species of the family Pasteurellaceae without generic affiliation (Piechulla et al., 1986).

TABLE 2. Bacteriological characteristics of A. seminis

Characteristic	Reactions[1]											
Reference[2]	1	2	3	4	5	6	7	8	9	10	11	12
Yellow pigment	-	-	-	v	-	*	-	*	-	v	*	-
Media enrichment	a	a	r	p	*	*	-	*	-	*	*	-
Capneic growth	p	p	p	p	*	*	-	*	-	p	-	*
Catalase	*	*	+	v+	+	-	+	+	+	v+	+	+
Oxidase	*	*	*	v-	d	-	+	+	-	v-	+	+
Hemolysis	*	-	*	-	-	*	-	*	-	-	-	-
Indole	-	*	-	-	-	*	v	-	-	-	-	-
Nitrate	*	*	+	v-	+	+	+	+	+	v-	+	*
Hydrogen sulfide	*	*	*	w	*	*	+	-	-	v+	*	*
Urea	-	*	-	-	-	*	-	v-	-	-	*	-
Lysine decarboxylase	*	*	*	*	d	*	*	-	-	*	-	*
Ornithine decarbox.	*	*	*	*	+	*	*	+	+	*	+	+
Arginine dihydrolase	*	*	+	*	w	*	*	-	-	*	-	*
Beta-galactosidase	*	*	*	*	*	*	*	-	*	*	-	+
Fermentation of												
Glucose	-	-	-	-	+	-	+	v+	+	+	*	+
Fructose	d	d	-	-	+	-	*	*	+	d	*	+
Xylose	-	-	-	-	s	-	*	*	+	+	*	+
Arabinose	d	d	-	-	d	-	*	v+	+	d	*	d
Lactose	-	-	-	-	-	-	*	*	-	-	*	*
Sucrose	-	-	-	-	*	-	*	-	-	-	*	-
Galactose	-	-	-	-	v	-	*	*	+	-	*	d
Maltose	-	-	-	-	d	-	*	*	+	+	*	d
Mannitol	d	d	-	-	+	-	*	v+	+	+	*	+
Mannose	-	-	-	-	-	-	*	*	+	d	*	-
Trehalose	d	-	-	-	-	-	*	*	-	d	*	*
Sorbitol	-	-	-	-	-	-	*	v-	-	-	*	-

[1]Symbols used: +, positive reaction; -, negative reaction; w, weak positive reaction; d, delayed positive reaction; v, variable reaction; v+, variable, usually positive; v-, variable, usually negative; *, not reported; r, required for growth; p, required for optimal growth; a, adaptable with subculture

[2]References: 1, Baynes and Simmons, 1960; 2, Livingston and Hardy, 1964; 3, Worthington and Bosman, 1968; 4, Van Tonder, 1979c; 5, Mannheim et al., 1980; 6, Dixon et al., 1983; 7, Stephens et al., 1983; 8, Erasmus, 1983; 9, Webb, 1983a; 10, Phillips, 1984; 11, Walker et al., 1986; 12, Piechulla et al., 1986

HISTOPHILUS OVIS

Recognition of the Species. In retrospect, the first reference to Histophilus ovis was made by Dodd and Hartley (1955) in New Zealand. They isolated a pleomorphic, Gram-negative, bipolar staining bacillus from cases of suppurative epididymitis in rams, and concluded it was unlike any previously described. Transmission experiments in rams supported its etiological role in ovine epididymitis.

The specific epithet Histophilus ovis was first proposed by Roberts (1956) to describe small, pleomorphic, Gram-negative bacteria isolated from the mastitic udder of a ewe in Australia. Because of an absolute requirement for undefined growth factors in blood or tissue, Roberts felt his isolate belonged in the tribe Haemophilae. However, given its specific atmospheric requirements, lack of motility, and independence of the specific growth factors hemin (X factor) and nicotinamide adenine dinucleotide (V factor), it could not be placed in existing genera. Therefore, Roberts proposed its classification in a new genus, and suggested the name Histophilus ovis to denote its predilection for tissue factors and its isolation from a disease of sheep.

Kater et al. (1962) isolated an organism from polytenosynovitis and pyemia in lambs and mastitis in a ewe that was culturally and morphologically indistinguishable from that of Dodd and Hartley (1955). They noted that Roberts had made a detailed comparison of their isolate

with his Hist. ovis and considered them identical. Experimentally, the organism could, regardless of its original source, cause epididymitis in rams, mastitis in ewes, and synovitis in lambs when introduced by an appropriate route.

Etiology. Histophilus ovis has since been reported as a cause of epididymitis in Australia (Claxton and Everett, 1966, Webb, 1983b), New Zealand (Bruere et al., 1977), the United Kingdom (Low and Graham, 1985), South Africa (Van Tonder, 1979a,b,c), and the United States (Walker et al., 1986). It is one of the most common causes of ovine epididymitis (Burgess, 1982), especially in immature rams (Walker et al., 1986).

Like A. seminis, it is frequently found in the preputial cavity of rams less than six months old, and may be a transitory part of the ovine genital flora (Walker and LeaMaster, 1986). Development of disease may occur as an ascending infection related to hormonal status (Jansen, 1980), the presence of other bacteria (Corbeil et al., 1985), or host immune status (Walker and LeaMaster, 1986).

Hist. ovis has also been recognized as a cause of synovitis, polyarthritis and neonatal deaths of lambs (Rahaley and White, 1977; Webb, 1983b), and of mastitis and abortion in ewes (Beauregard and Higgins, 1983; Webb, 1983b). It has been isolated from the vagina of clinically normal ewes (Higgins et al., 1981) and may be part of the normal vaginal flora (Walker and LeaMaster, 1986). However, Rahaley and

Edwards (1983) were unable to isolate Hist. ovis from the vagina of normal ewes. It has been isolated from the ovine brain (Stephens et al., 1983).

There is a single report of the isolation of Hist. ovis from cattle in association with pneumonia, abortion, vaginitis, and endometritis (Webber et al., 1983).

Description of the Organism. Histophilus ovis is a small, Gram-negative pleomorph ranging in length from 1 to 4 um (Claxton and Everett, 1966). Long rods and filaments may occur (Roberts, 1956). Cells occur singly, in pairs or in short chains (Roberts, 1956; Webb, 1983a). It is non-encapsulated and non-sporing (Webb, 1983a). Bi-polar staining has been reported (Dodd and Hartley, 1955; Rahaley, 1977) but not consistently (Webb, 1983a). It is non-acid fast and non-motile (Roberts, 1956).

Optimal growth requires increased CO_2 tension and serum or blood enrichment (Roberts, 1956; Webb, 1983a). Slight growth may occur aerobically or anaerobically, and adaptation to unenriched media has been reported (Rahaley, 1977). A growth response to thiamine monophosphate and satellitism around Staphylococcus aureus has been observed with some strains (Stephens et al., 1983). There is no X or V factor requirement (Rahaley, 1977; Stephens et al., 1983), and it does not grow on MacConkey agar (Walker et al., 1986).

Colonies of Hist. ovis are 1-2 mm after 24 hrs. at 37°C in 10% CO_2

(Stephens et al., 1983). They are smooth, moist, convex and entire (Roberts, 1956). Longer incubation results in increased size and a tendency to become umbonate (Stephens et al., 1983). Older colonies may develop a characteristic "fried-egg" appearance consisting of a central opaque area surrounded by a transparant peripheral skirt (Webb, 1983a). Colonies express a yellow pigment (Roberts, 1956; Stephens et al., 1983) that is not obvious on all media (Higgins et al., 1981). Most strains are non-hemolytic (Webb, 1983a), but alpha hemolysis has been reported (Swanepoel, 1984).

Carbohydrate metabolism is fermentative (Stephens et al., 1983), with consistent acid production reported from glucose, fructose, xylose, mannitol, and mannose (Roberts, 1956; Rahaley, 1977). Less consistent reactions have been reported for sorbitol, galactose, and maltose (Higgins et al., 1981; Webb, 1983a). Most authors have found Hist. ovis was catalase negative and oxidase positive (Roberts, 1956; Stephens et al., 1983; Low and Graham, 1985; Walker et al., 1986), but Webb (1983a) studied 17 strains, all of which were oxidase negative. Webb (1983a) also reported all strains were positive for ornithine decarboxylase and negative for both lysine decarboxylase and arginine dihydrolase. Other authors found that Hist. ovis was ornithine decarboxylase negative (Higgins et al., 1981) and the lysine decarboxylase reaction was variable (Walker et al., 1986).

Nitrate is reduced to nitrite, and indole is produced by most strains (Rahaley, 1977; Webb, 1983a; Stephens et al., 1983).

Production of H_2S has been reported (Stephens et al., 1983) but not consistently (Webb, 1983a). Urea is not decomposed (Rahaley, 1977; Webb, 1983a) and beta-galactosidase is not produced (Walker et al., 1986). The reported biochemical characteristics of Hist. ovis are summarized in Table 3.

The phenotypic similarity between Hist. ovis and A. seminis has been noted by a number of authors (Hughes et al., 1971; Dennis, 1974; Bruere et al., 1977), and they may represent biochemical variants of the same species (Webb, 1983a), however, other authors noted less similarity (Stephens et al., 1983), and Walker et al. (1986) differentiates the two based on the catalase reaction and indole production. Differences in carbohydrate fermentation reactions have also been noted (Webb, 1983a), and most A. seminis strains are not yellow-pigmented (Stephens et al., 1983). However, certain strains identified as A. seminis were catalase negative (Van Tonder, 1979c), produced indole (Stephens et al., 1983), and were yellow-pigmented (Phillips, 1984). In general, A. seminis is less fastidious than Hist. ovis (Webb, 1983a), but both require serum or blood enriched media and increased CO_2 tension for optimal growth (Roberts, 1956; Baynes and Simmons, 1960). They are also serologically cross-reactive (Rahaley, 1978; Webb, 1983a; Stephens et al., 1983).

Recent evidence suggests that Hist. ovis is indistinguishable from Haemophilus somnus and Haemophilus agni. Stephens et al. (1983) could not demonstrate a definitive difference between these organisms, and

preferred instead to "refer to these bacteria collectively rather than attempt to separate them into three species". That conclusion is supported by genotypic data (Walker et al., 1985). Piechulla et al. (1986) studied three strains of Hist. ovis and found no less than 92% DNA homology with H. somnus. Direct comparisons with H. agni were not done. Despite the phenotypic similarity between Hist. ovis and A. seminis (Webb, 1983a), they are not genotypically related (Piechulla et al., 1986). The mol % G + C of Hist. ovis is 38 (Piechulla et al., 1986).

At present, Histophilus ovis has received no official recognition (Kilian and Biberstein, 1984). While it satisfies the criteria for inclusion in the family Pasteurellaceae (Stephens et al., 1983; Piechulla et al., 1986), it does not fit precisely in any of the established genera. It might be best accommodated in a new genus (Webb, 1983a; Stephens et al., 1983), and it has been suggested the genus Histophilus be revived (Piechulla et al., 1986).

TABLE 3. Bacteriological characteristics of Hist. ovis

Characteristic	Reactions[1]									
Reference[2]	1	2	3	4	5	6	7	8	9	10
Yellow pigment	+	*	-	+	+	+	+	+	*	+
Media enrichment	r	r	a	r	r	r	r	-	p	p
Capneic growth	r	r	p	r	r	r	r	-	p	p
Catalase	-	*	-	-	-	-	-	-	-	v-
Oxidase	*	*	*	+	-	+	+	+	+	d
Hemolysis	-	*	-	-	-	-	*	-	-	v
Indole	+	-	v+	-	+	v-	v+	-	+	v+
Nitrate	+	+	+	+	+	+	+	+	+	+
Hydrogen sulfide	-	-	-	-	-	+	*	-	*	-
Urea	*	*	-	-	-	-	-	*	*	-
Lysine decarboxylase	*	*	*	-	-	*	*	*	v	-
Ornithine decarbox.	*	*	*	-	+	*	*	*	+	w
Arginine dihydrolase	*	*	*	*	-	*	*	*	-	-
Beta-galactosidase	*	*	*	*	*	*	*	*	-	-
Fermentation of										
Glucose	+	+	d	+	+	+	*	+	*	d
Fructose	*	*	d	+	+	*	*	*	*	v
Xylose	+	*	d	+	+	*	*	*	*	v
Arabinose	-	-	-	-	-	*	*	*	*	v
Lactose	-	-	-	-	-	*	*	-	*	-
Sucrose	-	-	-	-	-	*	*	-	*	*
Galactose	-	-	-	*	+	*	*	*	*	v
Maltose	-	+	d	+	-	*	*	+	*	*
Mannitol	+	+	v+	+	+	*	*	+	*	v
Mannose	+	+	d	*	+	*	*	*	*	d
Trehalose	-	*	-	-	-	*	*	-	*	*
Sorbitol	+	+	v+	-	+	*	*	+	*	-

[1]Symbols used: +, positive reaction; -, negative reaction; w, weak positive reaction; d, delayed positive reaction; v, variable reaction; v+, variable, usually positive; v-, variable, usually negative; *, not reported; r, required for growth; p, required for optimal growth; a, adaptable with subculture.

[2]References: 1, Roberts, 1956; 2, Claxton and Everett, 1966; 3, Rahaley and White, 1978; 4, Higgins et al., 1981; 5, Webb, 1983a; 6, Stephens et al., 1983; 7, Webber et al., 1983; 8, Low and Graham, 1985; 9, Walker et al., 1986; 10, Piechulla et al., 1986.

HAEMOPHILUS SOMNUS

Recognition of the Species. Kennedy et al. (1960) were the first to describe a small, Gram-negative coccobacillus isolated from meningoencephalitis in cattle. It required increased carbon dioxide tension and blood enriched media for primary isolation, and was described as "Haemophilus-like". Reports of similar organisms soon followed (Weide et al., 1964; Case et al., 1965; Panciera et al., 1968), although other authors described their isolates as "Actinobacillus sp." (Gossling, 1966) or "A. actinoides-like" (Bailie et al., 1966). Shigidi and Hoerlein (1970) compared a number of these and concluded they more closely fit the genus Haemophilus than Actinobacillus, and Bailie (1969) proposed the name based on its requirement for a growth factor that "could not be differentiated from the hemin-like or 'X' factor required by members of the genus Haemophilus". Although H. somnus has since been shown to grow independently of both X and V factors (Stephens et al., 1981), the name Haemophilus somnus has persisted in the literature. It has also been referred to as H. somnifer (Miles et al., 1972).

Etiology. Haemophilus somnus is most commonly associated with infectious thromboembolic meningoencephalitis (TEME) in cattle. The disease was first described in 1956 (Griner et al., 1956), and has also been referred to as "sleeper syndrome" (Bailie et al., 1966). It is characterized by acute onset, fever, lameness, incoordination, dyspnea,

rapidly progressing neurologic signs and, unless treatment is begun early, death (Griner et al., 1956; Kennedy et al., 1960; Bailie et al., 1966; Panciera et al., 1968). Although the characteristic lesions are usually confined to the central nervous system (Kennedy et al., 1960), focal areas of necrosis can be found in virtually any tissue (Stephens et al., 1981) including the cardiovascular, gastrointestinal, respiratory, reproductive and musculoskeletal systems (Griner et al., 1956; Kennedy et al., 1960; Bailie et al., 1966; Panciera et al., 1968; Brown et al., 1970). The widespread occurence of lesions suggests the disease is a septicemia (Kennedy et al., 1960; Panciera et al., 1968), and the clinical conditions associated with H. somnus have been referred to as the "Haemophilus somnus complex" (Brown et al., 1970). In addition to TEME, infections of the respiratory and reproductive tracts are common.

Respiratory disease involving H. somnus occurs in association with TEME (Humphrey and Stephens, 1983) or as an acute pneumonia of calves (Pritchard and MacLeod, 1977; Saunders et al., 1980; Corboz and Wild, 1981; Lancaster et al., 1984; Andrews et al., 1985; Corbeil et al., 1986). It has been reported, together with other species, as an agent in bovine "shipping fever" (Brown et al., 1970; Messersmith et al., 1972). The pathogenesis of H. somnus associated respiratory disease is obscure (Humphrey and Stephens, 1983). The organism has been isolated from normal respiratory tissues (Brown et al., 1970; Hall et al., 1977; Saunders and Janzen, 1980; Corboz and Wild, 1981), and may be part of

the normal flora of at least some cattle (Corstvet et al., 1973).

Isolations from the reproductive tract have also been reported from normal and clinically affected cattle (Miller, 1980; Stephens et al., 1981; Humphrey et al., 1982a,b; Slee and Stephens, 1985). It is frequently found in the preputial cavity in the absence of disease (Corbeil et al., 1985), and may constitute part of the normal flora (Humphrey et al., 1982a,b; Little, 1986). It has been associated with abortion (Chladek, 1975; Van Dreumel and Kierstead, 1975; Saunders et al., 1980), vulvitis/vaginitis (Ruhnke et al., 1978; Slee and Stephens, 1985), weak calf syndrome (Waldhalm et al., 1974), endometritis (Corboz and Nicolet, 1975; Miller, 1980; Corboz and Wild, 1981; Miller et al., 1983), and orchitis (Corboz and Nicolet, 1975). Haemophilus somnus has also been isolated from aborted fetal tissues and membranes (Miller et al., 1983; Slee and Stephens, 1985; Corbeil et al., 1986), and from the purulent ejaculate of bulls (Corboz and Nicolet, 1975; Corboz and Wild, 1981). Urinary excretion associated with TEME has been reported (Brown et al., 1970; Saunders et al.,1980).

Haemophilus somnus has been isolated in association with bovine mastitis (Armstrong et al., 1986), and can cause the disease experimentally (Hazlett et al., 1983). Other isolations have been from cattle with arthritis (Saunders et al., 1980), otitis (Nation et al., 1983) and conjunctivitis (Nation et al., 1983; Lamont and Hunt, 1982).

Although recognized primarily as a bovine pathogen, similarities between H. somnus and the ovine pathogens Hist. ovis. and H. agni have

been noted (Stephens et al., 1983). These organisms may constitute a single species (Stephens et al., 1983; Walker et al., 1986; Piechulla et al., 1986), making it difficult to judge the prevalence of H. somnus infection in rams. Walker et al. (1986) reported that one of the most common isolates associated with lamb epididymitis in the western United States was Hist. ovis, but that Hist. ovis and H. somnus were phenotypically indistinguishable. The specific nomenclatural choice would appear, therefore, to have been arbitrary, and other authors have identified isolates from ovine epididymitis as "Haemophilus-like" (Bruss et al., 1981) or H. somnus (Bulgin and Anderson, 1983). Haemophilus somnus has also been isolated from the reproductive tract of normal sheep, (Slee and Stephens, 1985), a fatal septicemia in rams (Groom et al., 1984), the arthritic joints of lambs (Slee and Stephens, 1985) and in association with ovine pneumonia (Canto and Biberstein, 1982).

Although biotypes of H. somnus have been described (Nivard et al., 1982; Corboz, 1981; Thompson and Little, 1981; Humphrey et al., 1982b; Ward et al., 1984), they are not correlated with pathogenicity. Humphrey et al. (1982b) were unable to distinguish between encephalitic, pneumonic and reproductive strains based on cultural or cellular characteristics. The serotypes described by Canto and Biberstein (1982) were also unrelated to pathogenicity.

On the other hand, virulence differences do occur among H. somnus strains (Miller et al., 1984). Humphrey et al. (1982a) established the existence of encephalopathic and non-encephalopathic strains, and Simonson et al. (1981) reported the susceptibility of isolates to normal bovine serum differed between pathogenic strains and those isolated from normal cattle. Ward et al. (1984) reported differences in the ability of certain strains to adhere to bovine cells in culture, suggesting the presence of specific adhesion factors. The virulence factors of H. somnus have not been defined (Humphrey and Stephens, 1983). Corboz and Wild (1981) considered all isolates pathogenic, and Little (1986) suggested that most biotypes of H. somnus existed in the bovine reproductive tract as benign commensals which, under unknown conditions, become invasive, causing pulmonary, genital, or systemic infection.

Haemophilus somnus has been isolated in the United States, Canada, Scotland, Germany, Italy, Switzerland, Holland, the United Kingdom, Poland, Romania, Russia, Japan, Australia, and South America (Pritchard and MacLeod, 1977; Humphrey and Stephens, 1983; Lancaster et al., 1984; Little, 1986).

Description of the Organism. Haemophilus somnus is a small, pleomorphic, Gram-negative bacteria ranging from coccoid to filamentous with occasional "bizarre forms" (Kennedy et al., 1960). Much of the pleomorphism is lost on subculture (Kennedy et al., 1960; Panciera et al., 1968), and may vary with growth conditions (Asmussen and Baugh,

1981). It is non-piliated, non-motile, and non-sporing (Bailie, 1969; Thompson and Little, 1981). Although there is a report to the contrary (Miller et al., 1975), most strains are not encapsulated (Kennedy et al., 1960; Bailie, 1969; Ward et al., 1984). It is non-acid fast (Bailie, 1969). Bi-polar staining has been reported (Van Dreumel et al., 1970) but not consistently (Kennedy et al., 1960).

Optimal growth occurs at 37°C in an atmosphere of 5-20% CO_2 (Shigidi and Hoerlein, 1970; Garcia-Delgado et al., 1977), however, Canto and Biberstein (1982) reported strains that grew aerobically on primary isolation, and most strains can be adapted to aerobic growth (Kennedy et al., 1960). Anaerobic growth is usually more abundant than aerobic (Bailie, 1969; Garcia-Delgado et al., 1977), but less than under capneic conditions (Humphrey and Stephens, 1983). Many strains fail to grow both aerobically and anaerobically (Kennedy et al., 1960). The optimum growth temperature is 37°C, but moderate growth has been reported between 25 and 43°C (Bailie, 1969; Shigidi and Hoerlein, 1970).

The nutritional requirements of H. somnus are complex. It grows readily on media supplemented with blood (Kennedy et al., 1960), and bovine blood has been reported superior to both lapine and ovine (Shigidi and Hoerlein, 1970). Some authors successfully replaced blood with serum (Bailie, 1969; Asmussen and Baugh, 1981), while others were unable to do so (Kennedy et al., 1960; Shigidi and Hoerlein, 1970). Optimal growth has been obtained using a combination of bovine blood and 0.5% yeast extract (Garcia-Delgado et al., 1977), although the latter is

not required (Bailie, 1969; Shigidi and Hoerlein, 1970). Haemophilus somnus does not grow on MacConkey agar (Stephens et al., 1983).

Bailie (1969) reported satellitic growth on a wide variety of media and concluded that growth was dependent on a factor present in animal or bacterial extracts. He further concluded that the growth factor could not be distinguished from the X factor (hemin) required by a number of Haemophilus species. Haemophilus somnus has since been shown to grow in the absence of X and V factors (Biberstein, 1981; Merino and Biberstein, 1982), and its X factor independence has been confirmed by its ability to produce porphyrins from delta-aminolevulinic acid (Stephens et al., 1983).

Corbeil et al. (1985) found a number of bacterial species among the normal flora of the respiratory and reproductive tracts that enhanced the growth of H. somnus. Other species were found to inhibit growth, and it was suggested the prevalence of certain species might account, in part, for the onset of disease.

The specific growth requirements of H. somnus have not been defined. Asmussen and Baugh (1981) reported that Isovitalex[1] was as stimulatory as the combination of bovine serum and yeast extract when added to brain-heart infusion broth. Component analysis suggested the required growth factor was thiamine pyrophosphate (cocarboxylase). Merino and Biberstein (1982) found that cocarboxylase was stimulatory but not required; the active ingredients in Isovitalex were the amino acids cysteine and cystine. Piechulla et al. (1986) reported that

growth in peptone-containing media was greatly enhanced by the addition of whole blood, fresh yeast extract, cocarboxylase, and cysteine. However, Humphrey and Stephens (1983) reported that neither cysteine or cystine were consumed during growth and suggested they must function in a non-nutrient manner. It was also noted that the only amino acid consumed was aspartic acid, and that soluble starch, while not hydrolyzed, enhanced growth. It was suggested starch acted as an absorbent, and growth might be limited as much by production of toxins as by lack of nutrients (Humphrey and Stephens, 1983). A marked increase in the pleomorphism of H. somnus cultured under minimal conditions has been noted (Asmussen and Baugh, 1981).

A selective medium for H. somnus incorporating vancomycin, neomycin, sodium azide, nystatin, and cyclohexamide in horse blood agar has been reported (Slee and Stephens, 1985). Inclusion of thiamine monophosphate was stimulatory.

Determination of the growth requirements of H. somnus are complicated by the report of atypical strains that grow aerobically on unsupplemented media (Canto and Biberstein, 1982). Also, H. somnus can adapt to media previously unable to support growth (Kennedy et al., 1960; Biberstein, 1981).

Under optimal conditions, colonies of H. somnus are small, convex, circular, and entire (Kennedy et al., 1960; Bailie, 1969; Shigidi and Hoerlein, 1970). At 24 hrs. they are pinpoint and transparent (Kennedy et al., 1960), developing to 1-2mm in 2-3 days (Bailie, 1969;

Garcia-Delgado et al., 1977; Stephens et al., 1983). Older colonies may become umbonate with an opague center and flattened, transparent periphery (Kennedy et al., 1960; Stephens et al., 1983). Most colonies are butyrous (Bailie, 1969; Garcia-Delgado et al., 1977; Stephens et al., 1983), but Corboz (1981) reported colonial variants ranging from rough to mucoid, and Nivard et al. (1982) described variants as translucent, small opaque and large opaque. Variation in colony type has been reported to correspond to variation in cell structure, pathogenicity in chick embryos, and adherence to bovine cells in tissue culture (Nivard et al., 1982; Ward et al., 1984). Garcia-Delgado et al. (1977) studied 68 strains of H. somnus but did not report differences in colony morphology.

Colonies of are yellow pigmented (Kennedy et al., 1960; Bailie, 1969, Shigidi and Hoerlein, 1970; Garcia-Delgado et al., 1977; Stephens et al., 1983) but pigmentation is not apparent on all media (Stephens et al., 1983), and may vary depending on colony type (Corboz, 1981).

Most strains of H. somnus are non-hemolytic (Kennedy et al., 1960; Gossling, 1966; Panciera et al., 1968; Kilian and Frederiksen, 1981), however, Bailie (1969) reported a "pronounced greenish lysis", and Shigidi and Hoerlein (1970) reported that colonies, while non-hemolytic, produced a "greenish discoloration of the blood in areas of confluent growth". Garcia-Delgado et al. (1977) observed a small zone of partial hemolysis in 50% of their strains, but it was not apparent unless

colonies were removed from the agar. Other authors reported clear zones of hemolysis (Humphrey et al., 1982b; Stephens et al., 1983), but the nature of the hemolysins was not determined (Humphrey and Stephens, 1983).

The reactions of H. somnus in standard bacteriological tests are variable (Table 4). In general, it is catalase negative and oxidase positive (Bailie, 1969; Shigidi and Hoerlein, 1970; Garcia-Delgado et al., 1977), although the opposite results have been reported (Gossling, 1966; Corboz and Wild, 1981). The atypical strains for which Biberstein (1981) reported aerobic growth on unsupplemented media were catalase positive, as were a number of his "H. somnus-like" isolates.

Consistently negative results have been reported for the methyl red/Voges-Proskauer, arginine dihydrolase, and citrate utilization reactions (Humphrey and Stephens, 1983). Hydrogen sulfide is produced by most strains (Bailie, 1969; Stephens et al., 1983) but its detection depends on methodology; Garcia-Delgado et al. (1977) demonstrated its production using lead acetate impregnated paper strips but not in TSI or SIM agars, and Bailie (1969) could demonstrate only trace amounts on lead acetate paper. Other authors failed to demonstrate its production (Gossling, 1966; Corboz and Wild, 1981).

Indole production and nitrate reduction tests are usually positive (Bailie, 1969; Shigidi and Hoerlein, 1970; Garcia-Delgado et al., 1977; Biberstein, 1981), although Panciera et al. (1968) reported both characteristics negative. Mannheim et al. (1980) reported weak

reactions for nitrate reduction, and Stephens et al. (1983) reported variability in the indole reaction. Although H. somnus is generally reported urease negative (Kilian and Frederiksen, 1981), a delayed positive reaction has been observed (Mannheim et al., 1980).

Garcia-Delgado et al. (1977) reported both lysine and ornithine decarboxylase were negative, but positive results have also been reported (Corboz and Nicolet, 1975; Mannheim et al., 1980; Kilian and Frederiksen, 1981).

Carbohydrate metabolism is fermentative (Stephens et al., 1983), but there is considerable variability with respect to specificity. Acid is usually produced from glucose, maltose, fructose, xylose, mannose, levulose, trehalose, mannitol and sorbitol (Humphrey and Stephens, 1983). Positive reactions have also been reported for sucrose (Kennedy et al., 1960), galactose (Bailie, 1969; Kilian and Frederiksen, 1981) and arabinose (Kilian and Frederiksen, 1981). Shigidi and Hoerlein (1970) and Panciera et al. (1968) were unable to demonstrate fermentation of carbohydrates.

Some authors reported that H. somnus was serologically homologous (Shigidi and Hoerlein, 1970; Garcia-DelGado et al., 1977), while others observed more diversity. Corboz (1981) identified both common and strain-specific antigens that varied with colony type. Canto and Biberstein (1982) observed common and specific antigens in a comparison of American and Swiss isolates; the latter were unrelated to pathological or anatomical factors.

Haemophilus somnus cross-reacts serologically with a wide variety of bacterial species (Miller et al., 1975; Humphrey and Stephens, 1983), including Hist. ovis (Stephens et al., 1983), H. agni (Canto et al., 1983), and A. seminis (Stephens et al., 1983). Canto et al. (1983) found the specificity of cross reactions varied with different antigen preparations and type of assay.

Although currently listed as a "species incertae sedis" in the genus Haemophilus (Kilian and Biberstein, 1984), H. somnus does not require either of the specific growth factors that define the genus, and "by current standards", does not qualify for inclusion (Kilian and Biberstein, 1984). Haemophilus somnus and H. influenzae exhibit little DNA homology (Gonzales and Bingham, 1983; Walker et al., 1986; Piechulla et al., 1986).

The appropriate taxonomic placement of H. somnus has not been determined (Humphrey and Stephens, 1983). Its mole % G + C ranges from 37.0 to 38.8 (Bailie et al., 1973; Piechulla et al., 1986); within the values reported for the family Pasteurellaceae (Mannheim, 1981). Although its inclusion is supported by phenotypic data (Mannheim et al., 1980; Piechulla et al., 1986), DNA homology with other members of the family is relatively low (Piechulla et al., 1986).

There is significant DNA homology between H. somnus, H. agni and Hist. ovis (Walker et al., 1986; Piechulla et al., 1986). Several authors noted similarities between these organisms (Kennedy et al., 1960; Higgins et al., 1981; Webber et al., 1983; Low and Graham, 1985),

and Stephens *et al*. (1983) were unable to differentiate between them, preferring instead to "refer to these bacteria collectively rather than attempt to separate them into three species". Piechulla *et al*. (1986) stated that the "*Histophilus ovis* group", which included *H. somnus*, did not belong in any of the established genera within the family *Pasteurellaceae*, and suggested it might be appropriate to revive the genus *Histophilus* to accommodate these organisms.

TABLE 4. Bacteriological characteristics of H. somnus

Characteristic	Reactions[1]										
Reference[2]	1	2	3	4	5	6	7	8	9	10	11
Yellow pigment	*	+	*	+	*	+	*	-	+	+	+
Media enrichment	a	r	r	r	r	r	*	*	r	r	p
Capneic growth	r	p	r	p	p	p	*	*	p	p	p
Catalase	*	-	*	-	-	-	-	*	-	v-	v-
Oxidase	*	+	*	+	+	+	*	+	v+	d	+
Hemolysis	*	-	-	g	g	v	-	-	v	v	v
Indole	w	+	-	+	+	+	+	+	v+	v+	v+
Nitrate	w	*	-	+	+	+	-	w	+	v+	+
Hydrogen sulfide	-	-	*	+	*	+	*	*	+	v	-
Urea	*	-	*	-	-	-	-	d	*	-	-
Lysine decarboxylase	*	*	*	*	*	-	-	-	*	v	-
Ornithine decarboxylase	*	*	*	*	*	-	+	+	*	v	+
Arginine dihydrolase	*	*	*	*	*	-	*	*	-	-	-
Beta-galactosidase	*	*	*	*	*	*	v	*	*	v	-
Fermentation of											
Glucose	w	+	-	+	-	+	+	d	+	+	d
Fructose	-	*	*	+	-	*	*	-	*	v+	v
Xylose	w	*	*	+	-	+	+	-	*	v+	v
Arabinose	w	*	*	-	-	-	v	-	*	-	v
Lactose	-	-	-	-	-	-	v	-	*	-	-
Sucrose	v	-	-	-	-	-	v+	*	*	v	*
Galactose	*	*	-	+	-	+	+	-	*	v	v
Maltose	w	+	-	+	-	+	+	-	*	v+	*
Mannitol	-	*	-	+	-	+	v	-	*	v+	v
Mannose	w	*	*	+	-	+	+	d	*	v+	d
Trehalose	*	*	-	+	-	+	v+	-	*	v+	*
Sorbitol	w	*	-	+	-	+	v+	-	*	v+	-

[1]Symbols used: +, positive reaction; -, negative reactions; w, weak postive reaction; d, delayed positive reaction; v, variable reaction; v+, variable, usually positive; v-, variable, usually negative; g, greenish lysis of blood agar; r, required for growth; p, required for optimal growth; a, adaptable with subculture.

[2]References: 1, Kennedy et al., 1960; 2, Gossling, 1966; 3, Panciera et al., 1968; 4, Bailie, 1969; 5, Shigidi and Hoerlein, 1970; 6, Garcia-DelGado et al., 1977; 7, Kilian and Fredericksen, 1981; 8, Mannheim et al., 1980; 9, Stephens et al., 1983; 10, Humphrey and Stephens, 1983; 11, Piechulla et al., 1986.

HAEMOPHILUS AGNI

Recognition of the Species. There are few reports concerning the isolation of Haemophilus agni. The first involved a severe, usually fatal, hemorrhagic septicemia of lambs (Kennedy et al., 1958). A similar organism was isolated from cattle suffering from infectious meningoencephalitis (Kennedy et al., 1960), but it did not cause disease when injected into susceptible lambs (Biberstein, 1981). There is a single report of the isolation of H. agni from cases of purulent orchitis in rams (Zeki et al., 1981).

Description of the Organism. Kennedy et al. (1958) isolated H. agni from the lung, liver, spleen, meninges, synovial fluid, cerebrospinal fluid and blood of affected lambs. It grew aerobically, but not as rapidly or abundantly as under capneic conditions. Colonies were semitranslucent, convex, and entire, ranging from 0.5 to 1.5 mm. Size did not increase appreciably after prolonged incubation, but colonies sometimes became flattened peripherally with contoured edges. Stephens et al. (1983) reported that colonies taken from blood agar were distinctly yellow when massed on a loop. Haemophilus agni is not hemolytic (Kilian and Biberstein, 1984).

Cellular morphology on primary culture ranges from coccobacillary to bacillary with occasional elongated forms (Kennedy et al., 1958).

All cellular forms occur in pure culture regardless of incubation or nutritional conditions, however, much of the pleomorphism is lost on subculture (Kennedy et al., 1958).

Satisfactory growth has been reported on blood, chocolate, and hemoglobin-cystine agars (Kennedy et al., 1958). Haemophilus agni does not require X (hemin) or V (NAD) factors (Kennedy et al., 1958), but satellitism around Staphylococcus aureus has been observed (Stephens et al., 1983). A growth response to thiamine monophosphate (TMP) has been noted, but no growth occurs on MacConkey, urea, citrate, or triple-sugar iron agars even when supplemented with TMP (Stephens et al., 1983).

Haemophilus agni is biochemically inactive. Carbohydrate metabolism is fermentative (Stephens et al., 1983) with weak acid reactions reported in arabinose, xylose, glucose, fructose, galactose, maltose, mannose, rhamnose, raffinose, mannitol, sorbitol and inositol (Kennedy et al., 1958). Kennedy et al. (1958) reported that fermentation was dependent on the addition of defibrinated blood to the media, and other authors observed less saccharolytic activity (Stephens et al., 1983). Piechulla et al. (1986) reported consistent fermentation of glucose and mannose, but never observed fermentation of rhamnose, sorbitol, or inositol. Variable results were obtained with galactose, fructose, xylose, arabinose, and mannitol.

Haemophilus agni is oxidase positive, catalase negative, and reduces nitrate to nitrite (Stephens et al., 1983; Piechulla et al., 1986). Indole production is negative (Kilian and Biberstein, 1984) or

weakly positive (Piechulla et al., 1986). Production of H_2S is inconsistent (Stephens et al., 1983; Piechulla et al., 1986). Ornithine decarboxylase is produced in contrast to both lysine decarboxylase and arginine dihydrolase (Mannheim et al., 1980; Piechulla et al., 1986). Urease production has been reported for some strains (Mannheim et al., 1980; Kilian and Biberstein, 1984).

The phenotypic properties of H. agni are similar to those of H. somnus (Stephens et al., 1983). Reported differential characteristics include indole production, growth requirements (H. agni is less dependent on CO_2), and pathogenicity in mice and guinea pigs (Biberstein, 1981). Some authors reported differences in ornithine decarboxylase production (Kilian and Biberstein, 1984), while others reported both species positive (Mannheim et al., 1980). Haemophilus somnus and H. agni cross-react (Stephens et al., 1983; Kilian and Biberstein, 1984), but are serologically distinct (Canto et al., 1983).

Stephens et al. (1983) were unable to differentiate between H. agni, H. somnus, and Hist. ovis. The phenotypic similarity between these organisms is reflected in genotypic studies; Walker et al. (1985) found they were related at greater than 80% DNA homology, close enough to possibly constitute a single species (Johnson, 1984). Piechulla et al. (1986) observed even greater homology, but also found one strain of H. agni with 91% DNA homology with A. seminis (ATCC #15768). The same strain exhibited nearly identical phenotypic properties with A. seminis.

The mol % G + C of H. agni varies from 36.8 (Kilian and Biberstein, 1984) to 37.7 (Piechulla et al., 1986). It is currently included in Bergey's Manual of Systematic Bacteriology as a "species incertae sedis", but it is stated that "by current standards", it does not qualify for inclusion in the genus Haemophilus (Kilian and Biberstein, 1984). With the exception of the strain noted above, Piechulla et al. (1986) referred to H. agni, Hist. ovis, and H. somnus as the "Histophilus ovis" group, and suggested they were a distinct species of the family Pasteurellaceae. They felt the genus Histophilus should be revived, but were unable to "give an unambiguous circumscription of the genus".

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II. BACTERIOLOGICAL CHARACTERISTICS OF THE GRAM-NEGATIVE PLEOMORPHS ASSOCIATED WITH OVINE EPIDIDYMITIS

Ovine epididymitis is most commonly associated with Brucella ovis (Burgess, 1982), but there is a correlation between etiological agent and reproductive status that suggests a distinction be drawn between the disease in rams and that in lambs (Bulgin and Anderson, 1983). While B. ovis predominates in mature breeding rams, the most common isolates from immature rams are a group of ill-defined, Gram-negative pleomorphic rods (GNPR's) (Bagley et al., 1985; Walker et al., 1986).

The identity of these "GNPR" isolates is unclear. Among the most common is Actinobacillus seminis (Baynes and Simmons, 1960; Van Tonder, 1979a; Walker et al., 1986), but some isolates in the western United States were identified as Actinobacillus actinomycetemcomitans (DeLong et al., 1979; Bulgin and Anderson, 1983; Bagley et al., 1985). The latter were unlike the type strain of that species (Bulgin and Anderson, 1983), and were probably A. seminis (Bagley et al., 1985; Healey et al., 1985). It remains unclear, however, whether the Actinobacillus sp. associated with lamb epididymitis constitute more than one species or more than one biotype of the same species (Walker et al., 1986).

Another GNPR isolate is Histophilus ovis (Walker et al., 1986). Originally isolated from the mastitic udder of a ewe (Roberts, 1956), Hist. ovis is phenotypically similar to A. seminis (Rahaley, 1977). It has been suggested they represent biochemical variants of the same species (Webb, 1983a), although they can be distinguished by cultural and morphological characteristics (Stephens et al., 1983), and are not genotypically related (Walker et al., 1985; Piechulla et al., 1986).

Phenotypic similarities have also been observed between Hist. ovis, Haemophilus agni, and Haemophilus somnus (Stephens et al., 1983). The latter are most commonly associated with hemorrhagic septicemia of lambs (Kennedy et al., 1958) and thromboembolic meningoencephalitis of cattle (Kennedy et al., 1960) respectively, but have also been associated with ovine genital disease (Zeki et al., 1981; Bulgin and Anderson, 1983). Moreover, the phenotypic similarity between these organisms is reflected in genotypic studies (Walker et al., 1985; Piechulla et al., 1986), and they probably constitute a single species (Stephens et al., 1983). The characteristics of that species have not been defined, but Hist. ovis, H. somnus, and H. agni have been referred to collectively as the "HH group" (Stephens et al., 1983) and the "Histophilus ovis group" (Piechulla et al., 1986).

Although not genotypically related, A. seminis is phenotypically similar to the "HH group" species, and they cross-react serologically (Rahaley, 1978; Webb, 1983a; Stephens et al., 1983). These similarities, plus the association of each with ovine epididymitis, makes differentiation both necessary and difficult. Although bacteriological characterizations have been reported (Kennedy et al., 1958; Bailie, 1969; Van Tonder, 1979c; Mannheim et al., 1980; Erasmus, 1983; Webb, 1983a; Stephens et al., 1983; Walker et al., 1986), the results are often inconsistent. Moreover, the lack of type strains for the "HH group" necessitates the phenotypic characterization of all strains available for study.

The purpose of this paper was, therefore, to define the bacteriological characteristics of available strains of A. seminis, Hist. ovis, H. somnus, and H. agni. For convenience, they will be referred to collectively as the AHH group or, with the exception of A. seminis, the HH group.

MATERIALS AND METHODS

Maintenance of cultures. Bacteria were received as lyophilized cultures or were clinical isolates at the Kansas State University Veterinary Clinic (Table 1). The latter had been frozen on glass beads in blood-enriched brain heart infusion (BHI) broth[1] at -70°C. All strains were initially plated on chocolate agar at 37°C in 10% CO_2; these conditions were used for routine maintenance. Strains received as lyophilized cultures were grown on glass beads in BHI broth supplemented with 10% ovine serum and stored at -70°C.

For daily use, each strain was subcultured every third day for up to four weeks, after which fresh cultures were taken from frozen storage. The identity of the last subculture and first fresh culture

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[1]Difco Laboratories, Detroit, MI

was confirmed by Gram-stain, colony morphology, and the catalase and oxidase reactions.

Cellular and colonial morphology. Cellular morphology was demonstrated by Gram-stain of growth from a 24 hr. culture incubated under maintenance conditions. Colony morphology was described after 48 hr. under the same conditions and, where appropriate, from blood agar.

Growth Requirements. Atmospheric requirements were determined at 37°C on chocolate agar[2]. Whether CO_2 was required (no aerobic growth) or preferred (reduced growth in air) was determined at the outset and again for each fresh culture taken from storage.

Nutritional requirements were demonstrated at 37°C on a base medium of BHI agar supplemented with thiamine monophosphate chloride[3] (Stephens et al., 1983) or 5% ovine or bovine serum. X and/or V factor requirements were determined on Haemophilus ID Quad Plates[2]. Growth on MacConkey agar was determined at 37°C in 10% CO_2.

Biochemical Reactions. Biochemical reactions were determined at 37°C in 10% CO_2. Catalase production was demonstrated by addition of 3%

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[2]REMEL (Regional Media Laboratories), Lenexa, KS

[3]Sigma Chemical Co., St. Louis, MO

hydrogen peroxide to growth from a chocolate agar culture. The immediate elution of bubbles was taken as a positive. The oxidase reaction was demonstrated by a similar method replacing hydrogen peroxide with either 1% N,N,N',N'-tetramethyl p-phenylenediamine dihydrochloride[1] or 1% p-aminodimethylaniline oxalate[2]. Reactions were scored as positive (color in 10 sec.), weak (color in 30 sec.), or negative.

Ornithine and lysine decarboxylase and arginine dihydrolase reactions were determined in Moeller Decarboxylase Base Media[1] to which the appropriate L-amino acid (1% w/v) and ovine serum (5% v/v) were added. Reactions were read after 72 hrs.

Indole production was determined in BHI broth supplemented with 5% ovine serum and 1% L-tryptophan. Reactions were read after 48 hrs. by addition of Kovac's reagent (Cowan, 1974). Decomposition of urea was demonstrated in commercial urea broth[1] supplemented with serum, and nitrate reduction in heart infusion broth[1] supplemented with 0.1% KNO_3 and ovine serum. Reactions were read after 48 hrs. by the step-wise addition of alpha-napthylamine in 5N acetic acid and sulfanilic acid in 5N acetic acid (Cowan, 1974).

Carbohydrate fermentation reactions were determined by a modification of the method of Stephens et al. (1983) in which 5% ovine serum was added to their base media.

API 20E Rapid Identification System. The commercial system[4] was used with minor modification as described by Erasmus (1983). Test strips were inoculated with growth from a 24 hr. chocolate agar culture suspended in sterile saline supplemented with ovine serum. Reactions were read using the manufacturers reagents after 48 hrs. aerobic incubation at 37°C. Homology values between strains were determined after elimination of reactions that were negative for all strains studied. The results of interstrain comparisons were tabulated in a similarity matrix, and the matrix was converted into a dendrogram by average linked cluster analysis (Goodfellow, 1977).

RESULTS

All AHH strains were small, non-motile, non-acid fast, Gram-negative pleomorphic bacilli (Fig. 1). Cellular morphology ranged from coccobacillus to bacillus with occasional filamentous forms. The latter were most prominent in older cultures. Bipolar staining was not observed.

The most striking difference in colony morphology was pigmentation (Fig. 2). Colonies of A. seminis were pale white with the larger colonies developing a grayish border; those of the HH group bacteria

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[4]Analytab Products, Div. of Ayerst Laboratories, Plainview, NY

were tan to yellow. Pigmentation was most obvious after 48-72 hrs. incubation on chocolate agar or when growth was massed on a loop; it was not readily demonstrable from blood agar. Haemophilus somnus appeared more pigmented, but quantitative studies were not done. Colonies of A. actinomycetemcomitans were white and easily distinguished from those of A. seminis.

Colony size varied from 0.5 to 4.0 mm. Colonies were round and entire although older colonies of Hist. ovis and H. agni sometimes developed an undulate edge. Colonies of H. agni were smaller and developed more slowly. Shape varied from flat (H. somnus 1048) to convex (A. actinomycetemcomitans). Colonies of A. seminis, Hist. ovis, H. agni and H. somnus (strain 1227) were low but convex. They sometimes became umbonate after 48-72 hrs. The type strain of A. seminis and strain 501 were butyrous, the other A. seminis strains varied from dry (504) to sticky (503). Strain 502 was variable, growing in small, dry colonies and larger, butyrous colonies. It was difficult to isolate stable variants of either type. Strains of Hist. ovis and H. agni were also butyrous, while H. somnus strain 1227 was sticky and strain 1048 was dry. ("Sticky" refers to a tendency of colonies to stick together and pull off the agar in a stringy fashion; it was unlike the stickiness associated with A. ligniersii and A. suis in which colonies were difficult to remove from the agar). With the exception of pigmentation, differences in colony morphology between the AHH strains were not absolute and not readily apparent in cultures less than 48 hours old.

Differences in growth requirements among the AHH group were a matter of degree; A. seminis was less fastidious than the HH group, growing aerobically and, to a limited extent, without added enrichment on BHI agar. Actinobacillus actinomycetemcomitans did not grow aerobically, and the HH group strains either required or drastically preferred increased CO_2 tension. The latter adapted to aerobic growth but it was never abundant.

All strains grew on blood agar, but colonies appeared sooner and were larger on chocolate agar. Using BHI agar as a base medium, only A. seminis grew to any appreciable extent. Slight growth was obtained with H. agni strain 1344 and A. actinomycetemcomitans. The addition of thiamine monophosphate to BHI agar did not enhance growth, but all strains showed a positive response to serum enrichment; the response of A. seminis was greater than that of the HH group strains.

There was no growth on MacConkey agar by any of the AHH group strains, nor did they require X or V factors.

Biochemical reactions are recorded in Table 2. All AHH strains were nitrate positive, oxidase positive and able to decarboxylate ornithine. The HH group bacteria were indole positive, but the reactions of H. agni and Hist. ovis were weak. Actinobacillus seminis was consistently indole negative. The catalase reaction was an important differential characteristic; among the AHH group bacteria, only A. seminis was positive. Actinobacillus actinomycetemcomitans was distinguished from A. seminis by its failure to decarboxylate ornithine.

All AHH strains were negative for urea decomposition, methyl red/Voges-Proskauer reactions, and arginine dihydrolase. Histophilus ovis strain 9L was weakly positive on two occasions for lysine decarboxylase. The known Actinobacillus spp. were catalase, oxidase, nitrate, and urea positive. They grew aerobically and did not decarboxylate ornithine; one strain produced indole (Table 2).

All AHH strains fermented sugars (Table 3), but the CO_2 requirement complicated the results due to a reversible drop in pH. This difficulty could be partially overcome by moving the tubes to an aerobic environment for several hours prior to reading the results; negative reactions were taken as development of the original pink color at the surface of the broth. In general, A. seminis fermented fructose, arabinose, and, usually, xylose. Only one strain produced acid from glucose under these conditions. The HH strains were positive for glucose, mannose, xylose, and, less consistently, mannitol.

Results of the API 20E Rapid Identification test system are presented in Table 4. They were generally consistent with those obtained by conventional methods. The exceptions were 1) the HH strains were consistently indole positive, 2) strain 9L did not decarboxylate lysine, and 3) there were variations in fermentation reactions, particularly the consistent fermentation of glucose by A. seminis and its failure to ferment arabinose. Except for H. agni, all AHH strains fermented inositol, and the HH strains fermented sorbitol and mannitol.

None of the AHH group bacteria produced beta-galactosidase (negative ONPG[5] reaction) or hydrogen sulfide.

DISCUSSION

As defined here, the AHH group consists of the nomenspecies Actinobacillus seminis (Baynes and Simmons, 1960), Histophilus ovis (Roberts, 1956), Haemophilus agni (Kennedy et al., 1958) and Haemophilus somnus (Bailie, 1969). They constitute a group in that all are associated with genital disease in rams (Van Tonder, 1979a,b; Zeki et al., 1981; Webb, 1983b; Bulgin and Anderson, 1983) and all exhibit bacteriological and serological similarities (Rahaley, 1977; Webb, 1983a; Stephens et al., 1983). While their taxonomic position is unclear, they do not belong in their currently assigned genera (Kilian and Biberstein, 1984; Phillips, 1984).

There is considerable variability in the published descriptions of the AHH group bacteria. For instance, Van Tonder (1979c) reported that A. seminis was catalase positive, oxidase negative, and usually failed to reduce nitrate. Variants existed, however, that were catalase negative and oxidase positive, and these were further characterized by slower, less luxuriant growth and a yellow pigment. Although many

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[5]ortho-nitrophenyl-beta-galactopyranoside

published reports for A. seminis list the nitrate reaction as positive (Worthington and Bosman, 1968; Mannheim et al., 1980; Dixon et al., 1983; Stephens et al., 1983; Erasmus, 1983; Webb, 1983a), Bergey's Manual of Systematic Bacteriology states that most strains do not reduce nitrate (Phillips, 1984), apparently on the strength of Van Tonders report (1979c). Moreover, the Manual states that some strains are yellow-pigmented, yet Van Tonder commented that these strains closely resembled Hist. ovis. Some of the most important characteristics observed here were nitrate reduction, catalase and oxidase production, and pigmentation; confusion such as that noted above makes it difficult to conclude that these characteristics can be used to differentiate species in the AHH group.

Actinobacillus seminis was characterized here by its reduction of nitrate, production of both catalase and oxidase (although the latter reaction was weak), and decarboxylation of ornithine. It did not require added enrichment or increased CO_2, but preferred both, and may, on primary isolation, require them. It was not yellow pigmented, did not produce beta-galactosidase, lysine decarboxylase, or arginine dihydrolase, and did not decompose urea. In contrast, Hist. ovis was indole positive, catalase negative, and sometimes decarboxylated lysine. Colonies were yellow, but that was not obvious on all media, and it required both media enrichment and increased CO_2 tension. In general, these results agree with those of other authors (Stephens et al., 1983; Walker et al., 1986). Although discrepancies exist in the literature

(Webb, 1983a), I am unaware of any yellow pigmented, catalase positive, Gram-negative pleomorphic bacteria having been isolated in association with ovine epididymitis.

Aside from the ability of one strain of Hist. ovis to decarboxylate lysine and relative differences in growth requirements (especially since these were not primary isolates), it was not possible to differentiate the HH group bacteria from each other. The results agree with those of Stephens et al. (1983), and have been supported by genotypic data (Walker et al., 1985). There is some question concerning subtypes within the HH group based on species of origin (ovine vs. bovine) (Walker et al., 1985). The H. somnus strains included in this study were isolated from cattle, and, relative to the ovine Hist. ovis and H. agni isolates, they were more pigmented, more consistently indole positive, and more dependent on CO_2 and blood enrichment. It is difficult to conclude, however, that these differences warrant establishment of subtypes. The original suggestion was made by Walker et al. (1985) and based primarily on DNA homology data: Hist. ovis and H. agni strains exhibited high levels of homology to a reference strain of H. somnus, but less than that observed between H. somnus strains. Piechulla et al. (1986) did not observe such definable differences. It would seem advisable to investigate the existence of subtypes as a function of pathogenicity in sheep and/or cattle.

Differences among the AHH strains in carbohydrate fermentation reactions were less conclusive and somewhat dependent on methodology.

While all A. seminis strains fermented glucose in the API 20E system, only one did so when tested by conventional techniques. Nearly opposite results were obtained for arabinose. The arabinose cupule had a tendency to turn blue- or yellow-green. Some authors have taken such a reaction as positive (Collins and Swanson, 1981), but, because it seemed to be a general tendency of that sugar, it was not done so here. That may account for the methodological differences in the arabinose reaction.

While the API 20E system was consistent under aerobic conditions, A. actinomycetemcomitans failed to ferment any sugar other than glucose. That could be related to its inability to grow aerobically; more active fermentation reactions have been reported for A. actinomycetemcomitans (Phillips, 1984).

It has been reported that A. seminis is unreactive in carbohydrate fermentation tests (Phillips, 1984), however, some authors observed greater reactivity (Baynes and Simmons, 1960; Mannheim et al., 1980), and the specific carbohydrates fermented agree with the results reported here. Walker et al. (1986) stated that the ability to ferment carbohydrates should not be used to exclude identification of an isolate as A. seminis. Actinobacillus seminis isolates may have been misidentified as A. actinomycetemcomitans based on their ability to ferment sugars. That could account for the association of the latter with ovine epididymitis.

The AHH group was distinguished from all other strains, including A. actinomycetemcomitans, by their ability to decarboxylate ornithine. Actinobacillus seminis and A. actinomycetemcomitans were excluded from the genus Actinobacillus by their inability to decompose urea or produce beta-galactosidase. Their failure to grow on MacConkey agar and lack of colonial "stickiness" would support their exclusion.

Although designed for differentiating the Enterobacteriacae, the API 20E Rapid Identification System is applicable to other organisms (Collins and Swanson, 1981). Using homology levels of 65% (genus) and 85% (species), Erasmus (1983) concluded that strains of A. seminis, A. actinomycetemcomitans, and Hist. ovis belonged to the same genus but to at least four species, and that most A. seminis isolates could be classified as either A. actinomycetemcomitans or Hist. ovis. It should be noted, however, that the A. actinomycetemcomitans used in that study was not a type strain; its source was listed as DeLong et al. (1979), and, given the difficulties discussed above, it was not necessarily identified correctly. In particular, Erasmus found it could decarboxylate ornithine, a reaction not observed here using the type strain. Other authors reported isolating A. actinomycetemcomitans in association with ovine genital disease, and later stated that their identification had been incorrect (Bulgin and Anderson, 1983). At least some of those strains closely resembled A. seminis (Bagley et al., 1985). Also, one of the A. seminis strains included in the Erasmus study was listed as V350 (Van Tonder, 1979c). That strain, which was

generally catalase negative and always yellow pigmented, closely resembled Hist. ovis.

Recognizing that the number of characters is limited, a dendrogram was prepared based on an average linked cluster analysis (Goodfellow, 1977) of interstrain comparisons. Including all 23 characters tested with the API 20E system, and using the homology levels for genus and species defined by Erasmus (1983), all 16 strains included in this study could be grouped in a single genus. However, since there were eight reactions negative for all strains (Table 4), which would automatically relate all species at 8/23 or 34.8%, it was felt it would be more appropriate to use the Jaccard similarity coefficient in which negative matches are excluded (Goodfellow, 1977). In this case, negative reactions were eliminated only when observed for all strains (i.e. all negative matches between any two strains were not necessarily excluded). As a result, the 16 strains were reduced to 8 species (Figs. 3 and 4). All A. seminis strains were included in a single species, as were all HH group strains. The two groups were related to each other at 73%, close enough to constitute a single genus. Also, A. actinomycetemcomitans was related to A. seminis at 83%, close enough to differentiate them as species but within the limits of a common genus.

Both A. actinomycetemcomitans and A. seminis were unrelated to known strains of Actinobacilli. Actinobacillus seminis does not qualify for inclusion in the genus (Phillips, 1984), and removal of A. actinomycetemcomitans has been suggested (Mannheim, 1981). However,

they are not genotypically related to each other (Piechulla et al., 1986), and can be differentiated by the ornithine decarboxylase reaction and colony morphology.

The Actinobacillus sp. [ATCC #27072], A. ligniersii and A. suis were related at 82%; close enough to belong in a common genus. They were differentiated from each other as species, and from the AHH group as genera. Both B. ovis and E. coli were alone in their respective genera.

SUMMARY

The AHH group bacteria were defined as small, Gram-negative, pleomorphic bacteria that required or preferred enriched media and a capneic atmosphere. They were nitrate positive, oxidase positive, and able to decarboxylate ornithine. Actinobacillus seminis was distinguished from the HH group strains by its production of catalase, lack of yellow pigment, less stringent growth requirements, and failure to produce indole. Actinobacillus seminis and A. actinomycetemcomitans were similar but could be distinguished by colony morphology and the ornithine decarboxylase reaction. It was not possible to clearly distinguish between Hist. ovis, H. agni, and H. somnus.

The reactions reported are consistent with the literature, but the confusion among the AHH group bacteria and A. actinomycetemcomitans makes it difficult to draw conclusions. For instance, whether certain

strains of A. seminis are yellow-pigmented, or those strains should be considered Histophilus ovis, has not been resolved. However, the type strain of A. seminis (ATCC 15768) was easily distinguished from both the HH group bacteria and A. actinomycetemcomitans. On that basis, the suggestion by Erasmus (1983) that most A. seminis strains could be classified as either A. actinomycetemcomitans or Hist. ovis does not seem appropriate. The results support the conclusion that the AHH group bacteria do not belong in the genus Actinobacillus or Haemophilus.

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TABLE 1. Bacterial strains used in this study

Species and strain	Site of origin	Source
A. seminis 15768	ovine semen	ATCC[1]
501	ovine semen	ATCC[2]
502	ovine epididymitis	KSU clinical isolate[3]
503	ovine orchitis	KSU clinical isolate
504	ovine semen	KSU clinical isolate
A. actinomycetem-comitans 29522	mandibular abcess	ATCC
Actinobacillus sp. 27072	porcine vagina	ATCC
A. lignieresii 202	bovine lumpjaw	KSU clinical isolate
A. suis 412	equine resp. disease	KSU clinical isolate
Hist. ovis A	ovine	Univ. of California[4]
9L	ovine vagina	Univ. of California[4]
H. agni 902	ovine septicemia	Univ. of California[4]
1344	ovine septicemia	Univ. of California[4]
H. somnus 1048	bovine semen	KSU clinical isolate
1227	bovine semen	KSU clinical isolate
B. ovis 25840	ovine	ATCC
E. coli Y1090		Promega Biotec[5]

[1] American Type Culture Collection, Rockville, MD
[2] On hand at KSU since March, 1979
[3] Kansas State University, College of Veterinary Medicine, Manhattan, KS
[4] Kindly supplied by Caroline Kirkham, Department of Veterinary Microbiology, Davis, CA
[5] 2800 S. Fish Hatchery Rd., Madison, WI

TABLE 2. Bacteriological characteristics of the AHH group bacteria and other species.

	A. seminis					other Actinobacilli[1]				*B. ovis*
	15768	501	502	503	504	29522	27072	202	412	25840
Pigmentation	gw	gw	gw	gw	gw	wh	gw	gw	gw	w
Aerobic growth	+	+	+	+	+	-	+	+	+	-
Growth on BHI agar	+	+	+	+	+	s	+	+	+	-
X or V factor req.	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	-
Indole	-	-	-	-	-	-	+	-	-	-
Nitrate	+	+	+	+	+	+	+	+	+	-
Urea	-	-	-	-	-	-	+	+	+	-
Ornithine decarb.	+	+	+	+	+	-	-	-	-	-
Lysine decarb.	-	-	-	-	-	-	-	-	-	-
Arginine dihydro.	-	-	-	-	-	-	-	-	-	-
MR-VP	-	-	-	-	-	-	-	-	-	-

continued on following page

TABLE 2. continued

	Hist. ovis		H. agni		H. somnus	
	A	9L	902	1344	1048	1227
Pigmentation	y	y	y	y	y	y
Aerobic growth	a	a	a	s	a	a
Growth on BHI agar	-	-	-	s	-	-
X or V factor req.	-	-	-	-	-	-
Catalase	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Indole	w	w	w	w	+	+
Nitrate	+	+	+	+	+	+
Urea	-	-	-	-	-	-
Ornithine decarb.	+	+	+	+	+	+
Lysine decarb.	-	v	-	-	-	-
Arginine dihydro.	-	-	-	-	-	-
MR-VP	-	-	-	-	-	-

[1]29522, A. actinomycetemcomitans; 27072, Actinobacillus sp.; 202, A. lignieresii; 412, A. suis.

Symbols: +, positive reaction; -, negative reaction; gw, gray-white; wh, white; y, yellow; s, slight growth; w, weak reaction; v, variable reaction; a, adaptable with subculture

TABLE 3. Carbohydrate fermentation reactions of the AHH group bacteria

	A. seminis					*Hist. ovis*		*H. agni*		*H. somnus*	
	15768	501	502	503	504	A	9L	902	1344	1048	1227
Glucose	-	-	-	-	w	+	+	+	w	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	w	+	-	w	-	-	-	-
Arabinose	+	-	w	w	+	-	-	-	-	-	-
Mannitol	-	-	-	-	+	w	-	w	-	w	-
Galactose	-	-	-	-	+	-	-	-	-	-	-
Mannose	-	-	-	-	-	w	+	+	w	+	w
Xylose	+	-	-	w	+	w	w	+	w	w	w

Symbols: +, positive (production of acid); -, negative; w, weak positive reaction

TABLE 4. Phenotypic characterization of the AHH group bacteria using the API 20E system.

	A. seminis					other Actinobacilli[1]			
	15768	501	502	503	504	29522	27072	202	412
B-galactosidase	-	-	-	-	-	-	+	+	+
Arginine dihydro.	-	-	-	-	-	-	-	-	-
Lysine decarb.	-	-	-	-	-	-	-	-	-
Ornithine decarb.	+	+	+	+	+	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	+	+	+
Tryptophane deam.	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-	-
Gelatin liq.	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	-	-	+
Mannitol	-	-	-	+	+	-	-	+	+
Inositol	+	+	+	+	+	-	-	-	-
Sorbitol	-	-	-	+	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-	+	+
Melibiose	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	+
Arabinose	-	-	-	-	-	-	-	-	-
Nitrate	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+

continued on following page

TABLE 4. continued

	Hist. ovis		H. agni		H. somnus		B. ovis	E. coli
	A	9L	902	1344	1048	1227	25840	Y1090
B-galactosidase	-	-	-	-	-	-	-	-
Arginine dihydro.	-	-	-	-	-	-	-	-
Lysine decarb.	-	-	-	-	-	-	-	+
Ornithine decarb.	+	+	+	+	+	+	-	-
Citrate	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-
Tryptophane deam.	-	-	-	-	-	-	-	-
Indole	+	+	+	+	+	+	-	+
VP	-	-	-	-	-	-	-	-
Gelatin liq.	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	-	+
Mannitol	+	+	+	+	+	+	-	+
Inositol	+	+	-	-	+	+	-	-
Sorbitol	+	+	+	+	+	+	-	+
Rhamnose	-	-	-	-	-	-	-	+
Galactose	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	+
Arabinose	-	-	-	-	-	-	-	-
Nitrate	+	+	+	+	+	+	-	+
Oxidase	+	+	+	+	+	+	-	-
Catalase	-	-	-	-	-	-	+	+

[1]29522, A. actinomycetemcomitans; 27072, Actinobacillus sp.; 202, A. suis; 412, A. ligniersii

FIG. 1. Cellular morphology of the AHH group bacteria. Gram-stained cells from chocolate agar cultures incubated at 37°C in 10% CO_2.

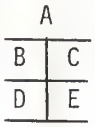
A. Actinobacillus seminis (ATCC strain 15768)

B. Histophilus ovis (strain 9L)

C. Haemophilus somnus (strain 1227)

D. Haemophilus agni (strain 902)

E. Escherichia coli (for comparison)



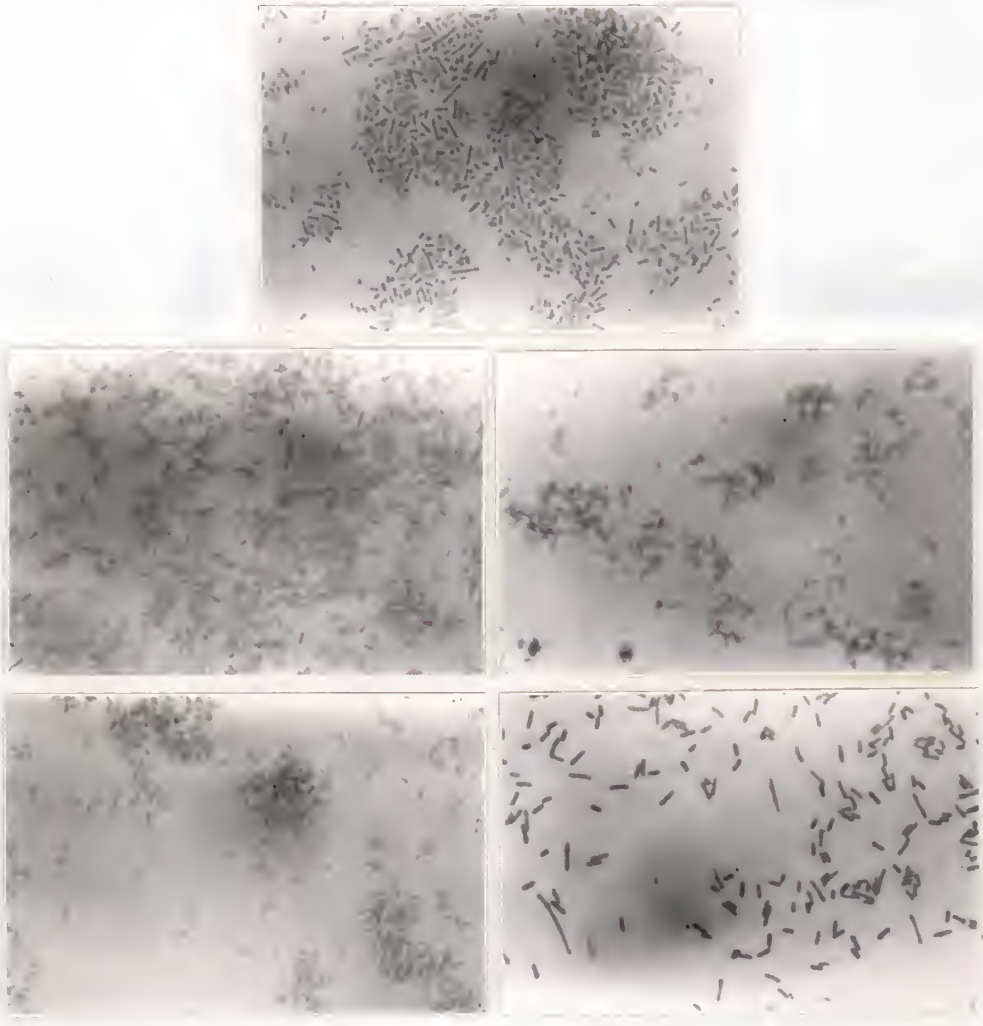


FIG. 2. Colony morphology of the AHH group bacteria. Photographs are of 24 h. chocolate agar cultures incubated at 37°C in 10% CO_2.

A. Actinobacillus seminis (ATCC strain 15768)

B. Histophilus ovis (strain A)

C. Haemophilus somnus (strain 1048)

D. Haemophilus agni (strain 1344)

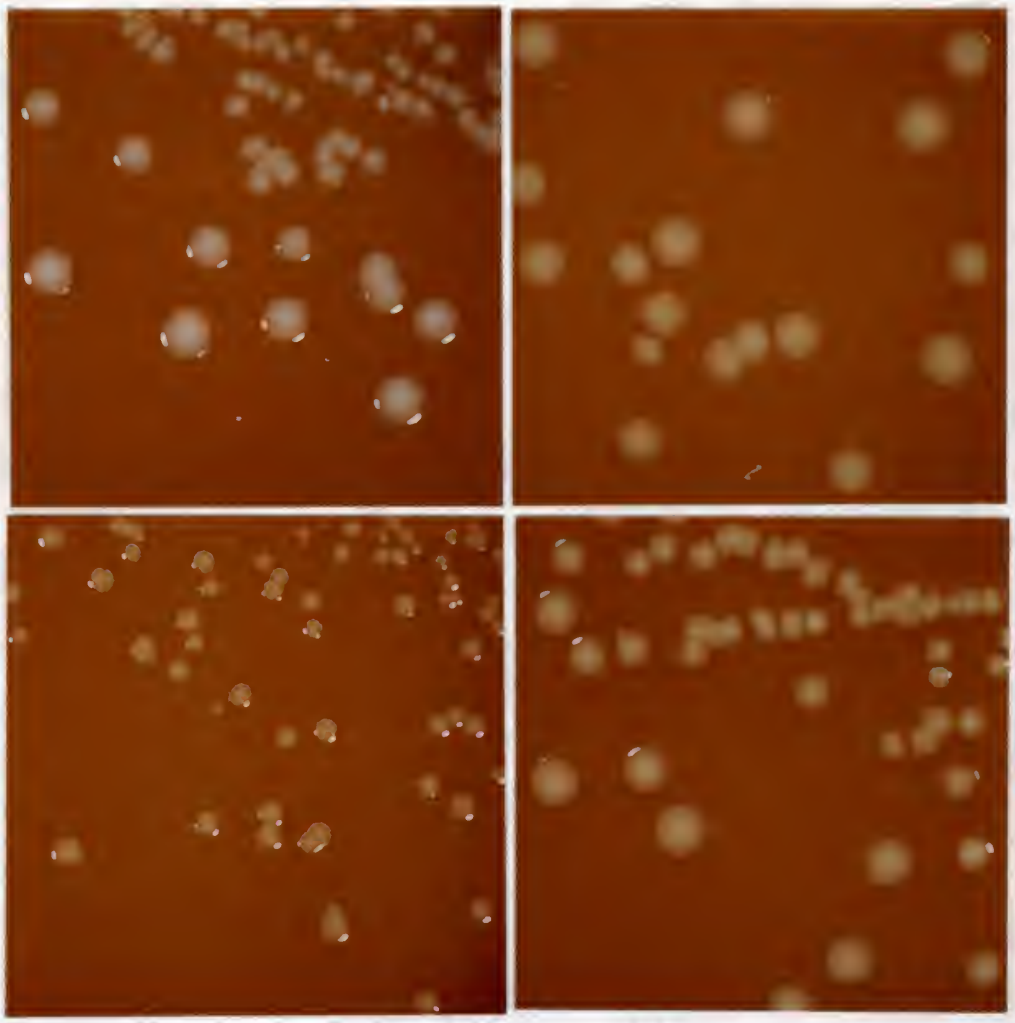
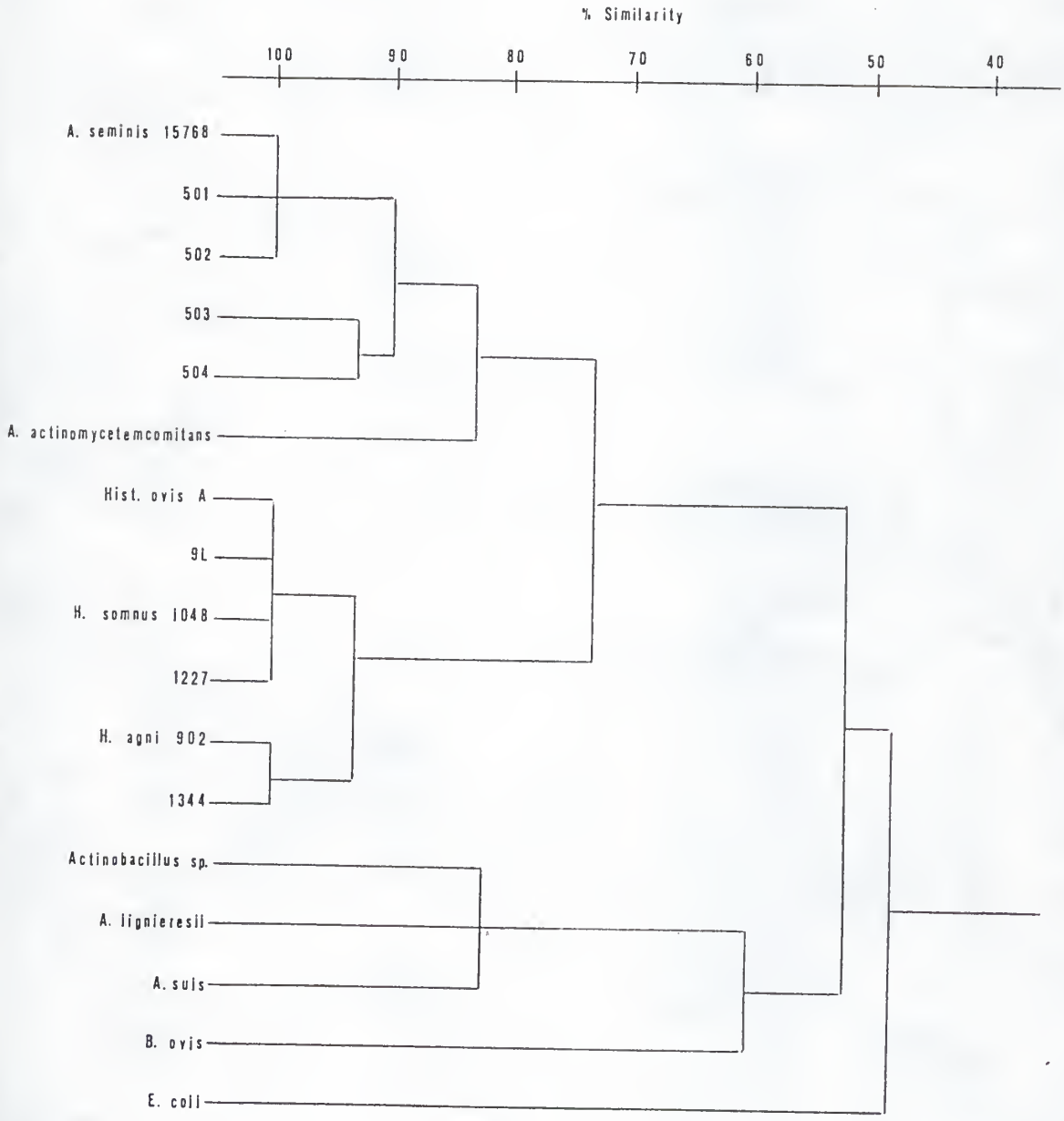


FIG. 3. Similarity matrix based on the API 20E Identification system. Values were calculated after elimination of all reactions negative for all strains. Strains: 15768, 501, 502, 503, and 504, A. seminis; A and 9L, Hist. ovis; 1048 and 1227, H. somnus; 902 and 1344, H. agni; 29522, A. actinomycetemcomitans; 412, A. suis; 202, A. lignierseii; Y1090, E. coli; 25840, B. ovis; 27072, Actinobacillus sp.

	% similarity with strain:															
Strain	15768	501	502	503	504	25922	A	9L	1048	1227	902	1344	27072	202	412	25840	Y1090
15768	100																
501	100	100															
502	100	100	100														
503	93	93	93	100													
504	87	87	87	93	100												
29522	87	87	87	80	73	100											
A	73	73	73	80	87	60	100										
9L	73	73	73	80	87	60	100	100									
1048	73	73	73	80	87	60	100	100	100								
1227	73	73	73	80	87	60	100	100	100	100							
902	67	67	67	73	80	67	93	93	93	93	100						
1344	67	67	67	73	80	67	93	93	93	93	100	100					
27072	67	67	67	60	53	80	40	40	40	40	47	47	100				
202	53	53	53	60	53	67	40	40	40	40	47	47	87	100			
412	53	53	53	60	53	67	40	40	40	40	47	47	73	87	100		
25840	67	67	67	60	53	80	40	40	40	40	47	47	73	60	47	100	
Y1090	40	40	40	47	53	53	53	53	53	53	60	60	33	33	47	47	100

FIG. 4. Dendrogram derived by average linked cluster analysis of similarity matrix based on the API 20E system.


% Similarity
100
90
80
70
60
50
40
A. seminis 15768
501
502
503
504
A. actinomycetemcomitans
Hist. ovis A
9L
H. somnus 1048
1227
H. agni 902
1344
Actinobacillus sp.
A. lignieresii
A. suis
B. ovis
E. coli

III. PROTEIN PROFILE HOMOLOGY AMONG THE GRAM-NEGATIVE PLEOMORPHS ASSOCIATED WITH OVINE EPIDIDYMITIS

Actinobacillus seminis (Baynes and Simmons, 1960), Histophilus ovis (Roberts, 1956), Haemophilus somnus (Bailie, 1969), and Haemophilus agni (Kennedy et al., 1958) constitute a group in that all are Gram-negative, pleomorphic bacteria associated with ovine epididymitis (Zeki et al., 1981; Burgess, 1982; Webb, 1983a; Bulgin and Anderson, 1983), particuarly in ram lambs (Bagley et al., 1985; Walker et al., 1986). Moreover, none of these species are recognized taxonomically (Kilian and Biberstein, 1984; Phillips, 1984), and it can be difficult to differentiate between them (Hughes et al., 1971; Dennis, 1974; Rahaley and White, 1978; Bruere et al., 1977; Webb, 1983a; Stephens et al., 1983). It has been suggested A. seminis and Hist. ovis are biochemical variants of the same species (Breure et al., 1977; Webb, 1983a), and that Hist. ovis, H. somnus, and H. agni belong in a single species (Stephens et al., 1983). Genotypic studies support the latter hypothesis (Walker et al., 1986; Piechulla et al., 1986), and these organisms have been referred to collectively as the "HH group" (Stephens et al., 1983; Walker et al., 1985) and the "Histophilus ovis group" (Piechulla et al., 1986). Although A. seminis does not appear to be genotypically related (Walker et al., 1985), its phenotypic similarity to the "HH group" makes their differentiation both necessary and difficult.

Historically, bacterial taxonomy has relied on cultural, morphological and metabolic characteristics (Zinneman, 1981). These

methods, while essential, are not always sufficient for differentiating closely related strains (Pohl, 1981), and do not necessarily reflect phylogenetic relationships (Johnson, 1984).

Since a limited portion of the bacterial genome is reflected by conventional phenotypic tests (Razin and Rottem, 1967; Mannheim, 1981), techniques aimed at a more comprehensive comparison have been developed. Among these are methods that demonstrate physical properties of DNA, most notably the relative proportion of guanine plus cytosine. This value, expressed as a mole percent (mol % G + C), is a constant parameter for any given species (Johnson, 1984), but is not specific enough to establish identity (Bailie et al., 1973), and does not reflect nucleotide sequence (Johnson, 1984). Therefore, while two closely related species are expected to have similar mol % G + C values, the opposite (that two species with similar values are closely related) is not true (Johnson, 1984).

The most definitive method for demonstrating taxonomic relationships is nucleic acid hybridization. The basic question is whether or not two strains have a nucleotide sequence sufficiently similar to allow the formation of stable DNA heteroduplexes or RNA/DNA hybrids (Johnson, 1984). DNA hybridization has proved more useful because it compares complete genomes (Johnson, 1984), but the technique is relatively complex and not readily applicable as a routine identification protocol (Mannheim, 1981; Lema and Brown, 1983).

The overwhelming committment of bacterial DNA to the production of mRNA transcripts, and the lack of extensive post-transcriptional modification (Burns, 1980), suggests the bacterial genome would be directly reflected in the protein content of the cell (Nicolet et al., 1980). Several authors successfully tested that hypothesis by polyacrylamide gel electrophoresis, and the results using reference strains generally agree with those obtained by other methods (Jarvis and Wolff, 1979; Sayed and Kenny, 1980; Seiter and Jay; 1980; Nicolet et al., 1980; Moore et al., 1980; Biavati et al., 1982; Lema and Brown, 1983). Cato et al. (1982) found that strains with greater than 80% DNA homology usually expressed identical protein profiles, while those with 70% homology had heterologous but similar patterns. Moreover, strains unrelated by DNA homology but phenotypically similar often expressed protein patterns more alike than strains with different phenotypic properties. The technique also allows identification of proteins of particular taxonomic or biologic interest.

The purpose of this paper was, therefore, to compare the cellular protein profiles of the Gram-negative pleomorphs associated with ovine epididymitis to determine whether taxonomic groups could be defined by the degree of homology between strains and, secondly, whether those groups conform to the results of conventional bacteriological tests.

MATERIALS AND METHODS

All strains were grown on chocolate agar at 37°C in an atmosphere of 10% CO_2 in air. Identity was confirmed by standard bacteriological methods (Section II). Pure cultures were expanded into lawn cultures, and cells were harvested after 48 hrs. by gently collecting the growth with a sterile, bent pasteur pipet. Cells were suspended in sterile physiological saline, mixed by vortexing, and recovered by refrigerated centrifugation at 10,000xg for 40 minutes. Pellets were resuspended in sterile saline, briefly sonicated to disperse the cells, and brought to visually equal densities with saline.

Soluble proteins were prepared by ultrasonic disruption[1] at 100% capacity for 5 min. in one min. cycles. Cells were held on ice during sonication, and cooled on ice for 10 min. between cycles. The debris was removed by refrigerated centrifugation at 10,000xg for 40 minutes. The supernate was sterililzed by filtering through a 0.22um filter, and the protein content determined using the BCA protein assay[2] with bovine serum albumin as the standard. Antigen preparations were standardized to 1.0 mg protein/ml by dilution with saline and stored frozen in aliquots at -70°C.

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[1]Sonifier Cell Disruptor, Heat Systems Co., Melville, NY

[2]Pierce Chemical Co., Rockford, IL

All gel reagents were electrophoresis grade[3]. Discontinuous gels (Laemmli, 1970) were prepared from a 30%T, 2.67%C polyacrylamide/N,N'-methylene bisacrylamide stock solution. Buffers were as follows: separating gel, 1.5M Tris-HCl (pH 8.8); stacking gel, 0.5M Tris-HCl (pH 6.8); running buffer, 0.025M Tris-HCl, 0.192M glycine, 0.1% SDS. Stacking gels (4%T,2.67%C) were made with 12.2 ml distilled H_2O, 5.0 ml Tris buffer (pH 6.8), 2.66 ml acrylamide/bisacrylamide stock, 0.2 ml 10% (w/v) sodium dodecyl sulfate (SDS), 0.1 ml 10% (w/v) ammonium persulfate (AMP), and 0.01 ml N,N,N'N'-tetramethylethylenediamine (TEMED). Separating gels 10%T,2.67%C) were made with 24.1 ml dist. H_2O, 20.0 ml acrylamide/bisacrylamide stock, 15.0 ml Tris buffer (pH 8.8), 0.6 ml 10% SDS, 0.300 ml 10% AMP, and 0.020 ml TEMED. Gel solutions were degassed for 30 min. before adding AMP and TEMED. Sample buffer consisted of 18.8 ml dist. H_2O, 4.0 ml Tris buffer (pH 6.8), 4.0 ml 10% SDS, 5.0 ml glycerol, and 10% (v/v) 2-mercaptoethanol. Samples were prepared by adding 100 ul antigen preparation to 75 ul sample buffer. The volume was brought to 200 ul by adding 20 ul (10 ug) of a 116 kilodalton (K) beta-galactosidase molecular weight marker[4] and 5 ul of a 1.0 mg/ml phenol red solution. The final bacterial protein concentration was 0.5 mg/ml; 80 ul samples added to each well constituted a total of 40 ug

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[3]Polysciences Inc., Warrington, PA

[4]Sigma Chemical Co., St. Louis, MO

bacterial protein/well. Samples were heated in a boiling water bath for 2 min., and allowed to cool before adding the tracking dye.

Gels (1.5 mm thick) were prepared on a Bio-Rad Model 220 electrophoresis unit[5]. Separating gels were poured in the afternoon and layered with distilled water. After 1-2 hrs., the water was replaced with an overlay solution consisting of 0.1% SDS in a 1:4 dilution of Tris buffer (pH 8.8). Polymerization was continued overnight at room temperature. The following morning, the overlay was removed, the gel surface was rinsed with buffer, and the stacking gel was poured. The stacking gel was allowed to set for 30 min. before the upper buffer chamber was filled with running buffer. Polymerization was continued for 60 min., at which time the running buffer was replaced, and the 20 well comb was removed. Wells were rinsed three times with running buffer before samples were loaded with a 100 ul Hamiliton syringe. All gels were run with the same pattern; the inner 14 wells contained bacterial protein solutions, and the outer three wells on either side contained molecular weight markers[4]. The two outermost wells were sometimes distorted and were ignored in the subsequent analysis.

Gels were stacked at 12.5 mA/gel and, once the dye front had entered the separating gel, run at 20 mA/gel. The total run time for the 8.5 cm gel was approximately 5 hrs. Gels were stained overnight at room temperature with 0.5% (w/v) Coomassie Brillant Blue in 25%

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[5]Bio-Rad Laboratories, Richmond, CA

isopropyl alcohol and 10% acetic acid, and destained for at least 12 hrs. in 10% acetic acid.

Gels were photographed prior to scanning. Scans were prepared by cutting each lane from the gel, laying the slice on edge along the side of a cuvette filled with water, and scanning at 578 nm in a Gilford Model 2400 spectrophotometer[6]. Optimal instrument settings were: slit width, .11-.12 mm; chart speed, 2 in./min.; and scan speed, 2 cm/min.

Gels were evaluated both visually from photographs and numerically from scans. Numerical analysis was done by two methods. The first was adapted from that of Seiter and Jay (1980), and based on relative mobility (R_f) values. Intragel variability was estimated from a gel in which all lanes contained the same extract (A. seminis 15768). Scans were made from each lane, and the R_f value of each peak was determined. Assuming that Rf values were normally distributed and the variance in each band was equal, a 95% confidence limit was constructed. Comparison of R_f values for the same band in different lanes of the same gel showed that, in order to conclude that two bands had different relative mobilities and, therefore, different molecular weights, their R_f values had to differ by .005 or more.

Bands were classified as major, intermediate, and minor on the basis of extinction coefficient (peak height). Classes were defined relative to a reference peak from strain 15768; on each gel, the height

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[6]Gilford Instruments Laboratories Inc., Oberlin, OH

of that peak was measured, and classes were assigned as major (greater than 50% of that height), intermediate (between 25 and 50%), and minor (less than 25%). In the subsequent analysis, two bands were scored as a positive match only if their R_f values were within .005 and they were within one class (i.e. an R_f match between a major and minor band was discounted). All interstrain comparisons were made. Homology values were determined using the Jaccard similarity coefficient (S_J) (Goodfellow, 1977; Seiter and Jay, 1980). Finally, homology values were organized in a similarity matrix, and a dendrogram was constructed by average linked cluster analysis (Goodfellow, 1977).

The second analysis was modified from that of Kersters and DeLey (1975). A horizontal baseline was drawn at the lowest extinction coefficient, and a vertical line through the peak of the marker was extended to meet this baseline. Each scan was reproduced as a transparency, and a numerical description of the peak profile was made by laying the transparency over a piece of graph paper scaled at 20 squares/inch (1.27 mm/square). The distance between the marker and dye front peaks was converted to 17.85-18.05 cm by scanning. Allowing for both the marker and dye front, the scan was converted into a series of 136 equidistant points. Scans were "normalized" (Kersters and DeLey, 1975) by aligning the midpoint of each scan at position 68. The extinction coefficients at each point was then read directly from the graph paper. The protein profile of each strain was thus converted into a sequence of 136 numbers, each reflecting the extinction coefficient at a given point. Profiles were compared by determining the Pearson

product-moment correlation coefficient (r) between all pairs of strains (Sokal and Rohlf, 1969). These values were recorded in a similarity matrix for construction of a dendrogram based on average linked cluster analysis (Goodfellow, 1977).

RESULTS

Gels were reproducible between runs. The requirement for peak consistency over three scans eliminated only minor peaks usually associated closely with proteins of similar molecular weight. The appearance of some peaks varied between scans in terms of extinction coefficient, but that was considered an artifactual result of sample preparation rather than a reliable indicator of relative concentration. Where a number of peaks occurred in close association with each other, there were sometimes minor differences in band resolution, with one that appeared as a well-defined peak on one scan occurring as a "shoulder" on another (Fig. 1). Such differences were more obvious from scans than from gels.

Numerical assessement of intergel variability was difficult because R_f values were not directly comparable. The molecular weight of each band from two strains was determined from each of three gels; the coefficient of variation ranged from 0.24 to 4.70 (average = 1.22). The S_J value between A. seminis strains 15768 and 501 was consistent whether determined from a single gel or from the average R_f values of three gels.

Visually, obvious similarities existed among both the HH group bacteria and the A. seminis strains (Fig. 2). Table 1 lists the molecular weights of proteins found to be consistent among different groups (Fig. 2, arrows). Of 21 bands listed, three (65.0K, 60.8K, and 37.2K) were common to all 11 strains of the AHH group. Eleven were shared by all A. seminis strains; 7 of those were unique to the species. The HH group was defined by 3 bands (81.6K, 71.7K, and 25.7K), and 3 additional bands were unique to H. somnus (51.7, 50.0, and 21.7). Taken together, two bands (27.5K and 23.0K) were unique to Hist. ovis and H. agni.

The similarity matrix derived from R_f values is shown in Fig. 4. Values ranged from .093 (H. agni 1344 vs. A. actinomycetemcomitans) to .579 (Hist. ovis A vs. Hist. ovis 9L). The associated dendrogram is presented in Fig. 5.

The Hist. ovis strains were most closely associated with each other (.579), and, although H. agni 1344 was more homologous with them (.562) than with H. agni 902 (.556), all four exhibited a relatively high degree of similarity (.514). The H. somnus strains were also most similar to each other (.514) and, secondarily, to the remainder of the HH group (.268).

A second, less homologous group consisted of the five A. seminis strains. Two subgroups were defined by this method: strains 502, 503, and 504 were most closely related to each other (.481), as were strains 501 and 15768 (.455). Homology between the two groups was lower (.291), although that between the type strain (15768) and all others was .354.

Intergroup similarity between A. seminis and the HH group bacteria was low (.165).

The protein profiles of Brucella ovis, A. actinomycetemcomitans, and the Actinobacillus sp. of Ross (strain 27072) were relatively unique. A. actinomycetemcomitans and strain 27072 were most closely related to each other (.271) and then to the A. seminis strains (.218).

Phenetic clusters defined by the Pearson product-moment correlation coefficient were strikingly similar to those described above (Figs. 6 and 7). A group was defined consisting of the H. agni and Hist. ovis strains (intragroup similarity = .688), however, the relationships within that group were different. Histophilus ovis 9L and H. agni 902 were most similar to each other (.873), and then to Hist. ovis A (.821). Haemophilus agni 1344 was more removed from the group by this analysis (.537). Likewise, the H. somnus strains were most similar to each other (.869), and to a lesser extent with the other members of the HH group (.410).

The A. seminis cluster was essentially identical to that described above. Strains 502, 503, and 504 constituted a group (.732) set apart from strains 501 and 15768 (.681). The homology between the two was .534.

The major difference between analytical methods was in the clustering of A. actinomycetemcomitans; based on correlation coefficients, it was more similar to A. seminis (.455) than to strain 27072 (.164). The intergroup similarity between the HH group and the "A. seminis group" (including A. actinomycetemcomitans) was low (.282),

and both B. ovis and strain 27072 were classified independently of either.

DISCUSSION

The method of preparing soluble extracts was chosen for its ease and because a similar extract prepared from Brucella ovis cells was reactive in an enzyme-linked immunosorbent assay for ovine antibodies (Rahaley et al., 1983). Although chemical analysis was not done, an antigen prepared by the same method contained principally proteins and nucleic acids (Afzal et al., 1984).

Centrifugation of harvested cells yielded a supernate that contained protein. Moreover, that from the yellow-pigmented Histophilus/Haemophilus strains had a yellow tinge, suggesting that strain specific proteins might be lost in washing. Since such proteins were likely to be associated with the outer membrane, and possibly of antigenic importance (Chapter 3), and since bacterial cells harvested from solid media are not necessarily contaminated by the media (Krieg and Gerhardt, 1981), it was felt that extensive washing was unnecessary and possibly detrimental. Whether or not the failure to extensively wash the cells prior to disruption affected the outcome is difficult to assess, however, there were no components detectable either visually or spectrophotometrically in all samples.

Pilot studies were done to ensure the addition of the molecular weight marker (beta-galactosidase) to each sample preparation did not

interfere with bacterial proteins. A sample was run consisting only of the marker in sample buffer in order to show that no contaminating components were introduced by either.

The observed intragel variability of R_f "plus or minus" .005 was unavoidable and probably due to limitations in scanning and measurement. Since molecular weight is a continuous characteristic, it is possible to have bands with very similar but distinct R_f values or, in contrast, to obtain slightly different R_f values for repeat samples of the same protein. Determination of variability by the method discussed is a statistical attempt to control the probability of concluding falsely that two bands had different molecular weights, and it allowed determination of overall similarity between different strains. It did not, however, allow the identification of individual proteins (i.e. bands).

Although most authors who used polyacrylamide gel electrophoresis as a taxonomic tool relied on visual comparison (Razin and Rottem, 1967; Morris, 1973; Daniels and Meddins, 1973; Sayed and Kenny, 1980; Nicolet et al., 1980; Moore et al., 1980; Biavati et al., 1982; Mocca and Frasch, 1982; Cato et al., 1982; Megraud et al., 1985), protein profile homology derived from R_f values and expressed as a similarity coefficient (S_J) has been reported. Seiter and Jay (1980) found that S_J values among species of Arthrobacter ranged from .207 to .345, while those of other coryneform bacteria, when compared to a type species of Arthrobacter, were lower (.079 to .185). The similarity between two

type strains of the same species was .571. Those values are not unlike the values obtained in this study.

Use of the Pearson product moment correlation coefficient has also been reported (Kersters and DeLey, 1975; Jarvis and Wolff, 1979; Lema and Brown, 1983). Both Kersters and DeLey (1975) and Jarvis and Wolff (1979) performed their analysis from tube gels, which required normalization and a process referred to as "compensation". Compensation was presumably required to correct errors due to something other than the overall length of gels (e.g. the exact mixture of gel ingredients, buffers, voltage, etc.). Since all comparisons in this study were made from the same slab gel, those undefined factors were controlled, and compensation did not seem to be necessary. However, the correlation coefficients reported by those authors were significantly increased by normalization and compensation (as opposed to normalization alone), and it is questionable whether the values obtained here are directly comparable.

In this study, no two strains were related at more than .880, and the type culture of A. seminis (15768) and a laboratory strain derived from it (501) were related at only .681. However, Jarvis and Wolff (1979) found that two strains had to be related at less than .800 before it could be concluded they were not the same strain, much less the same species.

The accumulated data in the literature suggests that H. agni, Hist. ovis and H. somnus are closely related, if not identical, species (Stephens et al., 1983; Walker et al., 1985; Piechulla et al., 1986).

The data presented here supports that conclusion. Stephens et al. (1983) reported that all members of the HH group contained major proteins with molecular weights of 75.0-95.0K, 34.0K, 28.0K, and 13.0K. Corboz and Wild (1981) reported that all H. somnus strains had major proteins with molecular weights of 53.0K, 36.2K, and 25.6K. The latter authors did not include strains of Hist. ovis or H. agni. Since minor differences in molecular weight can be accounted for by technical variation, it would appear that 5 proteins have been reported in association with the HH group bacteria: 75.0K-95.0K, 53.0K, 34.0-36.2K, 25.6-28.0K, and 13.0K. In this study, 13.0K was beyond the lower limit of resolution, therefore, it is not possible to confirm the existence of that protein. With regard to the others, the 53.0K protein reported by Corboz and Wild (1981) was observed here, and it was specific for H. somnus. Additional H. somnus-specific proteins were observed with molecular weights of 50.0K and 21.7K. A major protein with a molecular weight of 37.2K was also observed, however, it was common to all AHH strains including A. seminis. Stephens et al. (1983) did not report its presence in that organism. A 25.7K protein was observed that was specific for the HH group bacteria, as were two others with molecular weights of 81.6K and 71.7K. Although the latter are somewhat smaller than those reported by Stephens et al. (1983), the results reported here generally agree with the work of those authors.

Although highly related, H. somnus was distinguished from Hist. ovis and H. agni by specific proteins (Table 1), and by overall homology values (Figs. 5 and 7). With regard to the latter, the distinction was

arbitrary because there is no established level at which a species is defined. It is apparent, however, that these organisms exhibit similar protein profiles, and that Hist. ovis and H. agni are more related to each other than either is to H. somnus.

The A. seminis strains, while related most prominently to each other, constitute a relatively heterogenous group. There is some question in the literature concerning the existence of biotypes of A. seminis (Van Tonder, 1973; Walker et al., 1986), and the strains included here did fall into two reasonably well-defined groups on the basis of homology values. However, of 11 proteins common to all five strains of A. seminis, 7 were unique to the species (Table 1), and the results of earlier work failed to differentiate these organisms on the basis of bacteriological characteristics (Chapter 1).

Healey et al. (1986) reported the development of monoclonal antibodies to components of the outer membrane of A. seminis; three of these antibodies exhibited specificity for 10, 33, and 43K proteins. In this report, four A. seminis-specific proteins were observed ranging from 35.1 to 42.1K. Also, a major protein with a molecular weight of 37.2 was observed in common with the HH group bacteria. The biological significance of these proteins was not investigated, and which, if any, are important in the immune response is unknown.

Significant similarities have also been reported between A. seminis and members of the HH group. Webb (1983) suggested that A. seminis and Hist. ovis were biochemical variants of the same species. Piechulla et al. (1986) found over 90% DNA homology between the type strain of A.

seminis and one of two strains of H. agni. On the other hand, Stephens et al. (1983) were able to differentiate A. seminis from the HH group on the basis of several bacteriological tests, and Walker et al. (1986) found only 12.1% DNA homology between A. seminis and their strain of H. somnus. The results reported here agree with those of the latter authors; although several prominent proteins were identified that were common to all members of the AHH group (Table 1), the distinction between A. seminis and the HH group was supported both by overall homology values and the presence of specific proteins.

Lastly, certain isolates from ovine genital disease have been identified as A. actinomycetemcomitans (Bulgin and Anderson, 1983). Most have since been found to differ from the type culture of that organism, and it has been suggested they more closely resembled A. seminis (Bagley et al., 1985). It has also been suggested that many A. seminis strains could be classified as A. actinomycetemcomitans (Erasmus, 1983). The results presented here do not support that conclusion. Again, that is arbitrary in the absence of established homology levels, but it is supported by the results obtained in standard bacteriological tests (Chapter 1), and by genotypic data (Piechulla et al., 1986).

The technique itself has a number of advantages. It is relatively simple in that it allows investigation of any of a number of interstrain relationships in a single experiment without the need for isolating and radiolabelling nucleic acids. It could be useful, therefore, as a preliminary method for identifying taxonomic relationships that warrant more detailed investigation. Moreover, it allows identification of

significant proteins either unique to or shared between organisms. That is especially important when dealing with similar bacteria associated with the same or similar clinical conditions; characterization of such proteins could prove useful both diagnostically and therapeutically.

SUMMARY

The relationships existing among the AHH group bacteria were investigated by comparison of protein profiles obtained by polyacrylamide gel electrophoresis of soluble cellular extracts. The groups identified by this technique were consistent with the results of standard bacteriological tests; that is, the A. seminis strains were similar to each other and distinguishable from both the HH group strains and A. actinomycetemcomitans. Within the HH group, the Hist. ovis and H. agni strains were more similar to each other than either was to H. somnus. However, in the absence of established homology levels by which taxonomic divisions can be defined, it was not possible to divide the group further.

Protein bands were identified that were common to the AHH group and common to subdivisions within the group. The presence of those bands was consistent with overall homology values whether determined by band comparison (Rf values) or correlation coefficients. Although certain of these proteins could be of taxonomic or biologic interest, functional studies were not done.

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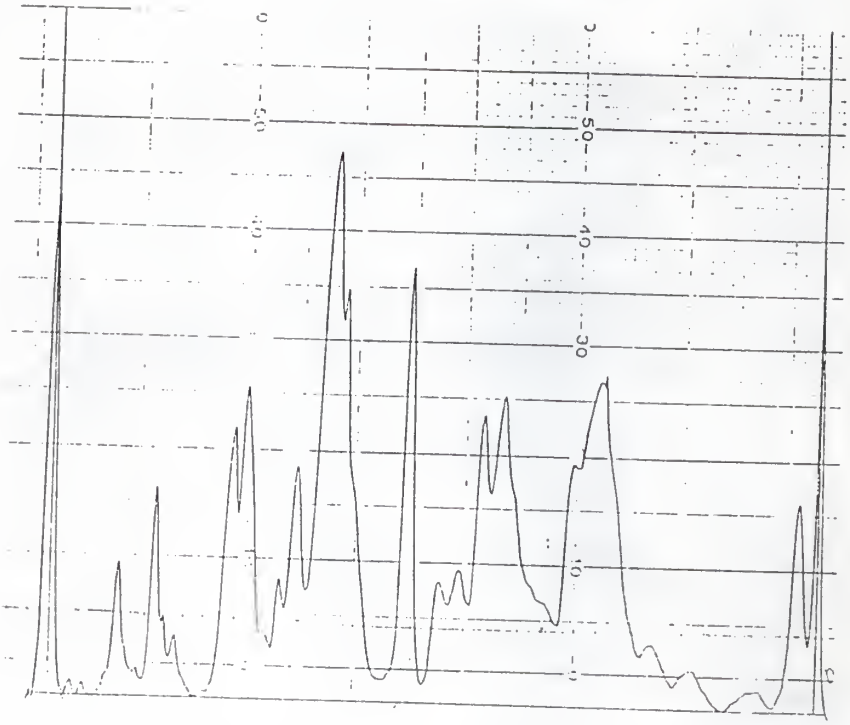
TABLE 1. Protein bands shared among species of the AHH group.

Molecular weight (x 1,000)	Species			
	A. seminis	Hist. ovis	H. agni	H. somnus
92.5	**********[1]			*********
82.9		**********		*********
81.6		**********	**********	**********
76.2	**********			
71.7		**********	**********	**********
65.0	**********	**********	**********	**********
60.8	**********	**********	**********	**********
55.0	**********			
51.7				*********
50.0				*********
48.0			**********	**********
42.1	**********			
40.9	**********			
39.5	**********			
37.2	**********	**********	**********	**********
35.1	**********			
27.5		**********		
25.7		**********	**********	**********
23.0		**********		
21.7				*********
16.7	**********			

[1]*, represents presence of a protein band of the indicated molecular weight in all strains of the species.

FIG. 1. Spectrophotometric scans of A. seminis (ATCC #15768) proteins fractionated by polyacrylamide gel electrophoresis. Scans from different gels are shown for comparison.



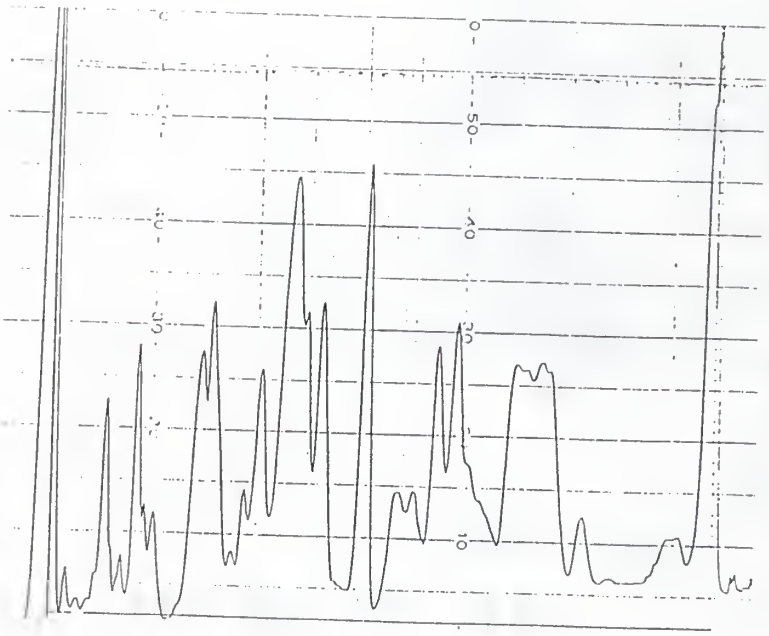


FIG. 2. Proteins of the AHH group bacteria and other species separated by polyacrylamide gel electrophoresis. Lanes: 1, B. ovis; 2, Actinobacillus sp. 27072; 3, A. actinomycetemcomitans; 4-8, A. seminis strains 504, 503, 502, 501, and 15768; 9-10, Hist. ovis strains A and 9L; 11-12, H. agni strains 902 and 1344; 13-14, H. somnus strains 1048 and 1227.

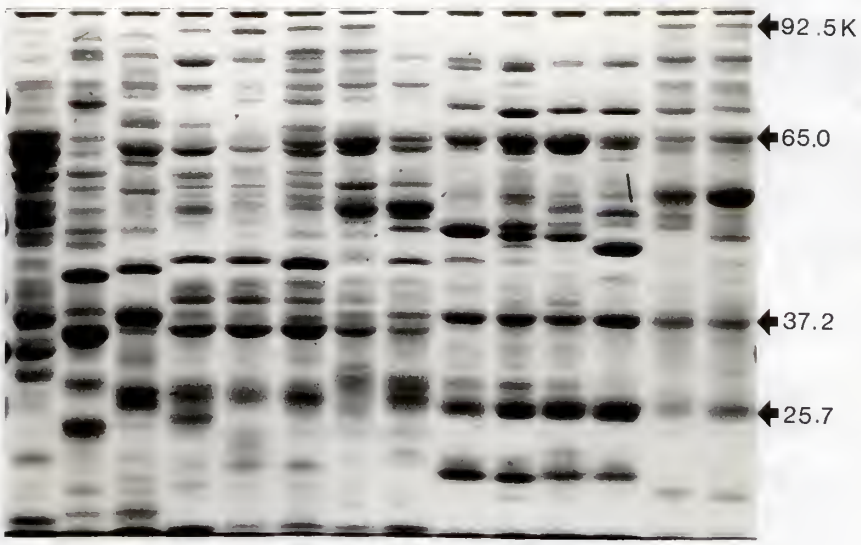
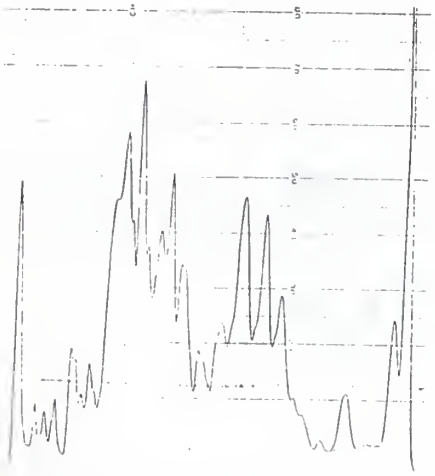
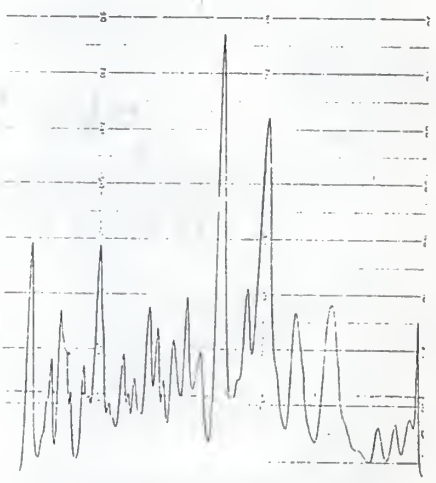

92.5K
65.0
37.2
25.7

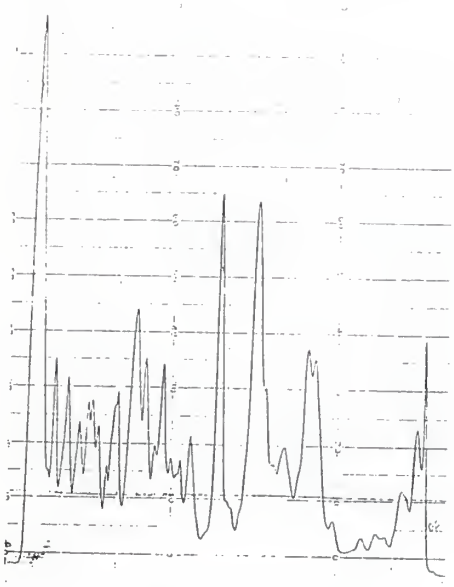
FIG. 3. Spectrophotometric scans of electrophoretically fractionated proteins of the AHH group bacteria and other species. Scans were produced by cutting each lane from the gel (Fig. 2) and scanning at 580 nm.



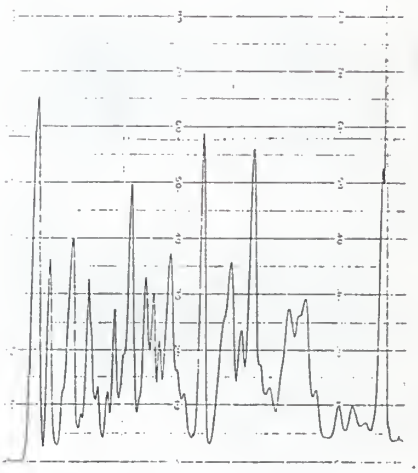
B. ovis



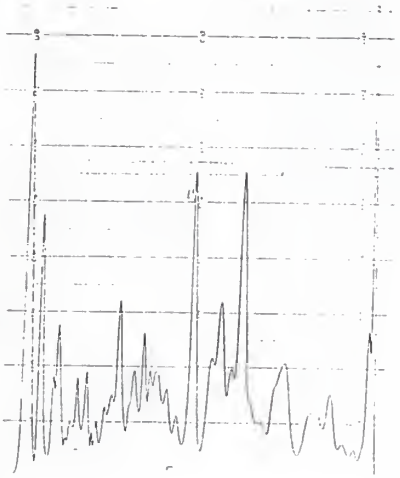
Actinobacillus sp. 27072



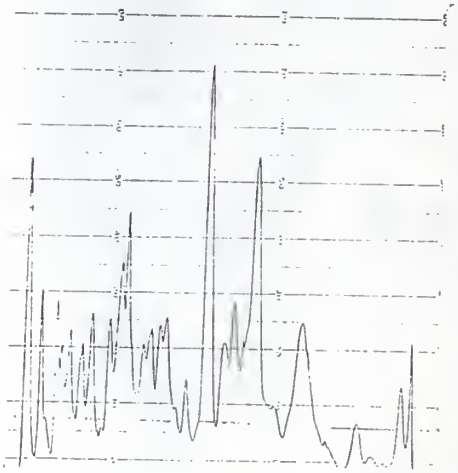
A. actinomycetemcomitans



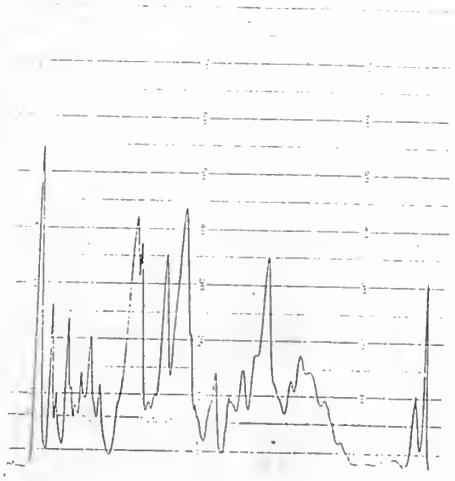
A. seminis 504



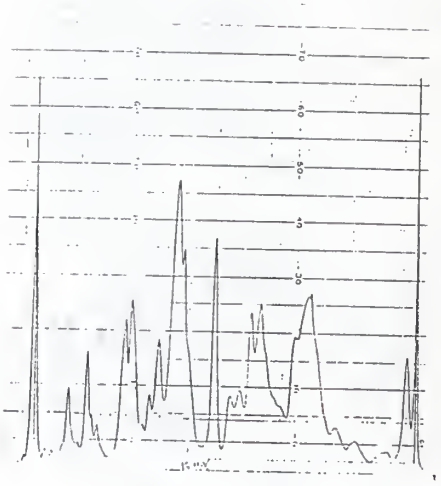
A. seminis 503



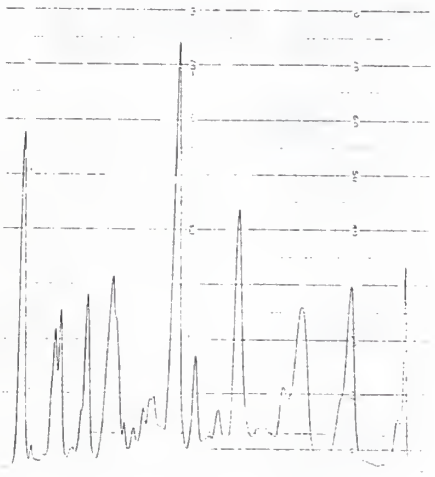
A. seminis 502



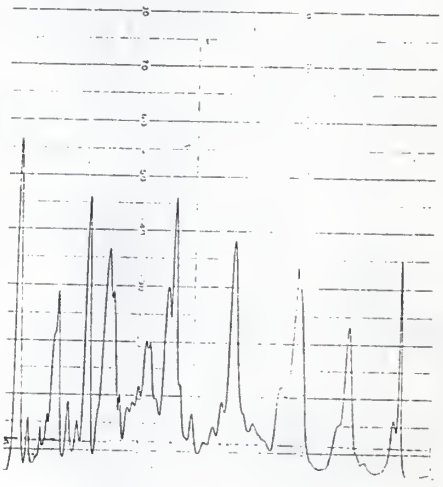
A. seminis 501



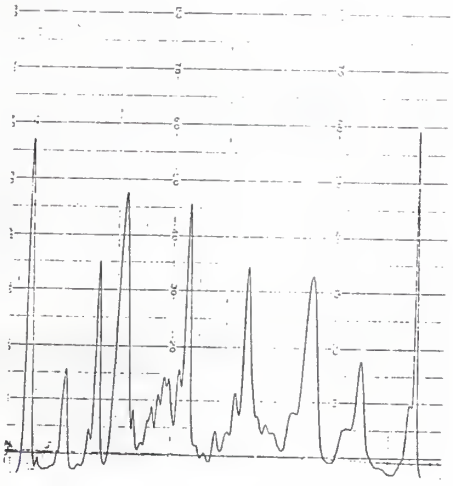
A. seminis 15768



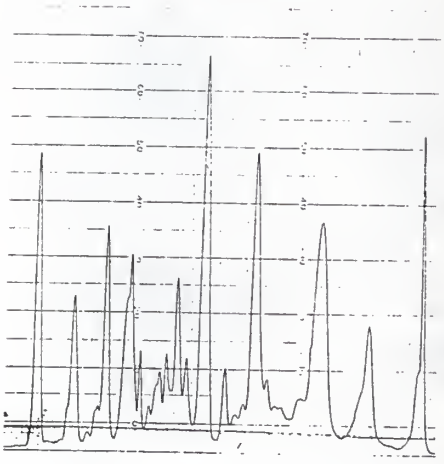
Hist. ovis A



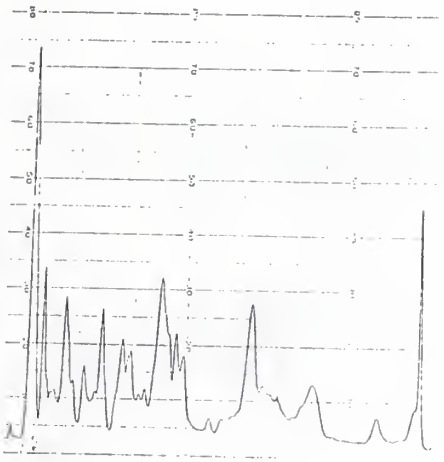
Hist. ovis 9L



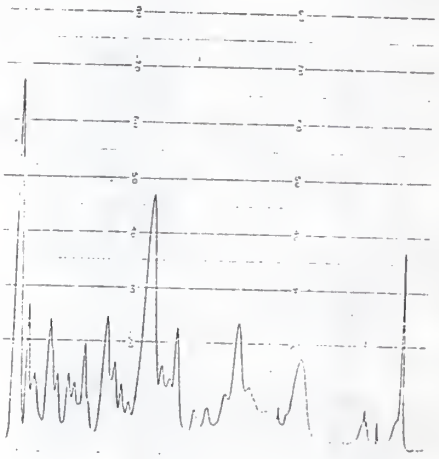
H. agni 902



H. agni 1344



H. somnus 1048



H. somnus 1227

FIG. 4. Similarity matrix based on protein profile homology derived from comparison of R_f values. Homology is expressed as the ratio of the number of shared bands over the total (S_J). Strains: 15768, 501, 502, 503, and 504, A. seminis; A and 9L, Hist. ovis; 902 and 1344, H. agni; 1048 and 1227, H. somnus; 25840, B. ovis; 29522, A. actinomycetemcomitans; 27072, Actinobacillus sp.

	Similarity with strain:													
Strain	A	9L	1344	902	1048	1227	25840	504	503	502	501	15768	29522	27072
A	1.000													
9L	.579	1.000												
1344	.571	.553	1.000											
902	.462	.525	.556	1.000										
1048	.326	.271	.333	.289	1.000									
1227	.279	.341	.138	.167	.514	1.000								
25840	.096	.371	.302	.184	.261	.098	1.000							
504	.140	.196	.167	.184	.137	.098	.184	1.000						
503	.156	.105	.189	.200	.111	.115	.176	.500	1.000					
502	.255	.145	.115	.132	.154	.137	.176	.500	.442	1.000				
501	.173	.204	.132	.176	.240	.148	.148	.292	.231	.280	1.000			
15768	.135	.189	.189	.176	.200	.261	.200	.227	.319	.248	.485	1.000		
29522	.200	.105	.593	.130	.151	.229	.196	.245	.189	.235	.300	.189	1.000	
27072	.239	.196	.273	.137	.184	.366	.137	.180	.176	.200	.265	.224	.271	1.000

FIG. 5. Dendrogram derived by average linked cluster analysis of protein profile homology based on Jaccard similarity coefficients (S_J).

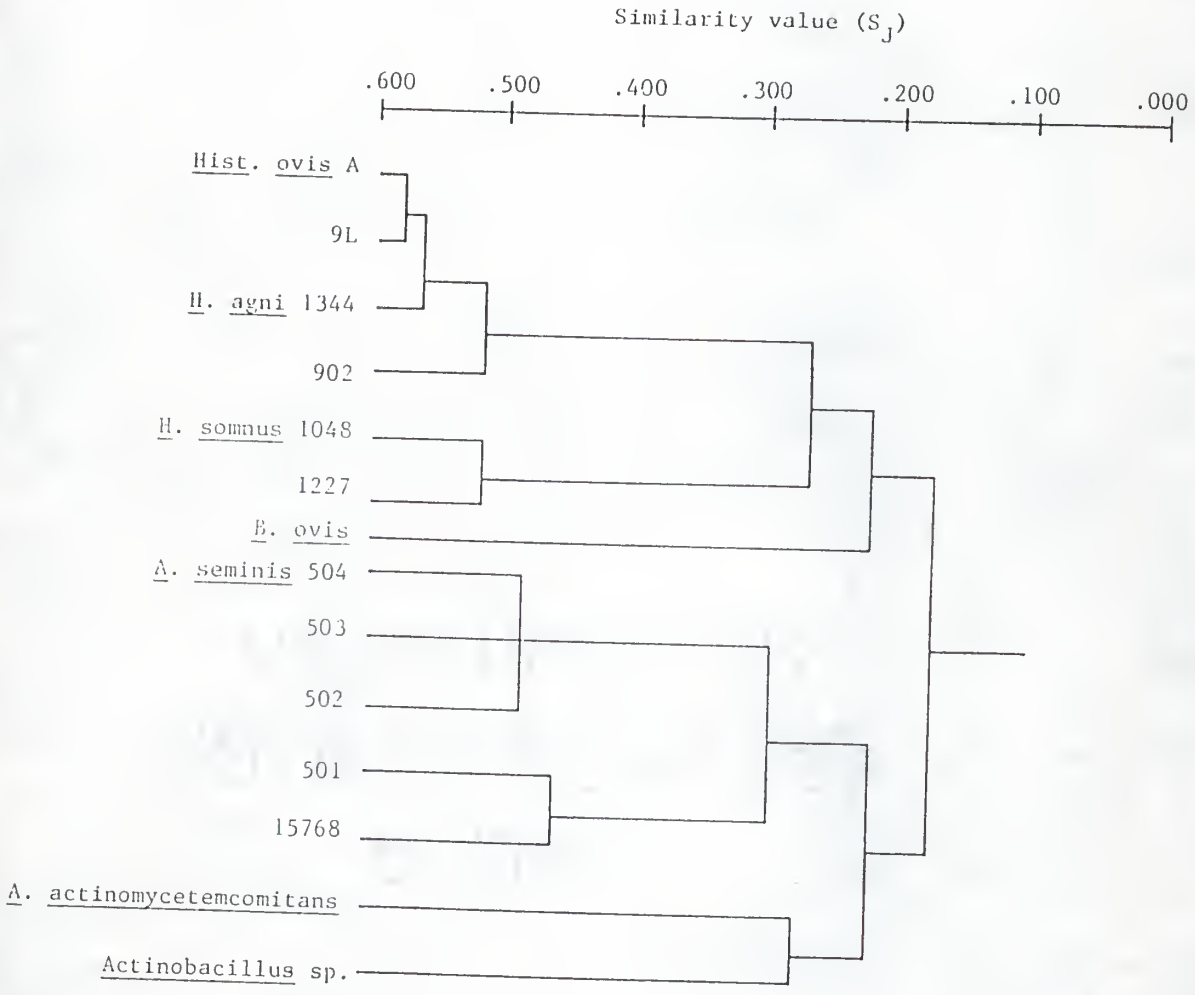
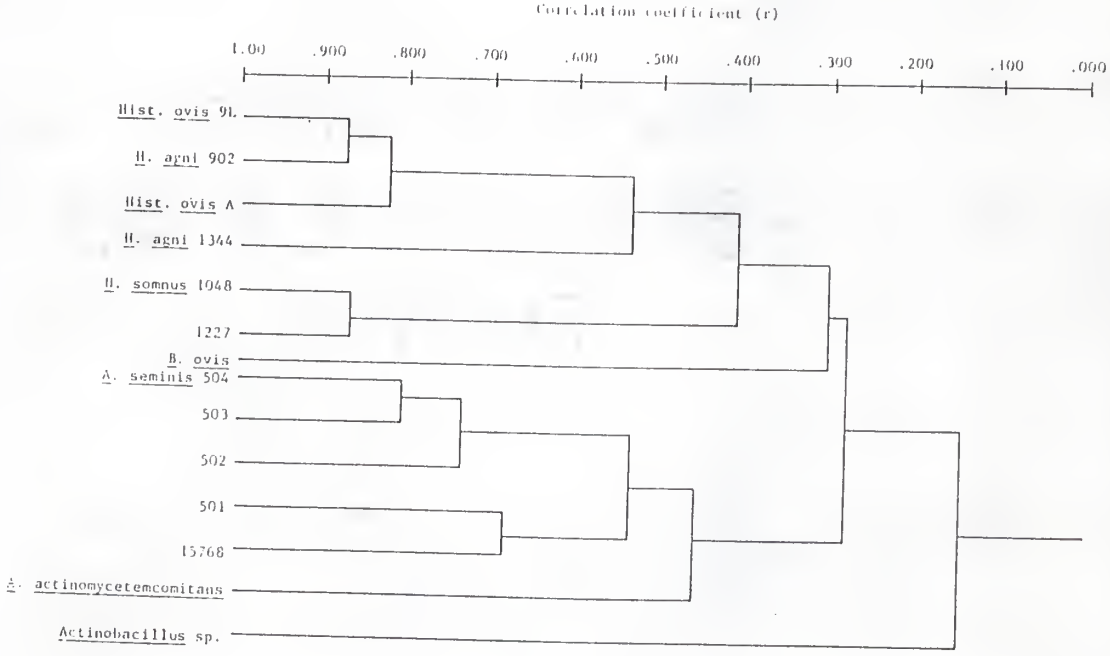

Similarity value (S_J)
.600
.500
.400
.300
.200
.100
.000
Hist. ovis A
9L
H. agni 1344
902
H. somnus 1048
1227
B. ovis
A. seminis 504
503
502
501
15768
A. actinomycetemcomitans
Actinobacillus sp.

FIG. 6. Similarity matrix based on protein profile homology derived from Pearson product-moment correlation coefficients. Strains are as described for Fig. 4.

	Similarity with strain:													
Strain	9L	902	A	1344	1048	1227	25840	504	503	502	501	15768	29522	27072
9L	1.000													
902	.873	1.000												
A	.830	.912	1.000											
1344	.582	.592	.437	1.000										
1048	.519	.415	.339	.348	1.000									
1227	.525	.450	.394	.289	.969	1.000								
25840	.350	.330	.294	.262	.327	.254	1.000							
504	.216	.242	.157	.217	.365	.298	.232	1.000						
503	.083	.124	.116	.138	.271	.204	.177	.804	1.000					
502	.212	.208	.197	.125	.380	.353	.208	.702	.742	1.000				
501	.336	.366	.183	.270	.651	.524	.329	.597	.457	.562	1.000			
15768	.356	.413	.294	.271	.413	.369	.339	.602	.443	.548	.681	1.000		
29522	.342	.270	.255	.163	.418	.389	.433	.406	.358	.597	.425	.487	1.000	
27072	.013	013	.017	.069	.182	.117	.116	.219	.291	.296	.223	.204	.164	1.000

FIG. 7. Dendrogram derived by average linked cluster analysis of protein profile homology expressed as a correlation coefficient (r).


Correlation coefficient (r)
1.00
.900
.800
.700
.600
.500
.400
.300
.200
.100
.000
Hist. ovis 91
H. agni 902
Hist. ovis A
H. agni 1344
H. somnus 1048
1227
B. ovis
A. seminis 504
503
502
501
15768
A. actinomycetemcomitans
Actinobacillus sp.

IV. IMMUNOREACTIVE PROTEINS AMONG THE GRAM-NEGATIVE PLEOMORPHS ASSOCIATED WITH OVINE EPIDIDYMITIS

Ovine epididymitis can be considered two diseases based on an association between etiological agent and the reproductive status of rams (Bulgin and Anderson, 1983). The classic disease occurs in mature breeding rams, is caused by Brucella ovis, and occurs most often in range flocks where a large number of rams are continually maintained (Bagley et al., 1985). The second form predominates in immature rams and is caused by one or more nonenteric, Gram-negative pleomorphic rods (Walker et al., 1986). The latter are a transient part of the genital flora of both rams and ewes, and give rise to disease in rams via an ascending infection of the genital tract (Walker and LeaMaster, 1986). Although the etiological distinction is not absolute (Walker et al., 1986), the predominance of specific organisms warrants differentiating between ram and lamb epididymitis (Bagley et al., 1985).

The most common isolates associated with lamb epididymitis are Actinobacillus seminis and Histophilus ovis (Walker et al., 1986), but many other species have been incriminated (Ekdahl et al., 1968; DeLong et al., 1979; Bagley et al., 1985). Most prominent among these are Haemophilus somnus (Bulgin and Anderson, 1983), and Haemophilus agni (Zeki et al., 1981). Actinobacillus actinomycetemcomitans has also been associated with lamb epididymitis (DeLong et al., 1979; Bulgin and Anderson, 1983), but the isolates were unlike the type strain of that organism (Bulgin and Anderson, 1983), and were probably A. seminis (Bagley et al., 1985; Healey et al., 1986).

Actinobacillus seminis, Hist. ovis, H. somnus, and H. agni (the AHH group bacteria), exhibit confusing bacteriological and serological

similarities (Hughes et al., 1971; Dennis, 1974; Rahaley, 1978; Van Tonder, 1979; Bruere et al., 1979; Webb, 1983; Stephens et al., 1983). Biotypes of A. seminis closely resembling Hist. ovis have been described (Van Tonder, 1979), and it has been suggested they represent biochemical variants of the same species (Webb, 1983). However, A. seminis and Hist. ovis can be distinguished phenotypically (Stephens et al., 1983), and they are not genotypically related (Piechulla et al., 1986). On the other hand, no definitive phenotypic differences between Hist. ovis, H. somnus, and H. agni have been demonstrated (Stephens et al., 1983), and DNA homology studies suggest they constitute a single species (Walker et al., 1985; Piechulla et al., 1986). Regardless of their relationships to each other, none of the AHH group bacteria belong in their currently assigned genera (Kilian and Biberstein, 1984; Phillips, 1984).

From an epizootiological point of view, the taxonomic status of the these organisms is less important than the accurate identification of affected rams. To date, the most reliable method is scrotal palpation (Healey et al., 1986), however, the incidence of subclinical infection can be high (Walker and LeaMaster, 1986), and early detection of infected rams is difficult (Healey et al., 1986). Serodiagnosis represents the most likely alternative, but is complicated by antigenic variability (Van Tonder, 1973; Canto and Biberstein, 1982; Humphrey and Stephens, 1983; Cardenas and Maki, 1986), and cross-reactivity among species of the group (Rahaley, 1978; Webb, 1983; Stephens et al., 1983). These complications warrant study of the serological relationships among the organisms associated with lamb epididymitis.

The purpose of this paper was, therefore, to investigate the antigenic profile of A. seminis and to identify cross-reactive components common to other members of the AHH group.

MATERIALS AND METHODS

The bacterial strains used and their phenotypic characteristics were reported in Section II. They were grown in lawn culture on chocolate agar at 37°C in 10% CO_2. Cells were harvested after 48 hrs. by suspension in sterile saline containing 0.6% formalin, washed once by centrifugation, and resuspended in sterile saline. Cell suspensions were standardized to 10% transmission in a Bausch and Lomb Spectronic 20[1], mixed 1:1 with Freunds Incomplete Adjuvant[2] and stored at 4°C.

Antisera were produced in rabbits by intramuscular injection of 1.0 ml every other week for 8 weeks. Sera were checked for antibody by immunodiffusion against a soluble extract (see below), and injections were continued until all rabbits had demonstrable titers. Each strain was inoculated into a pair of rabbits, and the serum from each pair was pooled for use.

In early immunoblotting experiments, anti-A. seminis was used to probe fractionated antigen from other species. Soluble antigens were

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[1]Bausch and Lomb, Rochester, NY

[2]DIFCO Laboratories, Detroit, MI

prepared by ultrasonic disruption of whole cells as described in Section III. In later experiments, A. seminis antigen (ATCC[3] strain 15768) was fractionated and reacted with antiserum directed against other organisms. In the latter, a second A. seminis antigen was used in which cells were more extensively disrupted (10 one-minute cycles). Antigen preparations were compared by polyacrylamide gel electrophoresis.

Immunodiffusion assays were done in 1% (w/v) Type IV agarose[4] in phosphate buffered saline containing 0.01% thimerosol. Gels were poured on GelBond Agarose Gel Support Medium[5] Incubation was at room temperature in a humid chamber; after 24 hrs., wells were refilled with the appropriate reagent, and incubation was continued for 48 hrs. Gels were washed by soaking for 72 hrs. in a large excess of distilled water, stained with 0.05% Coomassie Blue in 25% isopropyl alcohol and 10% acetic acid, and destained in 10% acetic acid.

Proteins were separated electrophoretically as described in Section III. Polyacrylamide gels were run on Bio-Rad model 220 or Bio-Rad Protean II electrophoresis systems[6]. The protein content of the second antigen preparation was greater (3.0 mg protein/ml); it was diluted 1:8 before mixing with sample buffer. A section of each gel containing both

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[3]American Type Culture Collection, Rockville, MD

[4]Sigma Chemical Co., St. Louis, MO

[5]FMC Corp., Marine Colloids Div., Rockland, ME

[6]Bio-Rad Laboratories, Richmond, CA

antigen and molecular weight markers was removed for staining while the remainder was transferred for immunoblotting. Staining was less sensitive than immunoblotting; antigen for stained gels was diluted 1:4.

Proteins were transferred to nitrocellulose electrophoretically (Burnette, 1981). Gels were equilibrated and transferred in Towbin's buffer (Towbin et al., 1979) using a Hoefer TE 42 Transphor Electrophoresis Cell[7]. Gels were transferred for 60-90 min. at 4°C.

Nitrocellulose transfers were developed by Western immunoblotting (Burnette, 1981; Bers and Garfin, 1985). All steps were carried out at room temperature. After rinsing in Tris-buffered saline (pH 7.5) with 0.1% Tween-20 (TBS-T), a section was removed for staining. The stain-destain solutions described above were used to stain proteins bound to nitrocellulose, but reaction times were reduced. Unoccupied sites on the remaining section were blocked by incubation overnight in TBS-T with 3% gelatin. Blocked transfers were washed three times in TBS-T before adding primary antibody. Antiserum was diluted 1:500 in TBS-T with 1.5% gelatin, and incubations were carried out at room temperature for 3 hrs. The wash cycle was repeated before adding secondary antibody (sheep IgG anti-rabbit IgG conjugated to horseradish peroxidase) diluted 1:2000 in TBS-T with 1.5% gelatin. After a 2 hr. incubation, transfers were washed twice in TBS-T and twice in TBS before development. Substrate was prepared by dissolving 60 mg

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[7]Hoefer Scientific Instruments, San Francisco, CA

4-chloro-1-napthol in 20 ml ice cold methanol and mixing with 100 ml TBS containing 60 ul 30% H_2O_2. Blots were developed for 10 min., rinsed with distilled water, and photographed. Identification of reactive bands was made by comparison to stained gels and nitrocellulose transfers.

RESULTS

In immunodiffusion tests using the more extensively extracted A. seminis antigen, six lines of precipitation formed with homologous antiserum (Fig. 1). Similar reactivity was observed with antiserum against other A. seminis strains; the number of lines of precipitation ranged from 4 (anti-502) to 6 (anti-501). Although several were connected at their tips, only one was present as an obvious line of identity between all strains.

Cross-reactions were observed when A. seminis antigen was reacted with anti-A. actinomycetemcomitans (ATCC 29522), and anti-Actinobacillus sp. (ATCC #27072); there was a single line of identity. A weakly reactive line was obtained with anti-Hist. ovis, and anti-H. agni. It was difficult to obtain significant titers to the Hist. ovis and H. agni strains; immunodiffusion assays against homologous antigens were also weak (data not shown). Strong reactions were observed with anti-H. somnus (Fig. 1); three lines of precipitation were observed, one of which was a line of identity with anti-A. seminis.

An immunoblot demonstrating the reactivity of anti-A. seminis with fractionated antigens of the AHH group bacteria is shown in Fig. 2. Three reactive bands with molecular weights of 64.5, 60.6, and 37.7K were common to all members of the group. A fourth (92.7K) was reactive with all strains except H. agni 1344, and the 60.6 fraction was shared by A. actinomycetemcomitans. Among the AHH group bacteria, a single band (45.4K) appeared specific for A. seminis. Weak but specific reactions were also obtained with 29.5, 27.6, and 22.8K fractions. The antigenic diversity of A. seminis was evident from differences in serological reactivity, especially with respect to proteins ranging from 37.5 to 60.6K (Fig. 3). Although less drastic, similar diversity was observed with fractionated extracts of the HH group bacteria.

An immunoblot demonstrating the reactivity of specific fractions of A. seminis is shown in Fig. 3. The increased cell disruption time and greater resolution obtained with a longer gel allowed identification of several additional bands; nineteen were reactive with anti-A. seminis serum, but one (16.7K) was also reactive with control serum and was excluded (Table 1). Of the remaining eighteen, 3 were inconsistently reactive. Two of the latter were closely associated with other proteins and may have been doublet bands not resolved on all blots. Of 15 consistently reactive A. seminis proteins, 9 cross-reacted with anti-A. actinomycetemcomitans, 11 with anti-H. somnus, 4 with anti-H. agni, and 5 with anti-Hist. ovis. Two bands (64.5 and 37.7K) were reactive with all antisera, and two (48.2, and 31.8) reacted consistently with anti-A. seminis and no others.

DISCUSSION

Actinobacillus seminis, Histophilus ovis, Haemophilus somnus, and Haemophilus agni are all associated with ovine epididymitis, especially in ram lambs (Bulgin and Anderson, 1983, Bagley et al., 1985; Walker et al., 1986; Walker and LeaMaster, 1986). Histophilus ovis, H. somnus and H. agni have been referred to collectively as the "HH group" (Stephens et al., 1983) and the "Histophilus ovis" group (Piechulla et al., 1986), and may constitute a single species (Walker et al., 1985). Although genotypically unrelated (Walker et al., 1985), A. seminis is similar bacteriologically (Webb, 1983a), and serological cross-reactions have been reported (Rahaley, 1978; Webb, 1983a; Stephens et al., 1983).

Scrotal palpation is the principal means of identifying infected rams (Healey et al., 1986), but the AHH group bacteria are often found in the genital tract of normal sheep (Walker and LeaMaster, 1986), and palpation is not always effective. An alternative method is serodiagnosis, but the antigenic relationships among these bacteria have not been investigated in detail. Cross-reactivity between A. seminis and Hist. ovis has been demonstrated by complement fixation (Rahaley, 1978), slide agglutination (Webb, 1983a), and immunodiffusion (Stephens et al., 1983), but the nature of that cross-reactivity was not determined.

Recently, Healey et al. (1985) produced a series of monoclonal antibodies to an Actinobacillus sp. isolated from the epididymis of an affected ram. Some exhibited specificity for outer membrane components,

and cross-reactions were observed with A. actinomycetemcomitans (Healey et al., 1986). Cardenas and Maki (1986) also demonstrated cross-reactivity between A. seminis and A. actinomycetemcomitans, but antigen characterization was not done.

In this study, four proteins common to all species of the AHH group bacteria (Section III) were shown to react with anti-A. seminis. Although there were a number of proteins of similar size, the molecular weights of these proteins were approximately 92.7, 64.5, 60.6, and 37.7K. Four reactive bands with molecular weights of 45.4, 29.5, 27.6, and 22.8K appeared specific for A. seminis; they were present in all A. seminis strains and no HH group strains. One (45.4K) was also present in Actinobacillus sp. (ATCC 27072), but that organism is not associated with ovine epididymitis, and its bacteriological characteristics (Section II) make it unlikely it would be confused with the AHH group bacteria.

Cross-reactions were also observed between fractionated A. seminis antigen and antiserum against the AHH group species and A. actinomycetemcomitans. Two bands (48.2 and 31.8K) were only weakly reactive with other antisera, but no A. seminis proteins showed an absolute specificity for anti-A. seminis, and most reacted strongly with one or more of the others. The four proteins shared by the AHH group bacteria were expected to react. The remainder could be accounted for by epitope specificity as opposed to protein specificity; proteins of different molecular weight may not be antigenically different with respect to all epitopes. It was not possible, therefore, to identify

the proteins present in the HH strains that induced production of antibody cross-reactive with components of A. seminis.

The only paper comparable to the results reported here is that of Healey et al. (1986). They reported mouse hyperimmune serum reacted with 27 A. seminis proteins ranging from 10 to 105K. Monoclonal antibodies were used to identify specific reactive components of the outer membrane with molecular weights of 10, 33, and 43K. Cross-reactions were observed with A. actinomycetemcomitans, but the proteins responsible were not identified.

Although the total number of reactive bands was lower, similar reactive fractions were observed here. Given variation in molecular weight due to technical differences, 6 reactive bands were identified ranging from 31.8 and 45.4K; one or more of these could correspond to the 33 and 43K fractions reported by Healey et al. (1986). Bands with molecular weights of 31.8, 42.1, and 45.4K either did not react with antiserum against A. actinomycetemcomitans or were only inconsistently reactive; although Healey et al. (1986) reported cross reactions with that organism, it was not observed with the monoclonal antibodies specific for the 33 and 43K fractions. It is possible, therefore, that these bands correspond to the outer membrane components identified by those authors. Since they did not include strains of the HH group bacteria, it is not known whether the monoclonal antibodies they developed were cross-reactive with those species.

The failure to induce significant titers to any of the four strains of Hist. ovis and H. agni is noteworthy. That difficulty was previously

reported (Rahaley, 1978), and may be a general characteristic If so, then the strong reactivity of antiserum against both H. somnus strains represents a definable and important difference between the HH group bacteria.

SUMMARY

Serological relationships among the AHH group bacteria were investigated by immunodiffusion and Western immunoblotting of soluble cellular extracts. Cross-reactivity was demonstrated between all members of the group as well as between A. seminis and A. actinomycetemcomitans. Four proteins with molecular weights of approximately 92.7, 64.5, 60.6, and 37.7K were common to all members of the AHH group and reactive with anti-A. seminis serum. The 60.6K protein was shared with A. actinomycetemcomitans. The occurence and reactivity of these proteins among all members of the group could make them useful as bacterins in a vaccination program for lamb epididymitis or as diagnostic reagents.

No A. seminis proteins were identified that did not react with antiserum against one or more of the HH group strains or A. actinomycetemcomitans. Two (48.2 and 31.8) were only inconsistently reactive with antisera against other strains, and given the sensitivity of the assay and the difficulties involved in precise identification of reactive bands, these could prove useful in a diagnostic assay specific for A. seminis.

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TABLE 1. Reactivity of specific fractions of A. seminis with rabbit hyperimmune serum against other species.

Molecular weight (x 1,000)	Antiserum against[1]				
	15768	1227	29522	902	A
92.7	+++[2]	+++	++	+	+++
84.2	+++	+++	+++		
82.6	+	+	+		
79.8	+++	+++			
76.2	+				
64.5	+++	+++	+++	+++	++
60.6	+++	+++	+++	+	+
55.0	+++	+++	+++		
48.2	+++	+			+
45.4	+++	+++			
44.4	++	+	++	+++	+
42.1	+++	++	+	+++	++
37.7	+++	+++	+++	+++	+++
34.5	+++	++	+++		
31.8	++	+	+		+
27.7	++	++	+	+	+
15.9	+	+	+		

[1]Strains: 15768, A. seminis; 1227, H. somnus; 29522, A actinomycetemcomitans; 902, H. agni; A, Hist. ovis.

[2]number of + signs indicates number of positive reactions in three trials.

FIG. 1. Immunodiffusion reactions between A. seminis antigen and antisera against other species. Wells: center, A. seminis antigen (strain 15768), 1, anti-A. seminis st. 15768, 2, anti-H. somnus st. 1227; 3, anti-Hist. ovis st. A; 4, anti-H. agni st. 902; 5, normal rabbit serum; 6, anti-A. actinomycetemcomitans st. 29522; 7, anti-A. seminis st. 15768; 8, anti-H. somnus st. 1048; 9, anti-Hist. ovis st. 9L; 10, anti-H. agni st. 1344; 11, normal rabbit serum; 12, anti-Actinobacillus sp. st. 27072; 13, anti-A. seminis st. 15768; 14, anti-A. seminis st. 501; 15, anti-A. seminis st. 502; 16, anti-A. seminis st. 503; 17, normal rabbit serum; 18, anti-A. seminis st. 504.

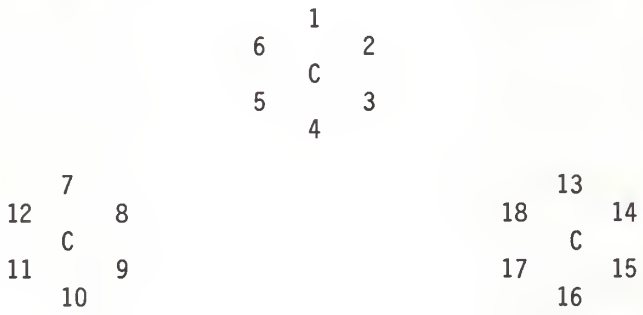


FIG. 2. Reactions of anti-A. seminis (ATCC #15768) with fractionated extracts of other bacteria. Lanes: 1, Brucella ovis; 2, Actinobacillus sp. st. 27072; 3, A. actinomycetemcomitans st. 29522; 4, A. seminis st. 504; 5, A. seminis st. 503; 6, A. seminis st. 502; 7, A seminis st. 501; 8, A. seminis st. 15768; 9, Hist. ovis st. A; 10, Hist. ovis st. 9L; 11, H. agni st. 902; 12, H. agni st. 1344; 13, H. somnus st. 1048; 14, H. somnus st. 1227

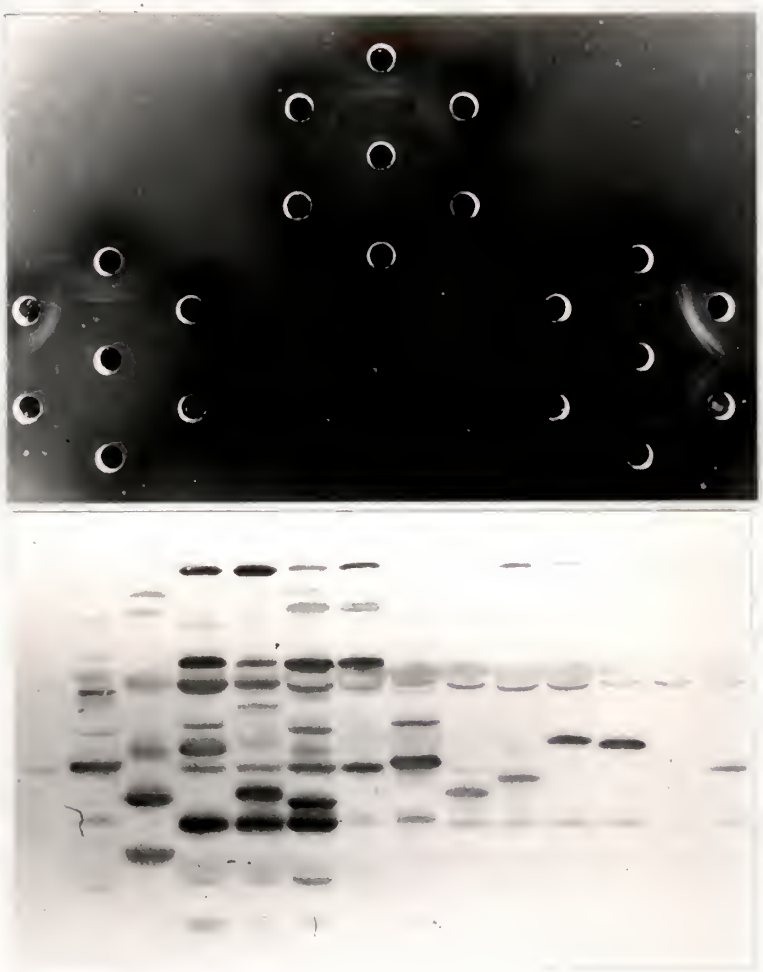


FIG. 3a. Reactions of fractionated A. seminis (ATCC #15768) antigen with antisera against other species. Lanes: 1, anti-A. seminis st. 15768; 2, anti-H. somnus st. 1227; 3, anti-A. actinomycetemcomitans st. 29522; 4, anti-H. agni st. 902; 5, anti-Hist. ovis st. A; 6, normal rabbit serum; 7, stained proteins on nitrocellulose.

FIG. 3b. Reactions of fractionated A. seminis (ATCC #15768) antigen with antisera against other species. Lanes: 1, anti-A. seminis st. 15768; 2, anti-H. somnus st. 1227; 3, anti-A. actinomycetemcomitans st. 29522; 4, anti-Hist. ovis st. A; 5, anti-H. agni strain 902; 6, normal rabbit serum.

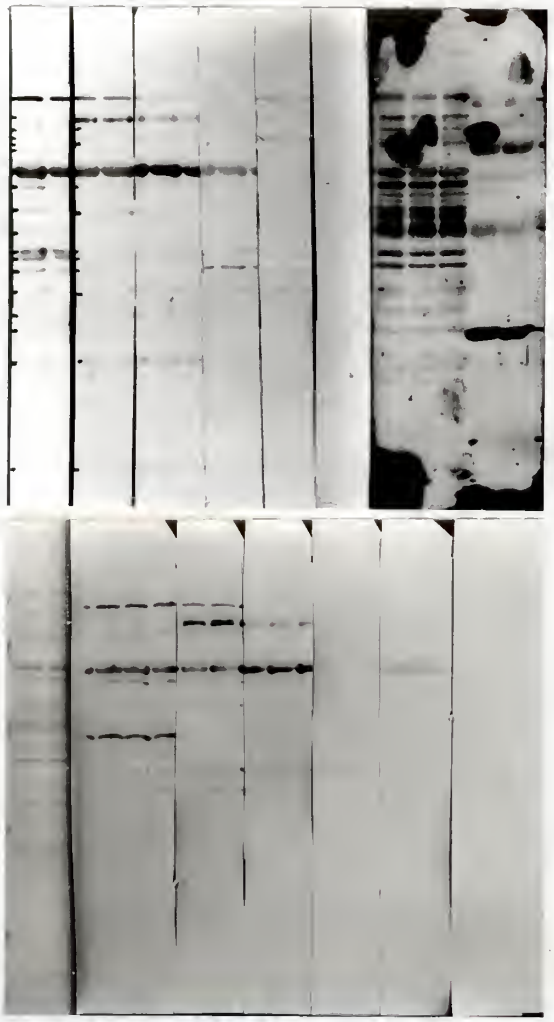
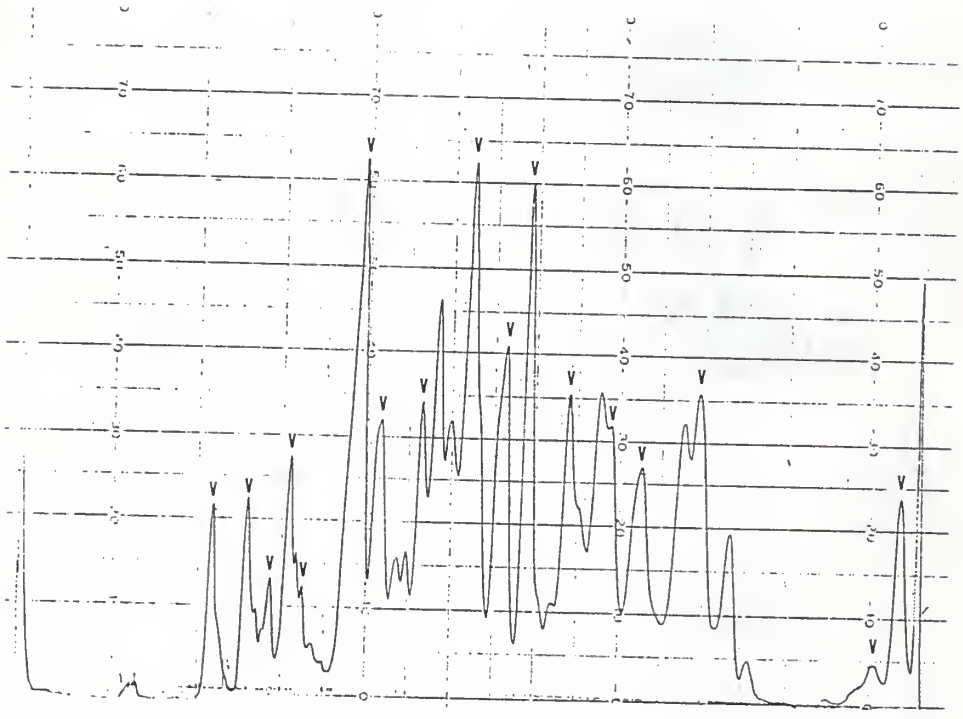


FIG. 4. Spectrophotometric scan of fractionated A. seminis antigen. Immunoreactive peaks are noted by arrows.



STUDIES ON THE GRAM-NEGATIVE PLEOMORPHIC BACTERIA ASSOCIATED WITH EPIDIDYMITIS IN RAMS

by

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AN ABSTRACT OF A THESIS

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The AHH group was defined as Actinobacillus seminis, Histophilus ovis, Haemophilus somnus, and Haemophilus agni. These unofficial species constitute a group in that all are associated with ovine epididymitis, particuarly in immature rams, and they exhibit confusing bacteriological and serological similarities. Their taxonomic status is uncertain, but they do not belong in their currently assigned genera.

The AHH group bacteria were small, Gram-negative, pleomorphic bacteria that required enriched media and a capneic atmosphere for optimal growth. They were nitrate positive, oxidase positive, and decarboxylated ornithine. Actinobacillus seminis was distinguished from the remainder of the group by its production of catalase, lack of yellow pigment, less stringent growth requirements, and failure to produce indole. No definitive difference was demonstrated between Hist. ovis, H. somnus, and H. agni. Although A. seminis was similar to A actinomycetemcomitans, they could be differentiated by colony morphology and the ornithine decarboxylase reaction.

Soluble cellular proteins of the AHH group bacteria were fractionated by polyacrylamide gel electrophoresis, and numerical techniques were used to derive homology values based on interstrain comparisons of overall protein patterns. The results were consistent with the bacteriological characterization of the group; A. seminis was distinguished from the others by the presence of specific proteins and by overall homology values. Strains of Histophilus ovis, H. somnus, and H. agni exhibited similar protein profiles, however, Hist. ovis and H.

agni were more similar to each other than either was to H. somnus. Specific protein bands were identified that were common to the AHH group and to subdivisions within the group. Specific bands were identified for A. seminis and H. somnus, but not for Hist. ovis or H. agni. The distinction between A. actinomycetemcomitans and the AHH group was confirmed by protein profile homology.

Serological relationships among the AHH group bacteria were investigated by immunodiffusion and western immunoblotting. When tested against antigen derived from the type strain of A. seminis, cross-reactions were observed with antisera directed against all species of the AHH group and against A. actinomycetemcomitans. Attempts to identify an A. seminis-specific fraction by western immunoblotting were unsuccessful. Fractions with approximate molecular weights of 92.7, 64.5, 60.6, and 37.7 kilodaltons were common to all AHH strains and were reactive with anti-A. seminis serum. The 60.6K fraction was shared by A. actinomycetemcomitans. A single reactive protein with a molecular weight of 45.4K was common to all strains of A. seminis and not found among other species of the AHH group, however, antisera directed against other species was reactive when tested against fractionated A. seminis antigen.

On the basis of phenotypic and genotypic data, it has been suggested that Hist. ovis, H. somnus, and H. agni constitute a single species. Although the characteristics of that species have not been defined, it would not belong in the genus Haemophilus based on nutritional factors. The AHH group organisms do appear to qualify for

inclusion in the family Pasteurellaceae, but do not fit precisely into any of the established genera. It has been suggested the genus Histophilus be revived to accommodate these organisms. Although A. seminis is not genotypically related, the results of this study support its inclusion in the genus based on bacteriologic and serologic properties, and pathogenicity in sheep.