INVESTIGATIONS INTO THE PATHOGENICITY AND BACTERIOLOGICAL CHARACTERISTICS OF A GRAM-NEGATIVE PLEOMORPHIC ORGANISM WITH THE SUGGESTED NAME *HISTOPHILUS OVIS* AND COMPARISONS OF IT WITH OTHER MEMBERS OF THE FAMILY *BRUCELLACEAE*

by

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PREFACE

This thesis reports the results of studies undertaken on the pathogenicity, bacteriological and serological characteristics of a Gram-negative pleomorphic organism with the suggested name of *Histophilus ovis* (Roberts, 1956). The work was undertaken as a part-time external student of the University of Sydney and was carried out during my period of employment by the New South Wales Department of Agriculture at the Colin Blumer District Veterinary Laboratory, Armidale.

Apart from collaboration with Dr A. R. B. Jackson on the experimental disease transmissions described in Chapters 4 and 5, the studies recorded are my own original work. The section on the pathology of polyarthritis in lambs was delivered personally as a paper to the Annual General Meeting of the Australian Veterinary Association at Perth in May, 1970.

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SUMMARY

Studies on the pathogenicity of *Histophilus ovis* in sheep were carried out over a seven year period from pathological material received at the District Veterinary Laboratory, Armidale. Four distinct disease syndromes were encountered from which *Histophilus ovis* was isolated in pure culture and accepted as the causative agent.

Mastitis in ewes caused by *Histophilus ovis* appeared as an extremely rare condition but was acute in nature and led to rapid loss of gland function, generalised toxaemia and death in untreated animals. Isolation and identification of the pathogen was essential for differential diagnosis and the correct choice of antibiotic for therapeutic use.

Epididymitis in rams due to *Histophilus ovis* was sporadic in occurrence with only small numbers of rams in a flock being affected. Lesions impaired fertility but as they were usually unilateral, complete sterility was rare. The disease was chronic in nature but differential bacteriological diagnosis was essential to distinguish the syndrome from other causes of epididymitis so that effective control measures could be undertaken.

Polyarthritis in lambs resulting from localisation of *Histophilus ovis* in the joints was acute in nature and resulted in exteme lameness and debility in untreated sheep. Lesions were non-suppurative in nature and differential diagnosis was assisted by the lack of any association of lameness with any managerial practices.

Abortion in ewes caused by *Histophilus ovis* was also extremely rare in occurrence but resulted from acute haemorrhagic

placentitis. Affected ewes soon recovered and did not appear to harbour infection into the next lambing season. However, the one outbreak investigated severely depressed lambing percentages when it occurred.

Isolates obtained from these four disease syndromes allowed for detailed bacteriological investigations. The organism described as *Histophilus ovis* is closely related to certain members of the Family *Brucellaceae* but various tests used routinely in diagnostic bacteriological laboratories allowed for its differentiation. Strains of the organism isolated from the different disease syndromes and from different geographical areas were found to give identical results to a wide range of characterization tests.

Serological studies on the organism showed the isolates to be antigenically homogeneous and also that they were closely related, if not identical, to the organism previously described as *Actinobacillus seminis*.

Chapter I

INTRODUCTION

In 1956 D. S. Roberts recorded the isolation of a Gram-negative pleomorphic organism from a case of severe acute mastitis in a ewe. He considered that the isolate had not been previously described and to it he gave the suggested name of *Histophilus ovis*. Previously, Dodd and Hartley (1955) had described the isolation of a similar organism from cases of specific suppurative epididymitis in rams. Subsequently, Kater, Marshall and Hartley (1962) described the isolation of a similar organism from lambs affected with polytenosynovitis in New Zealand.

Further reports in Australia of the isolation of this Gram-negative pleomorph were made by Hughes, Hartley, Haughey and McFarlane (1964) during a study of perinatal mortality in lambs and by Claxton and Everett (1966) from a single case of epididymitis in a ram.

Over the past five years, *Histophilus ovis* has been isolated at the District Veterinary Laboratory, Armidale, on a number of occasions during routine diagnostic bacteriological investigations. Isolates have been obtained from clinical field cases of epididymitis in rams, polyarthritis and polytenosynovitis in lambs, mastitis and abortion in ewes. These isolates have provided the opportunity to study in detail the characteristics of the organism and allow comparisons to be made with other similar organisms in the Family Brucellaceae (Breed, Murray and Smith, 1957).

Chapter II

MASTITIS IN EWES

2.1 Literary Review

Apart from dairy cattle and milking goats, sheep are the only other species of commercial livestock in which outbreaks of mastitis occur.

In Europe, there is considerable literature on the subject of mastitis in sheep. Gangrenous mastitis was first described by d'Arboval in France in 1823. In 1907, Dammann-Freese described contagious mastitis in sheep, and a short Gram-negative rod as the causative microorganism. In 1915, Joest found *Bacillus pyogenes* in the milk of mastitis cases, and described the pathology of the disease. Stephan and Geiger (1921) and Raebiger (1919) distinguished between two types of sheep mastitis, one caused by cocci and the other by bacilli. Schmidt (1923) reported the use of autogenous bacterin as an immunising agent. In 1929, Leyshon described mastitis in ewes as it occurred in England, with a bacteriological investigation of 38 cases. Various organisms were found, with *Staphylococcus sp.* predominating. In four cases on one farm, pure cultures of a small bipolar Gram-negative bacillus were obtained. In 1930, Bederke described outbreaks of sheep mastitis in the North Caucasus district, with 10% morbidity and 50% mortality. Haupt (1932) described as the causative agents of two enzootic forms of sheep mastitis, *Micrococcus ovis* Migula, and *Bacterium ovinum* n. sp., which corresponded closely to the organism described by Dammann-Freese.

In 1932, Marsh described a specific mastitis in range sheep in the western United States of America caused by infection with a *Pasteurella sp.*, which probably was the same organism described by Dammann-Freese (1907). Miessner and Schoop (1932) reported a detailed investigation of 27 cases of mastitis on eight farms, in which they found the Dammann-Freese organism 23 times. They proposed the name *Bacterium mastitidis*. In Russia, Milovzorov and Tchasovnikov (1933) reported effective immunisation with a formalin-killed bacterin. In 1935, Lesbouyries, Berthelon and Macrides described mastitis caused by *Bacterium mastitidis*, and discussed the close resemblance of this organism to *Pasteurella sp.* In 1936, Macrides described two types of enzootic mastitis of sheep in Greece - gangrenous mastitis, caused by the micrococcus of Nocard *(Streptococcus agalactiae)*, and contagious mastitis caused by *Bacterium mastitidis* of Dammann-Freese. In 1937, Weinberg, Forgeot and Moureu reported an outbreak of 18 cases of gangrenous mastitis in a flock of 150 ewes, with 12 deaths, with a diagnosis of *Clostridium perfringens* infection. In 1938, Rosati and Bertolino reported on mastitis in sheep and goats in France, Italy and Tunis, and incriminated *Corynebacterium pseudotuberculosis* as the causative microorganism. In 1941, Carpano reported on gangrenous mastitis in Albania, and Koens reported a highly fatal gangrenous mastitis in Holland. In 1943, Smith and Harnden reported Pasteurella mastitis from Oklahoma.

Tunnicliff (1949) continued Marsh's work in the United States and reported the occurrence of specific Pasteurella mastitis in the western states, having been identified in Texas, Wyoming, Utah, Montana and Oklahoma. One hundred and thirteen cases of one hundred and eighteen cultured were due to *Pasteurella mastitidis*. The remaining cases were due to *Staphylococcus sp.* (three), *Corynebacterium pyogenes* (one) and *Escherichia coli* (one).

The only surveys of the incidence of mastitis in ewes have been carried out in areas where ewe's milk is used extensively for human consumption. In such countries, Blood and Henderson (1968) state that *Streptococcus agalactiae* is the common cause whereas in most other areas either *Staphylococcus aureus* or *Pasteurella haemolytica* is the common pathogen. A survey carried out in Norway by Saeter and Eieland (1961) records a total incidenc e of mastitis in six thousand sheep at two per cent per year. Eighty six per cent of these cases were caused by *Staphylococcus aureus*, seventeen per cent by *Escherichia coli*, three per cent by *Streptococcus agalactiae* and one per cent by *Pasteurella haemolytica*. Tunnicliff (1949) states that his records over seventeen years in range sheep showed that 2-3% of the lambing ewes developed mastitis and the mortality in affected ewes was 21%.

The economic loss caused by ovine mastitis in Australia is difficult to assess but the absence of reports suggests that it is relatively rare. Carroll (1949) and Belschner (1959) describe mastitis in Australian sheep but give few details of the incidence of the disease. Moule (1954) reports on a survey of neonatal losses in lambs and states that 351 out of 1587 ewes examined had abnormal udders. Although he attributes some of these cases to chronic infection, no information on the number of such cases or aetiology is given.

Seddon (1965) states that the most common form of mastitis in ewes in Australia is caused by *Staphylococcus aureus* which gives rise to an acute gangrenous condition. It is usually sporadic in occurrence but its incidence in individual flocks is variable.

Pyobacillory mastitis due to *Corynebacterium pyogenes* sometimes occurs, the secretions being altered and containing pus. *Corynebacterium pseudotuberculosis* infection of the gland and supramammary lymph node commonly produces suppurative lesions and loss of gland function.

Simmons and Ryley (1954) investigated six outbreaks of mastitis in ewes in Queensland. Two outbreaks were caused by *Pasteurella mastitidis* and one by *Staphylococcus aureus*. In the remaining three outbreaks, *Pasteurella mastitidis* was associated with *Corynebacterium pyogenes* in one, *Staphylococcus aureus* in another and with *Streptococcus sp.* and *Staphylococcus sp.* in the third.

Laws and Elder (1969b) reported the isolation of *Actinobacillus ligniersi* from cases of severe mastitis in ewes on eight different properties in Queensland. Clinical signs usually appeared within seven weeks of lambing but up to three months post-partum. Morbidity ranged from 1% to 10% and mortality from 0.23-4.1% and the number of cases increased with the age of the ewe. Affected ewes had pyaemia, were depressed and appeared lame in the hind limb adjacent to the affected side of the udder. The gland was swollen, hard and painful and usually purplish in colour.

Watt, Bamford and Nairn (1970) described the artificial production of an acute gangrenous mastitis when overnight cultures of *Actinobacillus seminis* were injected into the teat canal of lactating ewes. The condition developed within three days of inoculation; death ensued quickly in one ewe whereas treatment with penicillin and streptomycin effected recovery in a second ewe.

2.2 Mastitis Due to *Histophilus ovis*

2.2.1 Introduction

The original isolate of *Histophilus ovis* was obtained by Roberts (1956) from a single case of acute gangrenous mastitis in a ewe found dead on a farm near Corrigin in Western Australia. The case occurred during the end of the lambing period and it was deduced from the appearance of the carcass and from the close scrutiny being kept on the flock that it had only been dead for a short time.

During the period of this project, routine bacteriological examinations undertaken on cases of mastitis in ewes referred to this laboratory has resulted in the isolation of *Histophilus ovis* from one field case of the disease (T.D. Killen, "Arida", Yarrowyck). This was apparently the only ewe in the flock noticed to be affected. Unfortunately, the apparent extreme rarity of mastitis in ewes in the area covered by this laboratory has prevented any comparative work but observations made on this case and on the experimentally induced disease will be used to define the syndrome.

2.2.2 Clinical observations

Affected ewes have only been found at the beginning of the lactational period. Clinical examination reveals the condition as being unilateral and the affected half of the udder is hot, swollen, tense and painful indicative of acute mastitis. The skin is tightly stretched over the surface of the affected side and has a patchy blue colour. The ewes usually have an altered gait, being very reluctant to move the hind leg immediately adjacent to the affected side, past the swollen udder. A systemic reaction comprising fever, toxemia, depression and anorexia is usually present with elevation of rectal temperature up to 41.5°C.

Milk expressed manually from the affected side is obviously abnormal, consisting of a cloudy watery fluid containing greenish-white flakes. Breed smears prepared from milk during the actue phase of the disease contain large numbers of polymorphonuclear inflammatory cells. If the ewes survive the first three to four days, large numbers of mononuclear cells become apparent. In one experimentally infected ewe, recovery was accompanied by fibrosis of the gland resulting in loss of function but as the condition is naturally unilateral in occurrence, recovered ewes could still raise a lamb from the unaffected side.

Histophilus ovis organisms are present in large numbers during the early phase of the disease and appear as Gram-negative pleomorphic rods in smears, prepared from the centrifuged deposit of the affected milk. Organisms are most easily detected when 1% carbol fuchsin is used as the counter-stain. Direct culturing of affected milk is best achieved by direct plating of centrifuged deposit onto 7-10% sheep blood agar plates which are incubated at 37°C under microaerophilic conditions. Growth is usually observable after 48 hours as pin-point colonies which develop into typical colonies after 5 days. In more chronic conditions where numbers of organisms are much smaller, primary inoculation of pathological material into nutrient broth enriched with 10% sheep serum, cooked meat medium or the yolk sacs of 5 to 7 day old embryonated chicken eggs increases the chances of isolation.

2.2.3 Gross pathology

In the typically per-acute form of the disease, the affected gland was found to be greatly enlarged and turgid. There was pitting oedema of the inguinal area, the adjacent flank and ventral midline.

The skin overlying the gland was tightly stretched, bluish in colour, exuded serum and was undergoing necrosis. When the affected side was incised, brownish purulent fluid gushed out. Gangrenous changes of the gland were usually seen in the teat and immediately adjacent portions of the udder, but occasionally involved the whole gland on the affected side. Pathological changes were typical of those associated with normal moist gangrene with an abundance of exudation but no gaseous formation.

In the more sub-acute cases which did not terminate fatally, multiloculated abscesses developed in the gland after 7 to 10 days with subsequent rupture to the surface and fistulation. There was evidence of interlobular fibrosis and involution but usually some or all of the affected side eventually sloughed off.

2.2.4 Histopathology

The reaction of the mammary gland to infection with *Histophilus ovis* was due to the obviously galactogenic route of infection with the insults primarily in the ductal system. Initially the pattern of inflammation was basically lobular but in the usual per-acute disease, there was evidence of direct centrifugal expansion from the initial focus of infection affecting very large sections of the gland involved. The small amounts of mammary secretion observed consisted of fibrinous inflammatory exudate which mechanically plugged and blocked the draining ducts and cistern. The parenchyma of the gland was intensely congested and contained areas of haemorrhage. Alveolar cells initially showed cytoplasmic degeneration, then pyknosis, karyorrhexis or karyolysis. Initially there were foci of necrosis affecting small groups of lobules but these often expanded and coalesced to involve almost the whole gland. Necrosis was due to thrombosis of veins and lymphatics and such areas were often replaced by haemorrhage.

In the more sub-acute form of the disease, histological changes were characterised by marked interstitial oedema. There were large numbers of polymorphonuclear inflammatory cells within this oedematous exudate and in the secretory acini. The stromal lymphatics were widely dilated and contained numerous leucocytes. The acinar epithelium appeared vacuolated and desquamated in some areas, whilst in others there was evidence of hyperplasia associated with underlying accumulations of macrophages and a few young fibroblasts.

Histophilus ovis organisms were detected in large numbers in Leishmann-stained sections both in the ducts and acini.

2.2.5 Pathogenesis

The portal of entry of *Histophilus ovis* into the mammary gland appears to be the teat orifice and canal. According to Jubb and Kennedy (1970), numerous factors control this invasion phase including the patency of the teat orifice, structural factors of both the orifice and teat canal as well as various chemical factors within the teat canal itself. Host immunity to infection is obviously of importance but is not an all-or-none phenomenon. The infection phase is that in which the organism is present within the cistern and ductule system. The third, or inflammatory phase is that which follows immediately on penetration of *Histophilus ovis* into the tissues from the ducts.

No specific environmental circumstances or managerial practices were detected during this project as being pertinent to the development and transmission of the disease. The condition appeared to develop at any time of the lactational period but more commonly during the first 3 weeks after lambing. Dumaresq (1949) states that injury to the teats by suckling lambs facilitates entry of the causative organisms of mastitis into the udder during early lactation while Seddon (1965) speculates that infection in ewes suckling lambs 2 to 4 months old follows trauma by the "bunting" of robust hungry lambs. Factors such as concentration of sheep on bedding grounds, mustering for routine managerial practices such as lamb-marking and vaccination, would obviously contribute to environmental contamination and spread of the organism. However, the only cases of ovine mastitis due to *Histophilus ovis* were very rare and sporadic in occurrence and no significant numbers of ewes were ever affected at any one time.

2.2.6 Differential diagnosis

Ovine mastitis due to *Histophilus ovis* is sporadic in occurrence and generally acute to gangrenous in nature. The ewes develop the severe condition and die in 3-7 days. Occasionally, ewes recover from the acute phase and the udder becomes fibrosed with abscess formation. In this respect, the mastitis is clinically and pathologically similar to that caused by *Staphylococcus aureus* (Seddon, 1965), *Pasteurella mastitidis* (Tunnicliff, 1949; Simmons and Ryley, 1954), *Pasteurella multiseptica* (Suveges, 1960) and *Actinobacillus lignieresi* (Laws and Elder, 1969b). Definitive diagnosis depends upon the isolation and identification of *Histophilus ovis* during the acute phase of the disease from either the milk or affected udder.

It appears that the organism described by Dammann-Freese (1907) was the same as that named *Bacterium ovinum* (Haupt, 1932), *Bacterium mastitidis* (Miessner and Schoop, 1932; Lesbouyries, Berthelon and Macrides, 1935; Macrides, 1936) and *Pasteurella mastitidis* (Marsh, 1932; Smith and Harnden, 1943; Tunnicliff, 1949; Simmons and Ryley, 1954). Furthermore, Laws and Elder (1969b) considered that their isolates of *Actinobacillus lignieresi* obtained from ovine mastitis could not be differentiated from these species. *Pasteurella haemolytica* appears to be the only other Gram-negative rod that has been isolated from ovine mastitis. Differentiation of *Histophilus ovis* from these organisms is best achieved by referring to Table 1.

TABLE 1: Differentiation of Histophilus ovis from similar organisms isolated from ovine mastitis

	Histophilus ovis	*Pasteurella mastitidis*	*Pasteurella haemolytica*	*Actinobacillus lignieresi*
Reference	Webb (1976) Roberts (1956)	Simmons & Ryley (1954)	Cowan & Steel (1974)	Laws & Elder (1969b)
Catalase activity	-	+	+	+
Indole production from tryptophane	+	-	-	-
Acid (no gas) production from sucrose	-	+	+	+

2.2.7 Experimental reproduction

(a) *Materials*

(i) *Sheep*: Six recently lambed and lactating ewes were obtained from the Shannon Vale Nutrition Station via Glen Innes and transported to concrete pens at this laboratory. One week was allowed for the ewes to settle in to their new environment and adapt to pen feeding of lucerne chaff and fresh water. At the end of the week, all ewes were eating well and no fluctuation in daily rectal temperature was detected. The ewes were randomly divided into 3 groups of 2.

(ii) *Culture:* The culture used was a 48 hour growth of *Histophilus ovis* in tryptone water containing 10% sterile sheep serum. Using the Miles and Misra (1938) technique, a total viable count revealed that the culture contained 1.67 x 10^8 organisms per ml.

(b) *Methods*

Group 1: Using a sterile, blunted 18 gauge 1½" needle, 5.0 ml of the culture was introduced into the teat cistern of the left mammary gland of two ewes.

Group 2: Using a sterile, 21 gauge 1½" needle, 2.5 ml of the culture was inoculated directly into the left mamary gland of two ewes.

Group 3: Using a sterile 18 gauge 1½" needle, 10.0 ml of the culture was inoculated intravenously into the left jugular vein of the final two ewes.

(c) *Results*

Group 1: Two days after the culture was inoculated, the left mammary gland of both ewes was swollen, hot and obviously painful. The milk expressed manually was small in amount and grossly altered, being watery in consistency and contained greenish mucoid plaques. Numerous organisms morphologically resembling *Histophilus ovis* were detected in direct smears of the milk and the organism was cultured from the centrifuged deposit of milk samples collected from the affected gland of both sheep. One ewe was destroyed 5 days after inoculation and showed pathological and histopathological changes of acute mastitis. The other ewe was destroyed 28 days after inoculation and showed typical pathological and histopathological changes of chronic mastitis with marked fibrosis and induration of the gland. *Histophilus ovis* organisms were isolated from the gland of the acute case at autopsy but not from the second ewe.

Group 2: Three days after inoculation, the left mammary glad of both ewes was slightly swollen and warm. The milk was altered, containing greenish stringy clots but changes were not as marked as for those ewes in group 1. One ewe was destroyed for autopsy 5 days after inoculation and examination revealed a fairly well defined acute abscess deep within the left mammary gland. The second ewe was destroyed for autopsy 28 days after inoculation. Examination revealed a small, well capsulated fibrotic abscess deep within the mammary tissue.

Group 3: No clinical abnormality was detected in either ewe for 28 days after inoculation. There was no evidence of pyrexia, mastitis or arthritis.

(d) *Discussion*

The production of acute mastitis by inoculating the culture into the teat cistern confirms the galactogenic route as the natural method of infection. The inability to cause the severe necrotizing per-acute disease could have either been due to the culture having had repeated sub-culture in the laboratory with a subsequent reduction in virulence, or to the fact that the culture used was one which had been obtained from a natural case of polyarthritis in lambs.

Alternatively, other factors may contribute to the natural occurrence of the disease; Lawrence (pers. comm. 1976) advised that in the experimental reproduction of "summer mastitis" in cattle, *Corynebacterium pyogenes* alone produces a milder condition than when *Streptococcus dysgalactiae* or anaerobic *Streptococci spp.* are introduced at the same time.

Chapter III

EPIDIDYMITIS IN RAMS

3.1 Literary Review

Epididymitis is one of the major causes of infertility in rams in Australia. Long before the part played by bacteria was established, Gunn, Sanders and Granger (1942) showed that epididymitis was present in 5.3% of 6420 rams examined clinically in western Queensland, and 5.7% of 3000 rams examined in New South Wales. Subsequent estimates of the incidence of epididymitis in samples of rams examined in Australia range from 3% to 18% (Moule, 1950; Miller and Moule, 1954; Watts, 1955; Osbourne, 1955; Ryan, 1964). Variations in the figures quoted are probably due to differences in incidence between stud and commercial flocks and between different breeds. Osbourne (1955) points out that data on average incidence grossly distorts the true position within individually infected flocks.

During recent years, numerous bacterial organisms have been isolated from clinical and pathological cases of ovine epididymitis. The majority of published information has been derived from work undertaken in Australia and New Zealand but specific aetiological agents have been described from cases in other countries.

Simmons and Hall (1953) first described the isolation of a Brucella-like organism from cases of epididymitis in Queensland. This organism was subsequently named *Brucella ovis* by Buddle (1956). Epididymitis caused by *Brucella ovis* is now known to occur in other states of Australia (Baynes and Simmons, 1960), New Zealand (Buddle and Boyes, 1953), California, U.S.A. (McGowan and Schultz, 1956; Kennedy, Frazier and McGowan, 1956), South Africa (Van Rensburg, van Heerden, Le Rouse and Snyders, 1958), Slovakia (Gdovin, Hrudka, Chladecky and Keppel, 1955; Biberstein and McGowan, 1958; Niznansky, Gdovin, Gamcick and Bogdan, 1961), Rumania (Tudoriu, Andrie, Cambir, Moldaveanu and Ionica, 1959) and Argentina (Szyfres and Chappel, 1963). According to Hughes and Claxton (1968) *Brucella ovis* is the most common pathogen isolated from epididymitis in rams in Australia. Hadju (1962) has reported an incidence of 30-54% of *Brucella ovis* in European Tsigai and Valachian rams compared with a 14% incidence in Merino rams. In Australia, observations indicate a higher incidence of *Brucella ovis* in British breeds of sheep compared to Merinos (Hughes and Claxton, 1968).

Baynes and Simmons (1960) described an apparently new organism from epididymitis in rams to which they gave the name *Actinobacillus seminis*. Further reports of this organism were made in Australia (Simmons, Baynes and Ludford, 1966; Galloway, 1966; Watt, 1966), U.S.A. (Livingstone and Hardy, 1964) and South Africa (Worthington and Bosman, 1968; van Tonder and Bolton, 1968). Other infectious causes of epididymitis have been described, including *Corynebacterium pseudotuberculosis* (Simmons and Hall, 1953; Clapp, Symons and Doolette, 1955; Christie, 1961), *Corynebacterium pyogenes* (Gamcick, 1960), *Pasteurella pseudotuberculosis* (Jamieson and Soltys, 1947; Simmons and Hall, 1953; Ekdahl, Money and Martin, 1968), and *Actinobacillus lignieresi* (Hughes and Claxton, 1968; Laws and Elder, 1969a). Miscellaneous bacteria isolated from epididymal lesions in rams by Ekdahl, Money and Martin (1968) included *Streptococcus spp.*, *Staphylococcus spp.*, *Pasteurella haemolytica*, *Pasteurella multocida*, *Bacteroides spp.*, *Brucella abortus* and *Brucella abortus* strain 19.

3.2 Epididymitis Due to *Histophilus ovis*

3.2.1 Introduction

A Gram-negative pleomorphic bacterium was described by Dodd and Hartley (1955) as being the cause of a specific suppurative epididymitis in rams in New Zealand. Ekdahl, Money and Martin (1968) isolated this same organism from 47 of 113 rams (41.6%) with palpable lesions and from which specific pathogens were isolated. Claxton and Everett (1966) isolated an apparently identical organism from a single ram in New South Wales and suggested that it was the same as the *Histophilus ovis* described by Roberts (1956).

During the period of this project, routine bacteriological examinations undertaken on cases of epididymitis in rams referred to this laboratory has resulted in the isolation of *Histophilus ovis* from 2 field outbreaks of the disease. On the first property (C. T. Say, Armidale Road, Glen Innes) 5 Dorset rams aged 5-8 months out of a flock of 75 developed acute unilateral epididymitis over a three week period. All 5 rams were negative to the complement fixation test for *Brucella ovis*. *Histophilus ovis* was isolated in cultures prepared from all of three testicles examined from 3 separate rams. On the second property (J. A. Livingstone, "Bullawarrie", Mungindi) 6 cases of unilateral epididymitis were detected in a mob of 98 unjoined two-tooth Merino rams. All 6 rams were negative to the complement fixation test for *Brucella ovis*; *Histophilus ovis* was isolated from the epididymis of two of these rams. These field cases provided the opportunity to examine in detail pathological aspects of epididymitis in rams caused by *Histophilus ovis*.

During the period of this project, other bacteria isolated from cases of epididymitis were *Brucella ovis*, *Actinobacillus seminis*, *Pasteurella multocida*, *Pasteurella haemolytica* and *Corynebacterium* *pyogenes*. These latter cases provided an opporunity to compare pathological changes with those due to *Histophilus ovis*.

3.2.2 Clinical observations

In the early acute stage, lameness or a straddled gait is often the first observable sign, and the affected side of the scrotum is hot, swollen and painful, the body temperature is raised, and the animal will not eat. These signs are followed by a marked increase in the size of the affected side and a rapid loss in the animal's condition, with death occurring in one to three weeks from the time the abnormality is first noticed. More commonly, rams recover from the systemic effects and commence eating again, although infection is still present in the scrotum. When the abscess has fully developed, palpation of the scrotum does not cause any acute pain reaction. In a few cases the abscess ruptures through the scrotal wall and discharges a sanguineous purulent material.

On palpation, the scrotal contents on the affected side are found to be firm, hard, immovable within the tunica vaginalis, and are covered by a greatly thickened layer comprising the tunics and the skin. If the scrotal wall has not ruptured, a large soft fluctuating swelling can be felt in the ventral part of the scrotum; the wall may be very thin where the abscess is pointing.

Semen was collected from eight affected rams by the electro-ejaculation method; the quantity of semen obtained and its quality was very variable. Smears of the semen stained by the Gram method showed large numbers of polymorphonuclear inflammatory cells and cell debris; spermatozoa were sometimes absent or sometimes present in large numbers. These smears were made primarily to detect organisms, but none could be detected with certainty. The semen was inoculated onto 10% sheep blood agar and incubated at 37°C in an atmosphere of approximately 10% CO_2 and an organism, subsequently identified as *Histophilus ovis*, was obtained from all cases. This will be referred to under Bacteriology.

3.2.3 Gross pathology

The commonest lesion encountered was a large multiloculated abscess affecting the tail of the epididymis of one testicle. The cavity contained much fluid and greenish-yellow flocculent odourless pus, often with lumps of inspissated necrotic debris. There were adhesions and gross fibrosis of the whole of the parietal and visceral layers of the tunica vaginalis and of the tunica dartos, these layers often being up to 1.5 cm in thickness. Parts of the epididymis dorsal to the abscess and parts of the adjacent vas deferens also showed fibrous thickening. In some cases there were also focal pus-filled cavities distending the lumen of the vas deferens. On one occasion, the abscess cavity did not directly affect the epididymis, but was situated ventral to the testicle apparently in the cavity of the tunica vaginalis; once again there was gross tunical fibrosis with adhesions. In early cases with epididymal abscessation, the vas deferens was usually patent and contained pus and spermatic fluid. In older cases, with no focal abscesses, the vas deferens was usually empty and had grossly fibrosed walls in the region adjacent to the epididymis. The testicle itself was rarely affected by the suppurative process, but was invariably smaller than the one on the unaffected side. In all cases showing gross thickening of the tunics, there was extensive subcutaneous oedema of the scrotal wall.

3.2.4 Histopathology

The epididymis in the earliest cases seen showed a severe suppurative inflammatory reaction of the epididymal tubular wall. The lumen was distended by semen and large quanitities of purulent material. In some places the infection was seen to be spreading to the adjacent interstitial tissues. Thionine stained sections showed numerous pleomorphic bacilli in the necrotic material in the epididymal tubule.

In older cases of epididymal abscessation there was, adjacent to the abscess cavity, an extensive interstitial chronic inflammatory reaction with much fibrosis; some tubules were dilated and contained necrotic cellular debris with many polymorphonuclear inflammatory cells, some showed focal accumulations of polymorphs in the tubular epithelium, and in others the lumen was empty and the epithelium showed hyperplasia.

In those cases showing focal lesions of the vas deferens, the lumen and most of the wall were replaced by a mass of caseous necrotic cellular debris. The suppurative inflammation of the wall was surrounded by granulation tissue and extensive fibrosis.

The testicle itself showed no evidence of the infective process; the only abnormality being complete abeyance of spermatogenesis, presumably due to the effect of adjacent pressure on normal function.

3.2.5 Pathogenesis

The disease does not appear to be related to any particular set of managerial practices, such as shearing, which could lead to wounds of the scrotum, and at present there is no evidence to suggest how the infection originates and why it localises in the scrotum. Ram hoggets only have been found affected and the infection has almost invariably been unilateral. Pathogenesis and pathological changes seen after infection with *Histophilus ovis* appear similar to those described for other aetiological agents. Jubb and Kennedy (1970) state that initial bacterial infection and localisation in the epididymis produces early oedema and lymphocytic infiltration in the cauda epididymis. This change also involves the vessels adjacent to the basal layers of the tubular epithelium which becomes oedematous and infiltrated with mononuclear inflammatory cells. Later, neutrophils appear in the exudate and the epithelium begins to show papillary hyperplasia and focal hydropic degeneration with the formation of intraepithelial cysts. Concomitantly, there is fibroplasia in the previously oedematous interstitial tissue. The combination of fibrosis and epithelial hyperplasia obstructs the lumen and causes stasis of its contents. In some instances in which the number of bacteria in the epithelium is large the infiltration of neutrophil leucocytes may cause destruction of the epithelium. This seems to be all that the bacterium itself is capable of provoking and these changes develop slowly over a period of months during which time large numbers of the organism are excreted in the semen. At this stage there may be some slight palpable enlargement and increased firmness in the tail of the epididymis. The subsequent development of the lesions depends on the extravasation of spermatozoa which occurs if the epithelium is destroyed by the infiltrating neutrophils and also by atrophy and rupture of the tubule at any point proximal to the obstruction. The majority of the extravasations occur immediately proximal to the obstruction in the tail but a small percentage may occur in the body or head of the organ. The resulting spermatic granuloma grossly resembles an abscess and the tail of the organ may be enlarged four or five times, largely by reactionary fibrosis. If the extravasated spermatozoa enter the cavity of the tunica vaginalis they provoke a severe inflammation from which dense adhesions develop. There is no primary orchitis. Testicular degeneration is secondary with stasis in the seminiferous tubules often leading to calcification, intratesticular spermatic granulomas of microscopic size, and fibrosis.

The affected organ is sterile. The lesion is usually bilateral in *Brucella ovis* (Jubb and Kennedy, 1970) or unilateral when *Histophilus ovis* is the causative agent. The ram may still be fertile when the condition is unilateral but they are capable of shedding the organism for many months.

3.2.6 Differential diagnosis

It was not found possible to differentiate the epididymitis caused by *Histophilus ovis* from that caused by the other recorded aetiological agents on clinical and pathological criteria. Differential diagnosis is dependent upon the isolation and identification of *Histophilus ovis* from either semen collected from the live affected ram or from the epididymal lesion presented after castration or at autopsy.

Of the previously mentioned 13 bacterial organisms recorded from cases of epididymitis, 2 are Gram-positive cocci *(Staphylococcus spp., Streptococcus spp.)* 2 are Gram-positive diptheroids *(Corynebacterium pyogenes* and *Corynebacterium pseudotuberculosis)* and one is a strict anaerobe *(Bacteroides spp.)*. *Brucella ovis, Brucella abortus* and *Brucella abortus* strain 19 are much slower to grow in cultures prepared from pathological specimens, produce a more regular colony on sheep blood agar, are more uniformly cocco-bacillary in Gram-stained smears and are positive to the modified Ziehl-Neelsen stain for semi-acid-fast organisms. There is thus no difficulty in differentiation from these causes.

Difficulties do occur, however, in differentiating epididymitis caused by *Histophilus ovis* from those due to *Pasteurella spp.* and

TABLE 2: Differentiation of Histophilus ovis from similar organisms isolated from ovine epididymitis

	Histophilus ovis	*Actinobacillus seminis*	*Actinobacillus lignieresi*	*Pasteurella multocida*	*Pasteurella haemolytica*	*Pasteurella pseudo-tuberculosis*
Reference	Webb (1976) Roberts (1956)	Baynes and Simmons (1960)	Phillips (1960)	Cowan and Steel (1974)	Cowan and Steel (1974)	Jamieson and Soltys (1947)
Growth on nutrient agar	-	+	+	+	+	+
Growth on McConkey agar	-	-	+	-	+/-	+
Motility	-	-	-	-	-	+
Bipolar staining	-	-	-	+	+	+
Acid (no gas) production from sucrose	-	-	+	+	+	+
Acid (no gas) production from sorbitol	+	-	-	+	+	-
Catalase activity	-	+	+/-	+	+	+
Indole production from tryptophane	+	-	-	+	-	-

Actinobacillus spp.

3.2.7 Experimental reproduction

(a) *Materials*

i) *Sheep*: Eight merino rams were acquired from the Shannon Vale Nutrition Station, via Glen Innes, for attempted reproduction of the disease. On arrival at the laboratory, the rams were placed in concrete pens and fed a well-balanced diet of lucerne hay and chaff and they had free access to fresh water. Rectal temperatures were taken at 4 p.m. daily and after seven days, all rams had adapted to their environment well. At the end of the first week, all rams were clinically examined and palpation of the scrotal contents did not reveal the presence of any detectable abnormalities. Semen samples were collected from all rams by electro-ejaculation and all samples showed excellent sperm density and motility. Direct smears prepared from the samples and stained by Gram, Leishmann and the modified Ziehl-Neelsen techniques did not show the presence of any bacterial organisms or inflammatory cells. Cultures prepared from the semen onto 7% sheep blood agar plates did not reveal the presence of any significant organisms after one week's incubation at 37°C in an atmosphere of approximately 10% CO_2 and were then discarded. Blood samples were collected from all rams and the sera submitted to the complement fixation tests for *Brucella ovis* and *Actinobacillus seminis* but with negative results. The eight rams were randomly divided into 4 groups of 2.

ii) *Culture*: A strain of *Histophilus ovis*, derived from a field case of epididymitis, was used for the transmission experiments. A 48-hour culture of the organism in tryptone water containing 10% sterile sheep serum was used and a total viable count, using the Miles and

Misra (1938) technique, showed it to contain 1.42 x 10^7 organisms per ml.

(b) *Methods*

Group 1: Using a 23 gauge 1½" sterile needle, 0.5 ml of the culture was injected into the tail of the left epididymis of two rams. At the same time, 0.5 ml of sterile tryptone water was injected into the tail of the right epididymis to act as a control.

Group 2: Using a 23 gauge 1½" needle, 1 ml of the culture was injected deep into the substance of the left testicle of two rams. At the same time, 1 ml of sterile tryptone water containing 10% sheep serum was inoculated into the right testicle.

Group 3: Using a sterile syringe, 15 mls of the culture was introduced into the prepuce of two rams. Immediately afterwards the urethral orifice was closed with a ligature which was left for 20 minutes before removal.

Group 4: Using an 18 gauge 1½" needle, 20 mls of the culture was injected intravenously into two rams.

(c) *Results*

Group 1: Two days after inoculation, both rams showed slight enlargement of the tail of the left epididymis; in each case the lesion was obviously painful and felt warm on palpation. By the third day, there was gross enlargement of the inoculated side, the dependent part of the left scrotal half being hot and extremely painful. Semen was collected from both rams by electro-ejaculation on the fourth day and *Histophilus ovis* was cultured from both samples. No palpable lesions could be detected in the tail of the right epididymis of either ram. Both rams showed slight elevation of rectal temperature (38.0°C to 39.5°C) but haematological determinations were within normal ranges.

One of the rams was destroyed seven days after inoculation and post mortem examination was undertaken in detail on the genital organs. There was an abundance of coagulated inflammatory oedema in the subcutaneous tissue adjacent to the tail of the left epididymis. Fibrinous adhesions were present between the parietal and visceral tunics. The tail of the left epididymis was grossly enlarged and sectioning revealed a cavernous lesion some 2.5 cms in diameter containing approximately 10 mls of reddish-green purulent material. The tail of the right epididymis was palpably normal but section revealed a small (0.75 cm) cavitation having the appearance of a sterile spermatocoele following traumatic damage. Changes were typical of that described under pathology for acute infection and *Histophilus ovis* was isolated in cultures prepared from the tunica vaginalis, head and tail of the left epididymis and the vas deferens. It was not isolated from the testicle, seminal vesicles, ampullae or bulbo-urethral glands.

The other ram was destroyed one month after inoculation. Autopsy revealed a small, multi-loculated abscess in the tail of the left epididymis. There were extensive fibrotic adhesions between the visceral and parietal tunics. The head of the left epididymis was swollen due to a cavity 2 cm in diameter containing creamy greenish fluid consisting of numerous inflammatory cells and degenerated spermatozoa. It was considered that this secondary lesion was due to the fibrosis in the tail which caused occlusion of the epididymal lumen and subsequent damming back of spermatozoa leading to rupture of the head and formation of a spermatocoele. Changes were in keeping with those described under chronic epididymitis and *Histophilus ovis* was isolated from the abscesses in the tail and head of the left epididymis.

Group 2: Four days after inoculation, the left testicle of both rams was slightly swollen and warm on palpation. The testicles remained swollen for the next 3 days. One ram was destroyed for autopsy seven days after inoculation and examination revealed an abscess in the testicle, 3.5 cm in diameter from which *Histophilus ovis* was cultured. There were no lesions of the epididymis, the rest of the genitalia appeared normal and were bacteriologically sterile. The other ram was destroyed one month later and the only lesion was a small fibrotic granuloma. Bacterial cultures were negative from this lesion and from the rest of the genitalia. Semen collected at regular intervals during that month did not contain *Histophilus ovis*.

Group 3: No detectable clinical lesions developed in either ram during the month following the introduction of the culture into the prepuce. One ram was then destroyed for autopsy but there were no detectable pathological changes in the genital organs. *Histophilus ovis* was not isolated in cultures prepared from the epididymis, testicle, seminal vesicle, ampulla or bulbo-urethral gland from both sides, nor from the prepuce.

Group 4: No detectable clinical lesions developed in either ram during the month following the intravenous inoculation of the culture. One ram was then destroyed for autopsy but there were no detectable pathological changes in the genital organs and cultures prepared from these were bacteriologically sterile.

Chapter IV

POLYARTHRITIS IN LAMBS

4.1 Literary Review

Arthritis is inflammation of intra-articular structures. According to Jubb and Kennedy (1970), arthritis in animals is almost always due to infection and occurs simultaneously with tendovaginitis, each condition being primarily a synovitis. Infective arthritis of animals has been recognised for many years. As long ago as 1928, Hare published an excellent treatise on the pathology of the condition and suggested a classification based upon the nature of the causative factor. He noted that sucklings of all animals, within their first month of life, are commonly affected with infective arthritis secondary in many cases to umbilical sepsis.

Arthritis in sheep most commonly arises from systemic infections (Jackson, 1969). A number of the specific arthritides follow an initial local infection such as navel infection at birth (e.g. *Sphaerophorus necrophorus*), grass seed penetration of the skin (e.g. *Staphylococcus aureus*) and infected wounds following shearing, marking and tailing (e.g. *Erysipelothrix insidiosa*). Others are unassociated with any obvious initiating factor (e.g. *Chlamydia spp.*, *Histophilus ovis*). Direct spread of microorganisms from adjacent bony or soft tissues following local infection or injury does, of course, occur but this represents an individual rather than a flock problem.

Numerous microorganisms have been recorded associated with polyarthritis in sheep. Broadly speaking, the resulting arthritis may be classified as suppurative, or non-suppurative according to the ability of the organism to produce a purulent or fibrinous inflammatory reaction.

The common pyogenic organisms of sheep can cause a severe suppurative arthritis or tendovaginitis. Cases usually follow umbilical or wound infection and their pyogenic and frequently permanent severe damaging character readily differentiates them from the non-suppurative types. Marsh (1947) recorded the isolation of three species of *Corynebacterium (C. pyogenes, C. pseudotuberculosis* and *C. equi)* from field cases in Montana, U.S.A. Blakemore, Elliott and Hart-Mercer (1941) isolated α-haemolytic *Streptocci* from cases of "joint-ill" in lambs in East Anglia, U.K. All isolates belonged to a well defined antigenic type closely related to Lancefield Group C. *Staphylococcus aureus* and *Sphaerophorus necrophorus* have also been isolated at this laboratory from cases of suppurative polyarthritis as mentioned by Jackson (1969).

Histophilus ovis causes a non-suppurative fibrinous polyarthritis. A number of other microorganisms have been recorded as causing a syndrome clinically and pathologically similar to it.

Erysipelothrix insidiosa was described by Poels (1913) as the cause of polyarthritis in sheep in Holland, and Wall (1914) reported similar cases. Christiansen (1919) reported the organism as a cause of acute septicaemia in very young lambs and Spiegl (1925) reported finding it in the umbilical vein, synovia and lungs of two lambs one week old, which had developed omphalophlebitis and polyarthritis. Krage (1923) isolated it from two outbreaks of septicaemia in lambs and Reinhardt (1924) from acute septicaemia in adult sheep. Biewener (1924) described a chronic arthritis in lambs seven to eight months old from which the organism was isolated. In England, Cornell and Glover (1925) reported an outbreak of arthritis in lambs due to *Erysipelothrix suis*. Harbour and Kershaw (1949) described two outbreaks of post-dipping lameness in sheep due to *Erysipelothrix insidiosa*. Edwards (1949) and Rowlands and Edwards (1950) described polyarthritis in lambs following the administration of lamb dysentery antiserum at birth or of pulpy kidney antiserum at three weeks of age, using serum that was contaminated with *Erysipelothrix insidiosa*. In 1927, Seddon and Carne described a specific arthritis in lambs in New South Wales caused by an organism they described as *B. pyogenes* which could have been *Erysipelothrix insidiosa*. According to Belschner (1959) and Seddon (1965) *Erysipelothrix insidiosa* is the most common organism associated with non-suppurative arthritis in lambs in Australia.

Following the demonstration of organisms of the *Chlamydia* group (Syn: *Bedsonia, Psittacosis - lympho-granuloma - venereum, Miyagawanella*) as causes of enzootic abortion in ewes in Scotland by Stamp, McEwen, Watt and Nisbet (1950) and of ovine enzootic pneumonia in California by McKercher (1952), they were subsequently recognised as a cause of polyarthritis in lambs (Mendlowski and Segre, 1960) and calves (Storz, Smart and Shupe, 1964). Epidemic polyarthritis of lambs due to *Chlamydia* is now well recognised in the United States. Mendlowski and Segre (1960) described the disease and its experimental transmission, and Mendlowski, Kraybill and Segre (1960) characterised the causative agent. Storz, Shupe, James and Smart (1963) describe isolation of *Chlamydia* from affected joints, internal organs and faeces of "stiff lamb disease" in Utah. Shupe and Storz (1964) describe a pathological study of the condition and Storz (1966) examined antigenic structures and interrelations of chlamydial agents associated with polyarthritis, enzootic abortions, intrauterine and latent intestinal infections. In Australia, *Chlamydia* have been isolated from non-suppurative polyarthritis in lambs by Jackson (1965) and Tammemagi and Simmons (1968).

Cordy, Adler and Yamamoto (1955) and Cordy and Adler (1960) reported the isolation of Mycoplasma species from a septicaemic and arthritic disease of goats. Experimental exposure of sheep to the goat arthritis Mycoplasma agent resulted in serositis and arthritis. However, no such organisms were isolated from field cases of arthritis in sheep.

Kennedy, Frazier, Theilen and Biberstein (1958) described a septicaemia of lambs caused by *Haemophilus agni*. Lambs that lived with this disease for twenty four hours or more subsequently developed fibrinopurulent arthritis, the changes being most easily recognised in the stifle joints. According to Pierson (1967) septicaemia in lambs due to *Haemophilus agni* and *Pasteurella haemolytica* may be characterised by stiffness, high body temperature, occasional arthritis and high mortality.

Pasteurella multocida, *Pasteurella haemolytica* and *Escherichia coli* are among other organisms recorded in New South Wales or elsewhere as causing a non-suppurative polyarthritis or synovitis in sheep (Jackson, 1969). Watt, Bamford and Nairn (1970) described the isolation of *Actinobacillus seminis* from lambs with polyarthritis in three flocks in Western Australia.

4.2 Polyarthritis due to *Histophilus ovis*

4.2.1 Introduction

Kater, Marshall and Hartley (1962) described a specific synovitis and pyaemia in lambs in New Zealand from which they isolated a Gram-negative pleomorphic organism which was indistinguishable from Roberts' (1956) isolate of *Histophilus ovis* from ewe mastitis and that recovered from ram epididymitis by Dodd and Hartley (1955).

During the period of this project, routine bacteriological examinations undertaken on cases of polyarthritis in lambs referred to this laboratory have resulted in the isolation of *Histophilus ovis* from sporadic field outbreaks of the disease. The condition has been seen usually in lambs from 1-6 weeks of age. The incidence of polyarthritis caused by *Histophilus ovis* is unknown but a recent abattoir survey conducted at this laboratory on an unusually high incidence of non-suppurative arthritis in slaughtered lambs showed *Erysipelothrix insidiosa* to be the major cause.

4.2.2 Clinical Observations

Affected lambs are obviously lame and reluctant to move. They show elevation of rectal temperatures up to 41.5°C during the acute phase of the disease. Lambs appear depressed, lethargic and anorexic and rapidly lose body condition. The affected joints of the limbs are usually swollen and warm to touch and lambs exhibit acute signs of pain when the joints are palpated.

A morbidity rate of 15% occurred in one outbreak investigated on a property on the north-western slopes of N.S.W. involving 700 crossbred lambs. There was no mortality but a number of the affected lambs were severely debilitated.

4.2.3 Gross Pathology

Arthritis in lambs due to *Histophilus ovis* was seen as essentially polyarticular in distribution with an associated tendo-vaginitis and bursitis. Lesions were more common in the carpal, tarsal, metacarpal and metatarsal joints than in those higher up the limbs or along the vertebral column but all joints have the potential to be involved. Kater, Marshall and Hartley (1962) noted involvement of the atlanto-occipital, atlanto-axial, costochondral, costosternal, costovertebral and intervertebral articulations in addition to limb joints.

The synovial fluid was increased in amount, distended the joint capsule, cloudy in appearance, mucoid in character and contained quite large greenish fibrin plaques. The synovial lining was hyperaemic and the villi were initially oedematous and later hyperplastic. The periarticular tissues were initially oedematous and congested and later fibrous, being invaded by a variety of inflammatory cells. There was no apparent ulceration of the synovial lining or necrosis of articular cartilages. The condition was initially described as a suppurative synovitis, but in the cases seen during this project the resulting polyarthritis was essentially fibrinous in appearance and more closely resembled that caused by *Erysipelothrix insidiosa* than the frankly purulent arthritides due to the common pyogenic organisms, and can thus be classified as a non-suppurative arthritis.

4.2.4 Histopathology

Histological sections prepared from affected joints showed hypertrophy and some hydropic degeneration of the synovial epithelium. There was intense vascular engorgement of the immediate underlying tissue and some small foci of haemorrhage. There were large numbers of migrating polymorphonuclear neutrophils particularly in the vicinity of the blood vessels and a moderate degree of inflammatory exudation was present. Numbers of mononuclear inflammatory cells were observed, many being almost fibroblastic in appearance. There was evidence of numbers of new capillary loops within the area of inflammation and in joints examined from lambs which had been affected for a week or more, numbers of fibroblasts and new vascularisations gave the typical appearance of new granulation tissue.

4.2.5 Pathogenesis

The synovial layer of the articular capsule (along with other papillary structures such as choroid plexuses of the brain and the ciliary processes of the eye) is a highly favoured site for the localisation of a variety of microorganisms. Most of these microorganisms are capable of proliferating in a variety of sites in the host and their local proliferation in synovial tissues implies opportunistic rather than selective localisation. A not too rapidly fatal bacteraemia will thus very frequently result in an arthritis. Arthritis then is usually haematogenous and polyarticular and associated with tendovaginitis and possibly bursitis. Initially probably most joints and tendon sheaths are involved but the inflammation subsides in some and progresses in others.

The mode of transmission of *Histophilus ovis* is not known, but there has never been any association with managerial practices such as lamb marking or dipping. Considering the age of affected lambs, navel infection following birth appears to be the most likely mode of infection but the possibility of intra-uterine infection cannot be dismissed.

4.2.6 Differential Diagnosis

The diagnosis of arthritis due to *Histophilus ovis* is dependent upon the isolation of the organism from affected joints, its identification, and its differentiation from other causes of non-suppurative arthritis. The causative organism is only readily recoverable from the more severely affected joints and even in those, it may be present in such small numbers in the synovial fluid that success in isolation is dependent upon initially inoculating synovial fluid into embryonated hen eggs or serum broth and then plating onto blood agar to demonstrate its presence.

Gram-negative rods which have been isolated from polyarthritis in sheep are *Haemophilus agni* (Kennedy, Frazier, Theilen and Biberstein, 1958), *Pasteurella haemolytica* (Pierson, 1967), *Pasteurella multocida* (Jackson, 1969) and *Actinobacillus seminis* (Watt, Bamford and Nairn, 1970). Differentiation of *Histophilus ovis* from these species may be achieved by reference to Table 3.

4.2.7 Experimental Reproduction

(a) *Materials*

(i) *Sheep:* Four 3-week-old lambs were obtained from the Shannon Vale Nutrition Station for use as experimental animals. These were the lambs out of the ewes used in the mastitis experiments. On arrival at the laboratory, the lambs were separated from their dams and fed three times a day with freshly prepared "Denkavite" milk mixture. After 4 days, all lambs were drinking the commercial food well and were regarded as clinically normal. They were then divided into 2 groups of 2 lambs.

(ii) *Culture:* The culture used was a dense 48 hour broth culture containing 1.8 x 10^7 viable organisms per ml as calculated by the Miles and Misra (1938) technique immediately prior to inoculation.

(b) *Methods*

Group 1: Using a 21 gauge 1" needle attached to a tuberculin syringe, two lambs were inoculated intravenously into the left jugular vein with 0.2 ml of the culture.

TABLE 3: Differentiation of Histophilus ovis from similar organisms isolated from ovine polyarthritis

	Histophilus ovis	*Haemophilus agni*	*Pasteurella haemolytica*	*Pasteurella multocida*	*Actinobacillus seminis*
Reference	Webb (1976) Roberts (1956)	Kennedy *et al.* (1958)	Pierson (1967)	Cowan and Steel (1974)	Watt *et al.* (1970)
Catalase activity	-	not tested	+	+	+
Indole production from tryptophane	+	-	-	+	-
Acid (no gas) production from sucrose	-	-	+	+/-	-

Group 2: Using a 25 gauge ½" needle attached to a tuberculin syringe, two lambs were inoculated directly into the left carpal joint with 0.1 ml of the same culture.

(c) *Results*

Group 1: Both lambs appeared slightly lame 2 days after inoculation but symptoms were far more obvious by the third day. Both lambs were lethargic, anorexic and extremely reluctant to move. Each lamb had a number of joints which appeared swollen, being particularly obvious in the carpal, tarsal and phalangeal joints. During this acute phase of the disease, rectal temperatures rose to 40.0°C and 41.3°C in the two lambs respectively.

One lamb (P) was destroyed 5 days, and the other (S) 7 days after inoculation for post mortem examination. Autopsy findings were identical to those described under pathological findings in field cases. The outstanding characteristic was the presence in all affected joints, bursae and tendon sheaths of soft greenish fibrin clots lying either free within the cavities or lightly adherent in places to the synovial membrane. In the lamb autopsied 7 days after inoculation, the clots appeared to be softening further and becoming almost caseous in appearance.

Stained smears were prepared from the synovial fluid of all limb joints and examined by Gram stain for the presence of organisms and by Leishmann stain for the presence of inflammatory cells. No organisms could be positively identified in direct smears and all joint fluids were inoculated onto 10% sheep blood agar and incubated at 37°C in an atmosphere of approximately 10% CO_2 for up to 5 days. The results of culturing and the presence of either polymorphonuclear (P) or mononuclear (M) inflammatory cells in the smears were as indicated in Table 4.

TABLE 4: Inflammatory cell response and culture results of joints in lambs artificially inoculated with Histophilus ovis intravenously

		Lamb P (5th day)		Lamb S (7th day)	
Limb	Joint	Smears	Culture	Smears	Culture
Right fore	Shoulder	P++++ M+	+ve	-ve	-ve
	Elbow	P- M+	-ve	P+ M-	-ve
	Carpus	P++++ M+	-ve	P++++ M-	+ve
	Phalangeal	P+ M+	-ve	-ve	-ve
Left fore	Shoulder	-ve	-ve	P- M+	-ve
	Elbow	P++++ M+	-ve	P+ M+	-ve
	Carpus	P++++ M+	+ve	P- M++	-ve
	Phalangeal	P+ M-	-ve	P+ M+	-ve
Right hind	Hip	-ve	-ve	-ve	-ve
	Stifle	P++++ M-	+ve	P- M+	-ve
	Tarsus	P++++ M-	-ve	P+ M+	-ve
	Phalangeal	P+ M++	-ve	P++++ M+	+ve
Left hind	Hip	P++ M-	-ve	P+ M+	-ve
	Stifle	P+++ M-	+ve	P+ M-	-ve
	Tarsus	P++++ M-	-ve	P- M-	-ve
	Phalangeal	P++ M-	+ve	P- M+	-ve

Group 2: Both lambs appeared lame the day after inoculation and by the second day the inoculated joints were grossly swollen, hot and obviously painful. No obvious systemic changes were apparent as there was no elevation of rectal temperature but there was a slight reduction in appetite. The lambs were destroyed 5 and 7 days after inoculation for post mortem examination. Autopsy revealed gross lesions in the inoculated joint only. Findings in this joint were identical to that previously described. There was no gross involvement of any other joints and smears and cultures prepared from all other limb joints failed to indicate the spread of the infection from the inoculated carpal joints.

Chapter V

ABORTION IN EWES

5.1 Literary Review

Congenital infection is only one of a multitude of factors listed by Hughes, Hartley, Haughey and McFarlane (1964) which contributes to the premature expulsion of the product of conception in ewes. Watson (1962) reviewed the world literature on this topic from 1873 and references cited by this author recorded normal incidence figures of 1-3% in individual flocks but up to and over 30% in the presence of a serious infectious outbreak.

McFarlane (1954) and Hartley and Boyes (1955) have shown that the infective agents which are responsible for abortion may also be associated with stillbirths (full term dead lambs) and the birth of full term lambs which die within a few hours of birth. Occasionally, lambs may be born from infected foetal membranes and survive. In a later investigation in New Zealand, Hartley and Boyes (1964) demonstrated that congenital infections were widespread at a low incidence on many properties and half of the accepted losses occurring in the ante-parturient, parturient and immediate post-parturient periods were associated with potentially pathogenic organisms.

In New South Wales, Australia, Hughes, Hartley, Haughey and McFarlane (1964) reported a low incidence of congenital infections in full term dead lambs. Haughey, Hughes and Hartley (1967) reported congenital infections in 28% of lambs lost in non-Merinos in the ante-parturient, parturient and immediate post-parturient periods. The same authors recorded that congenital infection was associated with at least 1% of all lambs born. Dennis (1965) demonstrated a low incidence of congenital infection in lamb losses in Western Australia.

The economic importance of abortion is difficult to ascertain as the majority of reports include such losses with those associated with parturient and post-parturient death of lambs, and the total of all these being referred to as the lamb contribution to the reason for low lambing percentages. According to Hartley (1968), observations made in all Australian states indicate that outbreaks of ovine infectious abortion are uncommon. However, as the majority of sheep are extensively grazed in this country, it is probable that abortions, if they occurred, would not be recognised.

The infectious agents associated with ovine abortion have been reviewed by Watson (1962).

Brucella abortus has been recorded in France (Carré, 1931), Greece (Ananiadés and Miaolis, 1931), Canada (Bruce, 1930), United States of America (Stoenner, 1951), and the United Kingdom (Young, 1953). *Brucella melitensis* has been recorded in France (Carré, 1931), Russia (Zdrodovskii, 1937), Japan (Itabashi and Watanabe, 1936; Watanabe, Tajima, Ito, Itabashi and Yoda, 1937) and in Germany (Karmann and Scholz, 1956; Geweniger, 1958). Reports of abortions associated with *Brucella ovis* have come from New Zealand (McFarlane, Jebson, Hartley, Salisbury, McClure and Osbourne, 1952; Buddle and Boyes, 1953), Australia (Keogh, Doolette and Clapp, 1958; Simmons, 1960) and the United States of America (Meinershagen, Frank and Waldham, 1974).

Salmonella abortus-ovis is the most common pathogen of the genus *Salmonella* in sheep and is the most common cause of ovine abortion in Germany (Endrejat, 1955). It has also been isolated in Great Britain by Bosworth and Glover (1925) and Greig (1936). Manley (1932) reported the infection in a flock in Cyprus, Lesbouyries, Dadot and Berthelon (1933) in France, Nikol'skii (1952) in Russia and in Italy (Pegreffi, 1934; Mura, Altieri and Contini, 1952). Occasionally other *Salmonella* serotypes have been associated with ovine abortion. Mlinac (1938) isolated a bacterium closely related to *Salmonella typhi* in Serbia. Vickers, Bierer, Atkinson, Mudge and Baker (1958) described the isolation of *Salmonella typhimurium* from a foetus, and Dennis and Armstrong (1965) report four outbreaks of gastroenteritis due to *Salmonella typhimurium* that were associated with abortion in Western Australia. Shearer (1957) and Watson (1960) reported *Salmonella dublin* as being associated with abortion and perinatal mortalities.

Vibrionic abortion in sheep was first recognised in England by McFadyean and Stockman (1913) as causing an abortion rate of 10% or more during a five year period. This condition has since been reported in the United States of America (Carpenter, 1920; Lee and Scrivner, 1938), in Germany (Knuth, 1925), in Sweden (Klarin, 1926), in France (Vinzent and Alloy, 1952), in Great Britain again (Buxton, 1931), in New Zealand (McFarlane, Salisbury, Osborne and Jobson, 1952), in Norway (Hoff, 1953; Hoff and Kaldahl, 1954; Eide and Helle, 1957), in Spain (Blanco Loizelier, Garcia Ferrero and G-Ochoa Juanes, 1955), in Poland (Czarnowski, 1956), and in Australia by Gardiner (1961).

Listeria monovytogenes has been isolated from aborted ovine foetus in Great Britain (Paterson, 1940), United States of America (Poppensiek, 1944; Eveleth, Gouldsby, Bolin, Halm and Turn, 1953), Sweden (Olson, 1945), India (Dhanda, Lall, Seth and Chandrasekariah, 1959), Hungary (Csontos and Pesti, 1955), and Bulgaria (Ivanov, 1956). An epidemic of abortion observed in Australia by Hindmarsh and Blumer (1932) was later considered to have been due to *Listeria monocytogenes*. Seddon (1965) mentions Listerial abortion in Queensland and Victoria, and Moule (1954) recovered it from ovine foetus.

Other bacteria have been isolated from foetus and have been considered to be related to their abortion. Howarth (1932) reported a coliform organism while Miessner (1929), and Miessner and Koser (1931) recovered *Escherichia coli* from ovine foetus. Sorum (1953) isolated a diphtheroid resembling *Corynebacterium pyogenes* in pure culture from aborted foetus. An organism classified as *Fusiformis fragilis* was isolated from an aborted foetus by Lindqvist (1956) and Horter (1956). Miessner and Koser (1931) and Hartley and Boyes (1955) isolated *Streptococci spp.* from aborted lambs. Lee and Scrivner (1938) and Manninger (1918) describe an unidentified Gram-negative bacillus. Hartley and Boyes (1955) describe *Pasteurella* organisms. Watson (1960) describes one instance of the isolation of *Pasteurella pseudotuberculosis* apparently associated with abortion; this organism has also been recorded in Australia by Hughes, Hartley, Haughey and McFarlane (1964). Leptospirosis has been described by Beamer, Harden Brook and Morrill (1953) in a flock of young ewes.

Stamp, McEwan, Watt and Nisbet (1950) detected the presence of elementary bodies resembling *Chlamydia* agents in foetal membranes and discharges of aborted lambs in Scotland. Later the condition was reported from Sardinia (Spanedda and Medda, 1951), France (Giroud, Roger, Vallée and Roger, 1956; Vallée, Roger and Roger, 1956; Moraillon, 1957; Faye, 1958), Germany (Mitscherlich, 1954; Horter, 1956; Monreal and Fritzsche, 1956; Enke, Liebermann and Schuckmann, 1959), Hungary (Romváry, 1958; Hadju, Ratalics, Szabo and Temesi, 1958), the United States of America (Young, Parker and Firehammer, 1958; Parker, 1960), Holland (Eisma and Terpstra, 1961), and Australia (Hughes, Hartley, Haughey and McFarlane, 1964; Kater, 1965).

Cole, Sanger, Farrell and Korrider (1954) were the first to associate ovine abortion with *Toxoplasma* infection. In the same year, Hartley, Jebson and McFarlane (1954) recorded lesions in aborted placental material and found organisms resembling *Toxoplasma gondii* in sections. Since then, abortion associated with *Toxoplasma* infection has been seen in Australia (Osborne, 1959; Studdert and Johnson, 1959; Smith 1961), United Kingdom (Beverley and Watson, 1959, 1961) and in Canada (Holland and Tobe, 1961; Fish, 1961).

Abortion may occur in pregnant animals acutely ill from any infectious disease and particularly in viral diseases such as Rinderpest, Sheep Pox and Foot and Mouth Disease. Oppermann (1920) reported abortion in 37% of a flock of ewes with Foot and Mouth Disease. Abortion due to Rift Valley Fever was noted by Daubney and Hudson (1931), Alexander (1951), Henning (1952) and van der Linde (1953). Wesselsbron virus caused abortion and neonatal deaths as reported by Weiss, Haig and Alexander (1956) as well as Nairobi Sheep Disease virus (Henning, 1952). Schultz and DeLay (1955) reported losses associated with the use of Bluetongue vaccine in pregnant ewes with lambs being stillborn, spastic and deformed. Stamp, Watt and Jamieson (1950) demonstrated that Tick-borne Fever can produce abortion and in the same year, Littlejohn (1950) recorded three outbreaks. The agent was classified as *Rickettsia phagocytophila* by Foggie (1951). Marmion and Watson (1961) described the isolation of *Rickettsia burneti* from placentae and aborted foetus.

5.2 Abortion Due to *Histophilus ovis*

5.2.1 Introduction

According to Hartley (1968) the commonest infectious agent involved in ovine abortion in Australia appears to be *Vibrio foetus*, followed by *Toxoplasma gondii* and *Listeria monocytogenes*. The other agents appear to be of minor importance and are listed by Hughes, Hartley, Haughey and McFarlane (1964) as *Brucella ovis* and *Chlamydiae spp*. During the period of this project, outbreaks of abortion in sheep were investigated on a number of occasions and the three commonest pathogens were detected in sporadic cases.

The one outbreak of abortion in ewes due to *Histophilus ovis* occurred on a property near Guyra, N.S.W., was investigated at this laboratory, and reported in Veterinary Notes, 1968. Approximately 50% of the ewe flock numbering 150 aborted their lambs.

5.2.2 Clinical Observations

Abortions occurred at approximately the 3-3½ months stage of pregnancy as judged by the cranial-rump length of the aborted foetus (Dun, 1955). Affected ewes did not appear to show any premonitary clinical signs of impending abortion. Some of the ewes did retain their foetal membranes for up to 3 days after aborting. All ewes failing to produce a lamb that year were subsequently culled so that no information is available on what effect the infection had on subsequent breeding performance.

5.2.3 Gross Pathology

The main pathological feature of abortion due to *Histophilus ovis* was a marked haemorrhagic placentitis involving both the cotyledons and the intercotyledonary areas; these areas were also slightly oedematous. Examination of the aborted foetus revealed excessive blood stained fluid in all body cavities and some subcutaneous oedema. There was advanced autolysis of visceral organs. One foetus examined showed some dehydration and changes suggestive of early mummification. There were no specific pathological changes in the foetus and no evidence of necrotic foci in the liver.

5.2.4 Histopathology

Sections prepared from the placenta were stained with haematoxylin-eosin. Examination revealed vascular engorgement of vessels within the cotyledons and intercotyledonary areas. Many of the placental cells were necrotic with pycnotic nuclei. There was mild oedema and inflammatory exudation which contained low numbers, but universally distributed, polymorphonuclear inflammatory cells. There were small areas of focal haemorrhage. Gram stained sections revealed what appeared to be Gram-negative pleomorphic organisms but differentiation from the inflammatory background was difficult.

5.2.5 Pathogenesis

There was no environmental condition or managerial practice detected that could be related to the abortion outbreak described. The rams which were used were not affected with epididymitis. Lambs born to the pregnant ewes which did not abort had no evidence of arthritis, bursitis or tendovaginitis.

The post mortem findings in the foetus of organ autolysis, early oedema, later dehydration and mummification, indicate initial intra-uterine death prior to expulsion. It was considered that foetal death was caused by the placentitis.

5.2.6 Differential Diagnosis

Diagnosis depends upon the demonstration, isolation and identification of *Histophilus ovis* from the aborted placenta. Organisms were demonstrated in Gram stained smears prepared from the placenta, using 10% carbol fuchsin as the counter stain, and isolated when inoculated onto 10% sheep blood agar and incubated under microaerophilic conditions at 37°C.

Amongst the numerous infectious agents previously listed as known to cause abortion in ewes, there does not appear to be any bacterial species with which *Histophilus ovis* could be confused on simple morphological characteristics and growth requirements. Differentiation from *Pasteurella sp.* provides the main bacterial diagnostic problem.

5.2.7 Experimental Reproduction

(a) *Experiment 1*

Two supposedly pregnant ewes were acquired from the Shannon Vale Nutrition Station, and housed at this laboratory as previously described. The isolate obtained from the abortion outbreak was inoculated into both ewes intravenously. Six weeks later, one of the ewes gave birth to a small, weak lamb which died shortly after birth. The placenta showed universal haemorrhagic placentitis. *Histophilus ovis* was isolated from the placenta but not from various foetal organs cultured. The second ewe was found to be non-pregnant.

(b) *Experiment 2*

Another two pregnant ewes were inoculated 3½ months after joining with an isolate obtained from a field case of epididymitis. Each ewe was inoculated intravenously with 10 cc of a 48 hour broth culture containing 1.3×10^7 viable organisms per ml. Six weeks later, both ewes produced a normal healthy lamb and a normal placenta. *Histophilus ovis* was not isolated from the placenta. This culture had undergone numerous passages on artificial media in the laboratory prior to use but, in an effort to increase its pathogenicity, it was passaged into the epididymis of one ram prior to use in this experiment. Other reasons why this experiment failed could be due to the fact that the ewes used were not at the correct stage of pregnancy for the infection to occur, or that isolates obtained from cases of epididymitis do not cause abortion. No abortions were observed in ewes which were running with affected rams on the properties where outbreaks of epididymitis occurred.

Chapter 6

BACTERIOLOGICAL INVESTIGATIONS OF HISTOPHILUS OVIS

6.1 Bacteriological Characteristics of *Histophilus ovis*

6.1.1 Introduction

Seventeen isolates of *Histophilus ovis* were used to determine the bacteriological characteristics of the organism. These isolates were derived from pathological cases of mastitis (one), epididymitis (three), polyarthritis (two) and abortion (one) submitted to the District Veterinary Laboratory, Armidale during the course of this investigation. In addition, Mr A. Corbould submitted eight isolates obtained at the Tasmanian Department of Agriculture's Mount Pleasant Laboratory, Launceston, from cases of polyarthritis and Mr R. Rahaley submitted two isolates obtained at the Victorian Department of Agriculture's Regional Veterinary Laboratory, Hamilton from cases of pyosepticaemia in young lambs. These isolates were used to determine the bacteriological characteristics of *Histophilus ovis*.

6.1.2 Growth Requirements

Optimum growth occurred when cultures were incubated at 37°C. There was no visible growth when cultures were incubated at 22°C or 45°C.

Optimum growth occurred when cultures were incubated under conditions of reduced oxygen tension, using the candle-jar technique. There was only slight growth in some strains under strictly aerobic or anaerobic conditions, whereas others did not grow. On this criterion, the organism was regarded as microaerophilic or facultatively anaerobic.

Growth did not occur in ordinary commercial nutrient agar, nor on MacConkey agar or in Koser's citrate medium. Oxoid brand peptone water or tryptone water broths did not support growth but good growth occurred when meat particles, 10% sterile sheep serum, or 10% sterile defrinated sheep blood was added to these media. The addition of either 1% glucose of 1% maltose to peptone or tryptone water did not produce growth but when 10% sterile sheep serum or meat particles was added, growth was adequate.

The addition of 0.0075% KCN to peptone water containing 10% sterile sheep serum prevented growth.

6.1.3 Growth Characteristics

On moist, freshly poured agar plates containing 10% sheep blood there is usually no evidence of growth of primary cultures until after 36-48 hours incubation microaerophilically at 37°C. At this stage, colonies are 0.5-0.75 mm in diameter, are circular in shape with an entire edge, are low convex in elevation with a smooth glistening surface and are almost transparent. After 72-96 hours incubation the colonies become differentiated into a central circular opaque area with a transparent peripheral skirt having an irregular margin giving the colony a characteristic "fried-egg" appearance. Individual colonies appear white-grey in the centre but when scooped up with a platinum loop or smeared onto white filter paper, they have a distinct yellow colour which is not apparent when growing on the agar. Colonies are butyrous in consistency and are easy to emulsify in physiological saline to form a stable suspension although occasionally there is fine granulation apparent with a 3 x eye lens. There is no evidence of haemolysis nor any pigment production either within the colony or diffusing in to the adjacent medium.

In tryptone water broth containing 10% sterile sheep serum, growth was scant to moderate. There was very little turbidity, the majority of bacterial mass being present in a granular deposit at the bottom of the bottle. This deposit disintegrated completely with mild shaking to form an even suspension but quickly settled again on standing. There was no evidence of surface growth nor any pellicle formation in either nutrient broth medium containing 10% sterile sheep serum nor in cooked meat medium. There was no apparent odour in broth cultures but a faint sickly odour was emitted by cultures on agar.

6.1.4 Bacterial Morphology and Staining

Histophilus ovis is a pleomorphic bacillus. In stained smears prepared from pathological material or from young cultures, the organism has the form of bacilli with parallel sides and rounded ends with a straight axis and 1-3μ in length by 0.4-0.5μ in width. In smears prepared from older cultures, more spherical forms are apparent with a diameter of 0.6-0.7μ. Usually a few filamentous forms are detected up to 6μ in length with tapering pointed ends and a slightly curved axis. In young cultures the organisms are usually arranged singly, but occasionally paired or in short chains in smears prepared from older cultures. Capsules are not apparent using the India-ink negative staining technique. Spores are not apparent in either Gram-stained smears or those using specific acid-fast and malachite green stains. The organisms are non-motile at 37°C and no flagellum is detectable using Leifson's method.

Histophilus ovis is a Gram-negative pleomorphic organism; it is seen best when 10% carbol fuchsin is used as the counter stain. The organism stains evenly and intensely with Leishmann's technique showing no evidence of bipolarity or metachromatic granules. In older cultures there are often some swollen forms which appear to take the stain mainly in their envelopes. It is non acid-fast to the standard Ziehl-Neelsen stain and is also negative when the modified technique of Stamp, Watt and Jamieson (1950) is used.

6.1.5 Biochemical characteristics

Ten per cent of freshly collected sterile sheep serum was added immediately prior to inoculation to all media used to determine biochemical characteristics.

The enzyme catalase was not detected when hydrogen peroxide was added to liquid cultures or when a loop full of colonies growing on sheep blood agar was emulsified in a drop of H_2O_2 on a glass slide. Cytochrome oxidase activity was not detected when colonies were removed from a blood agar plate using a bent glass rod and spread on a piece of filter paper previously moistened with a freshly prepared solution of 1% tetramethyl-p-phenylenediamine.

Carbohydrate fermentation studies were undertaken using a base medium of 1% "Oxoid" peptone water containing phenol red indicator at a final concentration of 0.01% to detect acid production, an inverted Durham tube to detect gas production and various carbohydrates at a final concentration of 0.5% in the media. There was acid but no gas production from the hexose monosaccharides glucose (dextrose), fructose (laevulose), galactose and mannose and the pentose monosaccharide xylose. There was also acid but no gas production from the sugar alcohols mannitol and sorbitol. It took from 3 to 7 days for the indicator to change. There was no evidence, after two weeks incubation, of fermentation of the pentoses arabinose and rhamnose, the disaccharides maltose, sucrose, lactose, trehalose, the trisaccharide raffinose, the polysaccharides inulin and dextrin, the sugar alcohols glycerol, adonitol, dulcitol and inositol nor the glycoside salicin. Using Hugh and Leifson's medium, it was deduced that acid production from glucose was fermentative.

Using "Oxoid Tryptone Water", a 72 hour culture was shown to produce indole from tryptophane by initially extracting with ether and then adding a small volume of Ehrlich's reagent.

The presence of the enzyme nitrate reductase was detected when an inoculated medium containing 0.02% KNO_3 was tested for nitrite after 72 hours incubation using sulphanilic acid and α-naphthylamine as the test reagents. There was no evidence of further breakdown of the nitrite.

The isolates were all negative to the methyl red test indicating their inability to sustain the acid conditions produced following the fermentation of glucose. Using Barritt's method, the cultures were all negative to the Voges-Proskauer test indicating their inability to form the condensation product acetyl-methyl-carbinol or its reduction product 2,3 butylene glycol from the pyruvate formed from glucose fermentation.

The production of hydrogen sulphide was not detected using either lead acetate paper strips held over nutrient broth cultures nor when isolates were inoculated into commercial SIM medium.

Formaldelyde-denatured gelatin discs containing finely powdered charcoal were not liquified when incubated for two weeks in nutrient broth cultures.

The organism did not split urea using Christensen's urea medium. No change was detected in litmus milk after 14 days incubation.

All isolates decarboxylated the amino acid ornithine but were negative for arginine and lysine decarboxylase.

Maintenance of viable cultures was initially a problem as both blood agar and cooked meat cultures usually died out in 5-7 days. Twice-weekly sub-culturing was essential until a satisfactory and reliable method of lyophilization was developed. Satisfactory preservation was achieved by harvesting the growth from blood agar plates, suspending a heavy concentration of the growth in a freeze-drying fluid consisting of 5% bovine albumen, 5% sucrose and 1% sodium glutamate in distilled water. 0.25 mls of this was placed into a pre-sterilised freeze-drying ampoule, shell-freezed in liquid nitrogen and then placed onto a previously cooled and evacuated Virtis freeze-drying apparatus and run for 6-7 hours at -50°C and a pressure of ten microns. Ampoules were then sealed under vacuum while still attached to the freeze-drier manifold.

There was no apparent loss of virulence after sub-culturing of strains as shown by the pathogenicity of repeatedly sub-cultured isolates for the ewe's mammary gland and the ram's epididymis when inoculated directly.

6.2 Comparison of *Histophilus Ovis* With Other Members of the Family *Brucellaceae*

On the basis of the cultural characteristics of his isolate, Roberts (1956) considered *Histophilus ovis* to be automatically placed in the family *Parvobacteriaceae* (Rahn, 1937) as described in the 6th edition of Bergey's Manual (Breed, Murray and Hitchens, 1948). The 7th edition of the Manual considered the new family name *Brucellaceae*, as being more acceptable for this group of organisms. They are described as being "small, coccoid to rod-shaped cells which occur singly, in pairs, in short chains or in groups; filamentous and pleomorphic forms are occasionally found. Motile and non-motile species occur, the motile species possessing from one to eight peritrichous flagella; with certain of the motile species, motility can be demonstrated only at lower temperatures (18° to 26°C). May or may not be encapsulated. May or may not show bipolar staining. Gram-negative. V(phosphopyridene nucleotide) and/or X (haemin) factors are sometimes required for growth; blood serum and similar enrichment materials may be required or may enhance growth. Increased CO_2 tension may also favour growth, especially on primary isolation. Gelatin usually not liquefied. Carbohydrates may or may not be attacked with the production of acid but no gas. Nitrites may or may not be produced from nitrates. Aerobic, facultatively anaerobic. Some invade living tissues; infection in some cases may take place by penetration of the organism through mucous membranes or through the unbroken skin. Parasites and pathogens which affect warm-blooded animals, including man, rarely cold-blooded animals".

From the findings of this project it would appear that all strains would satisfy the requirements of the family *Brucellaceae*.

Bergey's Manual (1957) divides the family into eight genera on the following criteria.

I. Non-motile at 37°C, but may be motile at lower temperatures.

A. Predominantly occur singly or in masses.

1. Cells predominantly occur singly and do not occur in masses.

a) Grow on peptone media but may require blood serum or similar enrichment materials for growth.

b) Show, or tend to show, bipolar staining.

c) Attacks carbohydrates.

Genus I. *Pasteurella*.

cc) Does not attack carbohydrates.

Genus II. *Bordetella*.

bb) Does not show bipolar staining.

Genus III. *Brucella.*

aa) Requires V (phosphopyridene nucleotide) and/or X (haemin) factors for growth.

Genus IV. *Haemophilus.*

2. Cells predominantly occur singly and show pleomorphism and/or occur in masses.

a) Growth occurs on ordinary media; increased CO_2 tension enhances growth, especially on primary isolation.

Genus V. *Actinobacillus.*

aa) Growth occurs on infusion media only after growth in chick embryo.

Genus VI. *Calymmatobacterium.*

B. Predominantly occur as diplobacilli.

Genus VII. *Moraxella.*

II. Motile at 37°C.

A. Optimum temperature for growth, 37°C. Litmus milk becomes strongly alkaline.

Genus II. *Bordetella.*

B. Optimum temperature for growth, between 28° and 30°C. Litmus milk unchanged.

Genus VIII. *Noguchia.*

Species within the genus *Pasteurella* (Trevisan, 1887) are described as being "small, ellipsoidal to elongated rods which show bipolar staining by special methods. Gram-negative. Gelatin not liquefied. Milk not coagulated. The majority of species ferment carbohydrates but produce only a small amount of acid; no or slight lactose fermentation; no gas production. Aerobic, facultatively anaerobic. May require low oxidation-reduction potential on primary isolation. Parasitic on man, other animals and birds". Findings from this project indicate many similarities of *Histophis ovis* to members of this genus. However, the most distinguishing features from this genus appear to be the total reliance of *Histophilus ovis* on either blood or serum in the medium for growth, its extreme pleomorphism and its even staining without bipolarity.

Species within the genus *Bordetella* (Moreno-López, 1952) are described as being "minute coccobacilli. Motile and non-motile species occur. Gram-negative. On primary isolation, some species are dependent on complex media; all are haemolytic. Carbohydrates are not fermented. Litmus milk becomes alkaline. A dermonecrotic toxin is produced. Parasitic. Cause whooping cough or an infection clinically resembling it". *Histophilus ovis* does not appear to be related to this genus as it is non-haemolytic, non-motile, attacks some carbohydrates, has no effect on litmus milk, and no dermonecrotic toxin was demonstrated.

Species within the genus *Brucella* (Meyer and Shaw, 1920) are described as being "short, rod-shaped to coccoid cells, 0.5 to 2.0 microns. Encapsulated. Non-motile. Gram-negative. Gelatin is not liquefied. No gas produced from carbohydrates. Urea is hydrolyzed, H_2S is produced and the species are positive to the modified Ziehl-Neelsen stain of Stamp, Watt and Jamieson (1950). Parasitic, invading all animal tissues and producing infection of the genital organs, the mammary gland and the respiratory and intestinal tracts. Pathogenic for various species of domestic animals and man". Buddle (1956) described the new species *Brucella ovis* which has pathological similarities to *Histophilus ovis* in causing ovine epididymitis and abortion. *Histophilus ovis* is not considered to be related to members of this genus as it is more pleomorphic, has different colonial morphology, does not split urea or produce H_2S and is negative to the modified Ziehl-Neelsen stain.

Species of the genus *Haemophilus* (Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith, 1917) are described as being "minute, rod-shaped cells which are sometimes thread-forming and pleomorphic. Non-motile. Gram-negative. Strict parasites, growing only in the presence of certain growth accessory substances. May or may not be pathogenic. Found in various lesions and secretions, as well as in normal respiratory tracts, of vertebrates". Findings from this project indicate similarities of *Histophilus ovis* to members of this genus. *Histophilus ovis* does appear to be more specific in its gaseous requirements for growth and requires different growth factors present in either serum or blood.

Species of the genus *Actinobacillus* (Brumpt, 1910) are described as being "small to medium sized, coccoid to rod-shaped cells which rarely grow into filaments; under special conditions the filaments may show some branching. Non-motile. There is a tendency to show bipolar staining. Gram-negative. Colonies may be mucous or stringy, especially when freshly isolated, and white, greyish white, yellowish or bluish in colour. Aerobic to facultatively anaerobic. Microaerophilic in primary cultures. Acid but no gas from carbohydrates, when fermented. Pathogenic for animals; some species attack man". It is apparent from the findings of this project that *Histophilus ovis* has characteristics resembling this genus although growth does not occur on unenriched media.

Species in the genus *Calymmatobacterium* (Aragão and Vianna,

1913) are described as being "pleomorphic rods which exhibit single or bipolar condensations of chromatin and which occur singly and in clusters. May or may not be encapsulated. Non-motile. Gram-negative. Growth outside of the human body occurs only in the yolk sac or amniotic fluid of developing chick embryo or in a medium containing embryonic yolk; after adaptation, growth may occur in meat infusion media. Pathogenic for man causing granulomatous lesions, particularly in the inguinal region". *Histophilus ovis* does not satisfy the criteria for this genus.

Species in the genus *Moraxella* (Lwoff, 1939) are characterised by being "small, short, rod-shaped cells which occur as diplobacilli and which are sometimes described as diplococci; occasionally occur singly. Non-motile. Gram-negative. Do not require V (phosphopyridine nucleotide) or X (haemin) factors for growth; growth is, however, dependent upon or improved with the addition of serum or ascitic fluid. Litmus milk is unchanged or becomes alkaline. Carbohydrates generally not attacked. Actively proteolytic, liquefying inspissated blood serum and even egg media. Oxidase-positive. Aerobic. Found as parasites and pathogens in warm-blooded animals, being especially found in association with diseases of the eye". The isolates of *Histophilus ovis* investigated in this project were not regarded as satisfying the requirements of this genus.

Species of the genus *Noguchia* (Olitsky, Syverton and Tyler, 1934) are characterised by being "small, slender rods, encapsulated, motile by means of peritrichous flagella. Gram-negative. Produce a mucoid type of growth which, on initial isolation, occurs with some difficulty on ordinary media. Aerobic to facultatively anaerobic. Optimum temperature between 28° and 30°C. Found in conjunctiva of man and animals affected by a follicular type of disease". *Histophilus ovis* is thus not regarded as a member of this genus.

Thus, it is felt that the organism described in this project, as well as the "Gram-negative pleomorphic organism" described by other Australian and New Zealand workers, closely resembles the species classified in the genera *Pasteurella*, *Haemophilus* and *Actinobacillus* of the family *Brucellaceae*. The recently published and radically altered eighth edition of Bergey's Manual of Determinative Bacteriology has dispensed with the family grouping *Brucellaceae* and lists these three genera amongst those of uncertain affiliation in Part 8 - the Gram-negative facultative anaerobic rods.

6.3 Comparison of *Histophilus ovis* With Species in the Genus *Pasteurella*

The number of species within the genus *Pasteurella* continues to grow smaller and in the eighth edition of Bergey's Manual, only four are recognised (*P. multocida*, *P. pneumotropica*, *P. haemolytica* and *P. ureae*). Two additional species are included as being of uncertain status (*P. anatipestifer* and *P. pfaffi*). Marsh (1932) and Smith and Harnden (1943) described what they felt to be a new species of the genus, suggesting the name *Pasteurella mastitidis*, from a specific mastitis in ewes.

Pasteurella multocida is the type species of the genus and has been isolated from pathological cases of polyarthritis (Jackson, 1969) and epididymitis (Ekdahl, Money and Martin, 1968) in sheep in Australasia, under which conditions, confusion with *Histophilus ovis* may occur. However, during this project, *Pasteurella multocida* was found to be much easier to isolate, colonies were easily detectable on sheep blood agar after 24 hours incubation, growth requirements were far less exacting and biochemical characterisation tests made differentiation easy between the two species.

Pasteurella haemolytica has been isolated from cases of epididymitis (Ekdahl, Money and Martin, 1968), mastitis (Blood and Henderson, 1968) and polyarthritis (Pierson, 1967; Jackson, 1969) in sheep. Furthermore, it is a well recognised cause of septicaemia in lambs (Bruner and Gillespie, 1966). Although described initially as haemolytic, strains recovered during this project were generally non-haemolytic but this was probably due to the fact that sheep blood was used entirely, whereas the original reports by Jones (1921) and Newsom and Cross (1932) used horse and cow blood. Confusion on the taxonomy of this species does occur as Bruner and Gillespie (1966) consider it to be a variant of *Pasteurella multocida* and Smith (1974) regards it as being strongly related to the genus *Actinobacillus*. Certainly difficulties were experienced in differentiating this species from other members of the genera *Pasteurella* and *Actinobacillus* but the ease and rapidity of growth on primary isolation allows differentiation from *Histophilus ovis*.

Pasteurella mastitidis has been described in Australia by Simmons and Ryley (1954) following its original description by Marsh (1932) and Smith and Harnden (1943). It causes a specific acute gangrenous mastitis in ewes. Bacteriological investigations by Simmons and Ryley (1954) showed that their isolate attained a colony size of 2-3 mm following incubation at 37°C for 24 hours on 10% sheep blood agar. Colonies were round and entire and had a zone of haemolysis beneath the colony. These factors readily allow early differentiation from *Histophilus ovis*. Biochemical tests showed similar results but did differ as outlined in Table 5.

TABLE 5: Differentiation of Histophilus ovis from Pasteurella mastitidis

		Pasteurella mastitidis (Simmons and Ryley, 1954)	*Histophilus ovis* (Webb, 1976 Roberts, 1956)
Indole production from tryptophane		-	+
Inositol	Acid (no gas) production	+	+
Glycerol	Acid (no gas) production	+	-
Raffinose	Acid (no gas) production	+	-
Lactose	Acid (no gas) production	+	-
Sucrose	Acid (no gas) production	+	-
Mannose	Acid (no gas) production	-	+
Arabinose	Acid (no gas) production	+/-	-

On the basis of colony morphology and growth requirements it is suggested that *Histophilus ovis* does not satisfy the requirements of the genus *Pasteurella*. The species *Pasteurella mastitidis* appears to be indistinguishable from *Pasteurella haemolytica* and may well be a strain of it.

6.4 Comparison of *Histophilus ovis* With Species in the Genus *Haemophilus*

There are 14 well recognised and described species of the genus *Haemophilus* in the eighth edition of Bergey's Manual. Each of these species has well defined specific requirements for the growth factors X (haematin) and/or V (Coenzyme I or II) and these requirements are used to identify the individual species. As neither of these factors, either singly or combined, support the growth of *Histophilus ovis*, these 14 species will not be discussed further. Amongst the 5 species of "uncertain status" included in the genus is *Haemophilus ovis* which appears to have been described only once (Mitchell, 1925). In addition, Kennedy, Frazier, Theilen and Biberstein (1958) described what they considered a new species of the genus for which they proposed the name *Haemophilus agni*. These latter two species do have similarities to *Histophilus ovis*.

Haemophilus ovis was described by Mitchell (1925) as a new species following its isolation from a single outbreak of a disease thought initially to be haemorrhagic septicaemia in sheep. Clinically affected sheep showed prostration, difficult breathing, cyanosis, initial elevation of body temperature and chest auscultation revealed that signs were due to lung involvement. Autopsy examination revealed bilateral peracute broncho-pneumonia. The organism was isolated from the lungs, bronchi, pleural and pericardial cavities. Mitchell considered that the isolate was a species of *Haemophilus* on the basis of "its behaviour on serum agar and its faint staining characteristics". This supposition was strengthened by the "better growths obtained when cultures were made on chocolate agar". From his description, it is apparent that *Haemophilus ovis* could not be distinguished from *Histophilus ovis* on the basis of colony morphology, growth requirements, bacterial morphology and staining reactions. Furthermore, carbohydrate fermentation studies gave similar results (see Table 6) and the only difference appears to be in the production of indole from tryptophane.

Haemophilus agni was described by Kennedy, Frazier, Theilen and Biberstein (1958) as a new species following its isolation from a single outbreak of septicaemia in a flock of 6-7 month old lambs. Clinically, affected lambs were depressed, febrile and reluctant to move. Autopsy examination revealed multiple haemorrhages throughout the carcass, swollen spleen and multiple foci of liver damage. Lambs

TABLE 6: Results of fermentation studies on Histophilus ovis, Haemophilus ovis and Haemophilus agni

	Histophilus ovis (Webb, 1976)	*Haemophilus ovis* (Mitchell, 1925)	*Histophilus agni* (Kennedy *et al.*, 1958)
Acid (no gas) production from:			
Glucose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Sorbitol	+	+	+
Inositol	-	-	+
Raffinose	-	+	+
Arabinose	-	-	+
Lactose	-	+/-	-
Mactose	+	+	+
Mannitol	+	+	+
Mannose	+	+	+
Xylose	+	+/-	+
Rhamnose	-	-	+

which survived 24 hours or more developed a fibrino-purulent arthritis. All lambs showed pulmonary congestion and oedema, some dying from apparent asphyxiation. Kennedy, Frazier, Theilen and Biberstein (1958) considered their isolate to be in the genus *Haemophilus* as it "failed to grow in the absence of blood or blood constituents". However, Cruickshank (1970) states that "if the term haemophilic is used in a broad sense to designate organisms which require blood for their growth it would embrace a number of heterogeneous species and the generic name *Haemophilus* is therefore restricted to those organisms which are dependent on one or more of the growth factors required by *Haemophilus influenzae*". Kennedy, Frazier, Theilen and Biberstein (1958) simply state that V factor is not required for *Haemophilus agni*. From the description of *Haemophilus agni*, it is apparent that there are many similarities between this species, *Histophilus ovis* and *Haemophilus ovis*. Fermentation studies gave similar results (see table) but Kennedy makes the point that these are "unimportant, due to the notorious variability in biochemical behaviour of different strains". No mention is made in Mitchell's paper of how many strains of *Haemophilus ovis* he examined and all of Kennedy's 5 strains of *Haemophilus agni* originated from one flock during one outbreak. Growth characteristics and requirements appear similar between *Haemophilus agni* and *Histophilus ovis* and only appear to differ from *Haemophilus ovis* in factors not specified by Mitchell (e.g. enhanced growth under 10% CO_2 atmospheric conditions). Morphological and staining characteristics of *Haemophilus agni* are indistinguishable from the other two species.

It is thus felt that findings from this project indicate that the organism with the suggested name of *Histophilus ovis* cannot be unequivocably differentiated bacteriologically from the species previously described as *Haemophilus ovis* and *Haemophilus agni*. Furthermore, it is apparent from the recorded descriptions of the latter two organisms that their inclusion in the genus *Haemophilus* is on rather dubious grounds and if the definition of the genus as stated by Zinnemann and Biberstein (1974), and by Cruickshank (1970) are to be accepted, then neither species qualifies for inclusion.

6.5 Comparison of *Histophilus ovis* With Species in the Genus *Actinobacillus*

The eighth edition of Bergey's Manual contains only two species as acceptable within the genus *Actinobacillus*, the type species

Actinobacillus lignieresi and the equine species which has had such a chequered taxonomic career, *Actinobacillus equuli*. Five additional species are listed as being of uncertain status within this genus. Baynes and Simmons (1960) isolated an apparently new species to which they gave the suggested name *Actinobacillus seminis*.

Actinobacillus lignieresi was first described by Lignieres and Spitz (1902) as the causal agent of a specific granulomatous disease of cattle. Phillips (1960) published an excellent paper on the characterization of 225 strains of this organism and was subsequently responsible for the definition of this genus in Bergey's Manual. It has been isolated from cases of mastitis in ewes in Queensland (Laws and Elder, 1969b) and epididymitis in rams (Laws and Elder, 1969a). In their report, Laws and Elder (1969b) considered that the strains obtained by them were indistinguishable from *Pasteurella mastitidis* and other Gram-negative pleomorphs isolated from cases of ovine mastitis. On this basis it is apparent that *Histophilus ovis* has some characteristics which align it to the type species of this genus.

Actinobacillus seminis was first described by Baynes and Simmons (1960) as a new species following its isolation from cases of ovine epididymitis. Subsequently, Watt, Bamford and Nairn (1970) isolated it from 3 natural outbreaks of polyarthritis in lambs, and experimentally produced mastitis in ewes with their isolate. These authors considered that Roberts' (1956) isolate of *Histophilus ovis* could well have been *Actinobacillus seminis*. There does appear to be a number of reasons why this species does not satisfy the requirements of the genus *Actinobacillus*. It does not grow on MacConkey agar nor split urea; furthermore, Phillips (1974) states that "failure to ferment any of a wide range of carbohydrates excludes this species from the genus". *Histophilus ovis* could be confused with *Actinobacillus* *seminis* as the two species are similar on bacterial morphology, staining reactions, most growth requirements and colony morphology.

From the findings of this project, it is considered that *Histophilus ovis* does not satisfy the requirements of the genus *Actinobacillus*. No extracellular slime or capsules were detected in india ink preparations, cultures on blood agar were not noticeably sticky and were easily removed from the surface, hydrogen sulphide was not produced, urea was not decomposed but indole was produced from tryptophane; no growth occurred on MacConkey agar. These are all opposite to the characters listed in the definition of the genus. Furthermore, no evidence of "granulomatous lesions containing club colonies in the form of sulphur-like granules" were detected in the pathological cases described.

Eight strains of an organism named *Actinobacillus seminis* were obtained from the Veterinary Research Station, Glenfield to compare their bacteriological characteristics with those of *Histophilus ovis*. Most of the characteristics tested gave identical results, differences only occurring in the characters listed in Table 7.

Actinobacillus seminis appears less exacting for growth requirements but this could have been due to repeated laboratory sub-culturing and adaptation to media and conditions used. The addition of 10% sheep serum to sugar media enhanced the ability of the strains of *Actinobacillus seminis* to ferment the "sugars" glucose, fructose, maltose, arabinose, mannitol, xylose and mannose. In Baynes and Simmons (1960) original descriptions of failure to produce acid from these sugars, no mention is made as to whether serum was added to the media or not.

Thus, *Histophilus ovis* is not regarded as satisfying the requirements for inclusion in the genus *Actinobacillus*. It is felt that the organism is closely related to the species *Actinobacillus seminis* and that this latter species also does not satisfy the requirements of the genus.

TABLE 7: Differentiation of Histophilus ovis from Actinobacillus seminis

	Actinobacillus seminis	*Histophilus ovis*
Growth on Trypticase Soya agar without blood	+	-
Growth in Peptone water without serum	+	-
Growth in Tryptone water without serum	+	-
Growth in Tryptone water with serum @ 22°C	+/-	-
Growth on 10% sheep blood agar aerobically	+	+/-
Growth on 10% sheep blood agar anaerobically	+	+/-
Catalase activity	+	-
Indole production from tryptophane	-	+
Acid, no gas production from:		
Maltose	+	-
Sorbitol	-	+
Arabinose	+	-

Chapter VII

SEROLOGICAL INVESTIGATIONS OF HISTOPHILUS OVIS

7.1 Introduction

Serological studies were undertaken on some strains of the organism previously described as *Histophilus ovis* in an attempt to achieve a number of objectives. Firstly there was a basic need to develop a serological test to assist in the diagnosis of the naturally occurring diseases described in sheep; this is of paramount importance in the cases of epididymitis in rams and polyarthritis in lambs as the differential diagnoses would greatly influence control measures and antibiotic therapy administered in these two conditions. The second objective was to examine the antigenic components of the various strains recovered at Armidale and to ascertain if they were antigenically homogeneous. It was also considered desirable to compare these strains antigenically with similar organisms described from other geographic areas and also with strains of the closely related species *Actinobacillus seminis* to ascertain if there were any antigenic differences. The third and final objective was to develop hyperimmune serum against *Histophilus ovis* so that it could be used to assist in the identification of new field isolates.

7.1.1 Antigen

Eight strains of *Histophilus ovis* and two strains of *Actinobacillus seminis* were used for the preparation of antigen for serological studies. The strains of *Histophilus ovis* were derived from natural outbreaks of the four disease syndromes experienced at the District Veterinary Laboratory, Armidale, from cases of polyarthritis in lambs experienced by Mr A. Corbould at Launceston, Tasmania and from cases of pyo-septicaemia in lambs by Mr R. Rahaley at Hamilton, Victoria. The two strains of *Actinobacillus seminis* were obtained from cases of ovine epididymitis by Mr P. Claxton at the Veterinary Research Station, Glenfield. Initial problems were experienced in the preservation of cultures and necessitated twice-weekly sub-culturing on 10% sheep blood agar but eventually a satisfactory freeze-drying technique was developed as described previously so that all antigens used for serological tests were derived from freeze-dried ampoules of the various strains and all were handled in the same way.

The contents of a freeze-dried ampoule were suspended in approximately 0.3 ml of sterile nutrient broth. The suspension was then inoculated onto 10% sheep blood agar and also into nutrient broth containing 10% sterile sheep serum and freshly prepared cooked meat medium to increase the chances of isolation. After 72 hours incubation at 37°C, the two broth cultures were sub-cultured onto 10% sheep blood agar. Single colonies were picked off the agar and inoculated into sterile tryptone water containing 10% sterile sheep serum. These were incubated at 37°C for 72 hours and then used as the parent cultures to inoculate the production medium.

To produce a sufficient amount of antigenic bulk devoid of either sheep serum or blood, the following technique was used. The medium used was B.B.L. Trypticase Soy agar containing 1.5% agar to which was added 10% sterile defibrinated sheep blood and 1% Oxoid Ionagar No. 2 to produce "stiff" blood agar. Approximately 15 mls were distributed into medical flat glass bottles and allowed to set. All media were then incubated for 24 hours and any bottles which supported the growth of contaminants were discarded.

Using a sterile Pasteur pipette, approximately 2 mls of the 72 hour Tryptone Water culture was delivered onto the surface of each stiff agar bottle. Even wetting of the surface was achieved by manipulation and the culture was allowed to sit for 5 minutes. Excess tryptone water culture was then drained and removed with a Pasteur pipette and the stiff agar bottles incubated, with the agar uppermost, for 96 hours at 37°C.

To harvest the antigen, a number of small sterile glass beads were introduced onto the surface of the stiff agar, together with 5 mls of sterile phosphate-buffered saline. By gentle rocking of the bottle, the colonies were dislodged and washed off the surface of the agar so that a suspension of organisms in phosphate-buffered saline was obtained. This was washed by centrifuging and re-suspending in phosphate-buffered saline three times at which stage it was considered that no foreign protein derived from either sheep blood or serum remained. Antigen suspensions were preserved using 1:10,000 merthiolate. The end product provided the "whole-cell" antigen for the techniques described below. Owing to the use of sonicated antigen in the diagnostic serological test for *Actinobacillus seminis* infections, some of the above antigens were sonicated in the same way, that is placed in a MSE disintegrator for 1 minute at maximum power (2 amps).

7.1.2 Antiserum

Serum used in the tests described were derived from 2 sources; from rabbits hyper-immunised with various strains of antigen described and from sheep that were artificially infected with these strains.

Whole-cell live antigens were used to hyper-immunise the rabbits. Antigen was prepared as described above, except that merthiolate was not added. A Miles and Misra (1938) viable count was undertaken on the suspension used immediately prior to use. This gave some indication of the amount of antigen administered but as counts varied between different strains (from 3.2×10^5/ml to 1.7×10^8/ml) it may not have given a particularly reliable result as many of the organisms may not have been viable but still have contributed to eliciting an antibody response. Pairs of rabbits were used for each strain and antigenic suspensions were inoculated intravenously into the ear vein using a ½" 26 gauge needle attached to a tuberculin syringe. Inoculations were administered weekly on four occasions with a graded increase in dose rate (0.5, 1.0, 1.5 and 2.0 ml). Blood samples were collected from the rabbits 2 weeks after the 4th injection, allowed to clot and the serum harvested the following day following centrifugation. All rabbits used were Large New Zealand Whites and obtained from the Veterinary Research Station, Glenfield. They were fed on commercial dried rabbit-food pellets and daily-supplied fresh lucerne and water.

Sheep sera were obtained from animals used in the experimentally produced disease syndromes at the time of destruction for pathological studies.

7.2 Serological Tests

7.2.1 Slide agglutination tests

To undertake the slide agglutination test, a few colonies were taken off a pure culture on 10% sheep blood agar and emulsified in a drop of sterile normal saline on a glass microscope slide. Usually an even suspension was achieved but occasionally some strains auto-agglutinated, the latter taking the form of fine granularity. A loop-full of serum was gently mixed into this bacterial emulsion and then the slide was gently rocked for 3 minutes in a warm environment.

Strong positive agglutination, consisting of the formation of large granules, was obtained when hyperimmune serum produced in rabbits was tested against the homologous antigen of *Histophilus ovis* used for the hyperimmunisation. No perceptible difference in the degree of agglutination was observed when different strains of *Histophilus ovis* derived from either Armidale, Launceston or Hamilton were tested with sera produced from heterologous strains or from the two strains of *Actinobacillus seminis*. Sera obtained from sheep experimentally infected with the organism did not produce any observable agglutination to this test.

It would thus appear that the use of a slide agglutination test using hyperimmune serum to assist in the initial identification of *Histophilus ovis*, when the latter is isolated from pathological samples, may have diagnostic possibilities. The apparent antigenic homogeneity of the various strains used, from different disease syndromes and geographical locations, suggests that serum produced in rabbits from one strain could have wide application. The lack of agglutination using serum obtained from infected sheep negates the use of this technique for the diagnosis of *Histophilus ovis* infection.

7.2.2 Gel diffusion tests

Gel diffusion tests were initially undertaken in standard 85 mm sterile plastic petri dishes using 15 mls of Oxoid Ionagar No. 2 at a final agar concentration of 1%. Wells were cut using a template consisting of seven aluminium thermometer cases spaced equidistantly from one another by the use of an outer sleeve of thick rubber tubing. A seven-hole pattern was used as shown in Figure 1 and wells were numbered as shown. Wells were filled using a standard 40-dropper Pasteur pipette.

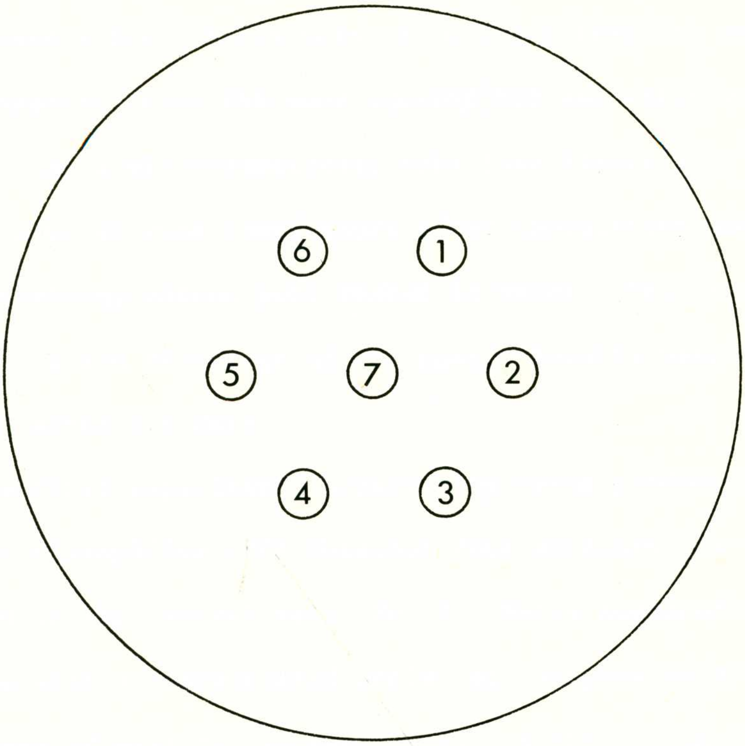

Fig. 1.

FIGURE 1: Plate pattern for gel-diffusion tests

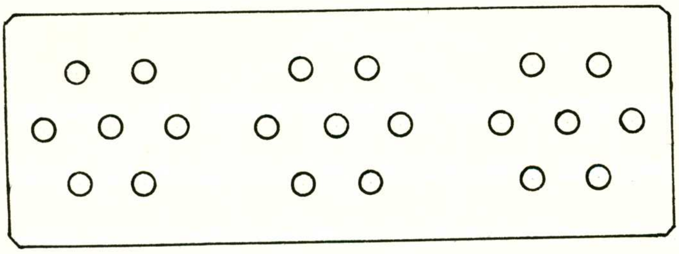

Fig. 2.

FIGURE 2: Slide pattern for gel-diffusion tests

In an effort to economise on antigen and serum, a micro-technique was developed using 4 mls of Oxoid Ionagar No. 2 on a standard 75 mm x 25 mm glass slide. Wells were cut using a template made from seven 13 gauge needles, with the ends squared off and wells were filled by capillarity using a microhaematocrit tube (see Figure 2). All plates and slides were kept at room temperature after inoculation and placed in plastic bags containing cotton wool soaked in water. This was done to prevent dehydration and shrinkage of the agar. Results were distinct and easy to read after 3-4 days.

In the first experiment, hyperimmune serum produced in rabbits from a strain of *Histophilus ovis* obtained from epididymitis in rams (E.2) was placed in the central well (No. 7). Wells numbered 2, 4 and 6 were then filled with a concentrated whole-cell suspension of antigen derived from cases of mastitis in ewes (M.1), arthritis in lambs (L.2) and abortion in ewes (A.1), respectively.

As can be seen in the diagrammatic representation of the result (Figure 3) a strong heavy distinct central precipitin line was obtained between the wells containing serum and homologous antigen. On either side of this was a faint line of precipitation, the inner one being more diffuse than the outer one. Similar lines were obtained between the well of serum and those containing other antigenic strains (L_2, A, and M_1).

This experiment was repeated using serum produced in rabbits against the other three strains of *Histophilus ovis* antigen. Almost identical results were obtained, the main central line of precipitation being always obtained but the peripheral lines, especially the outer one, being somewhat irregular in appearance. From these results, it was concluded that all strains of *Histophilus ovis* isolated at the District Veterinary Laboratory were antigenically homogeneous.

EXPERIMENT 1.

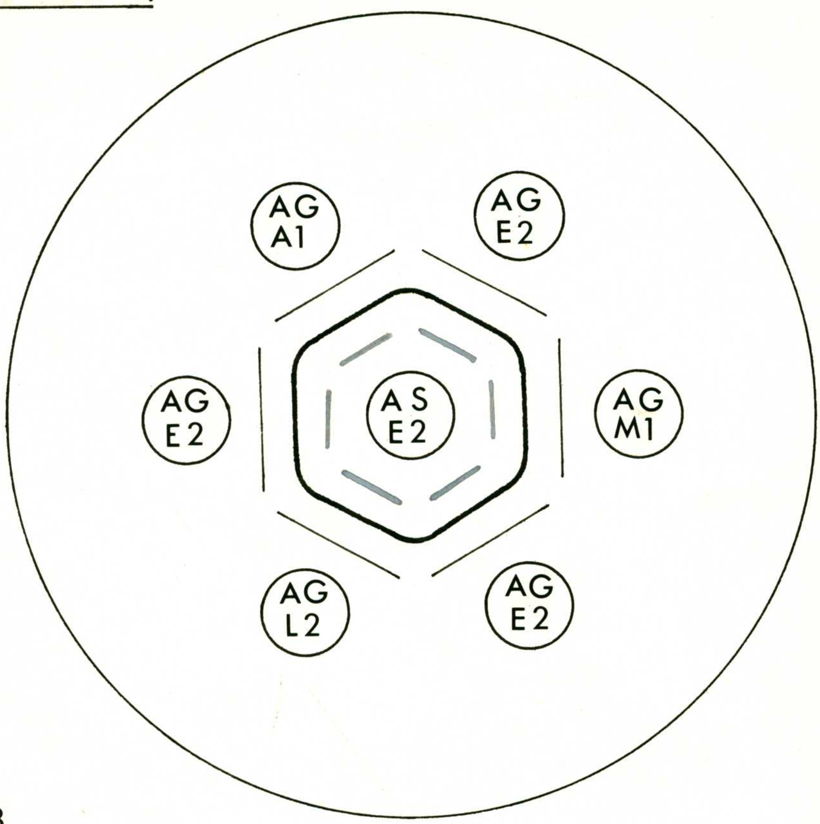

Fig. 3.

FIGURE 3: Results of gel-diffusion test using antiserum E_2 against *Histophilus ovis* antigens E_2, M_1, L_2 and A_1

EXPERIMENT 2.

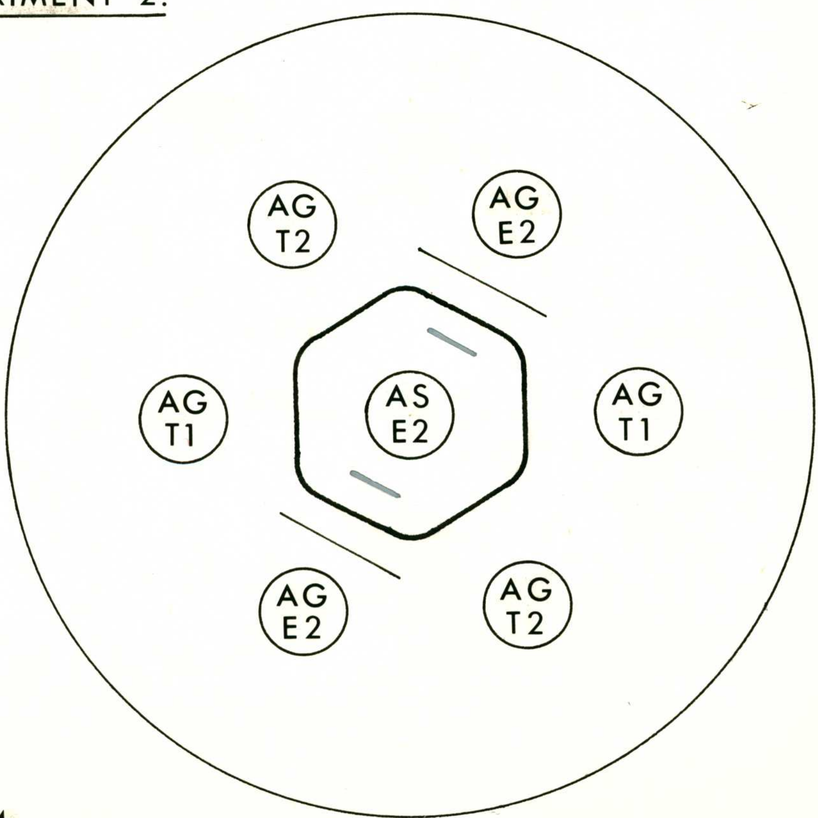

Fig. 4.

FIGURE 4: Results of gel-diffusion test using antiserum E_2 against *Histophilus ovis* antigens E_2, T_1 and T_2

The second experiment was undertaken using hyperimmune serum E2 against two strains of antigen obtained from cases of polyarthritis in lambs in Tasmania (T_1 and T_2).

From the results obtained (see Figure 4), it was concluded that not only were the two strains obtained from Tasmania antigenically identical but that they were also identical to the strains of *Histophilus ovis* obtained from epididymitis in rams at Armidale.

In the third experiment, antiserum E_2 was tested against two antigenic strains from cases of pyosepticaemia in lambs in Victoria (H.1 and H.2).

As indicated in Figure 5, the results obtained demonstrate that the 2 strains from Hamilton appear antigenically identical and show lines of identity to the type strain, E_2 from epididymitis in rams in Armidale.

The three experiments were repeated using fractionated antigens but no precipitation lines were observed using either homologous or heterologous antiserum prepared in rabbits using whole-cell antigen. It was thus concluded that the precipitation lines detected in the above experiments were due to cell wall antigen - specific antibody combination.

In the final experiment, antiserum E_2 was tested against the two antigenic strains of *Actinobacillus seminis* obtained from Glenfield (G_1 and G_2). The same major precipitation line was detected and both strains showed lines of identity to E_2 as shown in Figure 6.

It was thus concluded from the gel diffusion experiments that a strong specific antibody response was elicited in rabbits using whole-cell antigen and that this response was exclusively to cell wall antigen. Furthermore, other geographic strains of an apparently identical organism

EXPERIMENT 3.

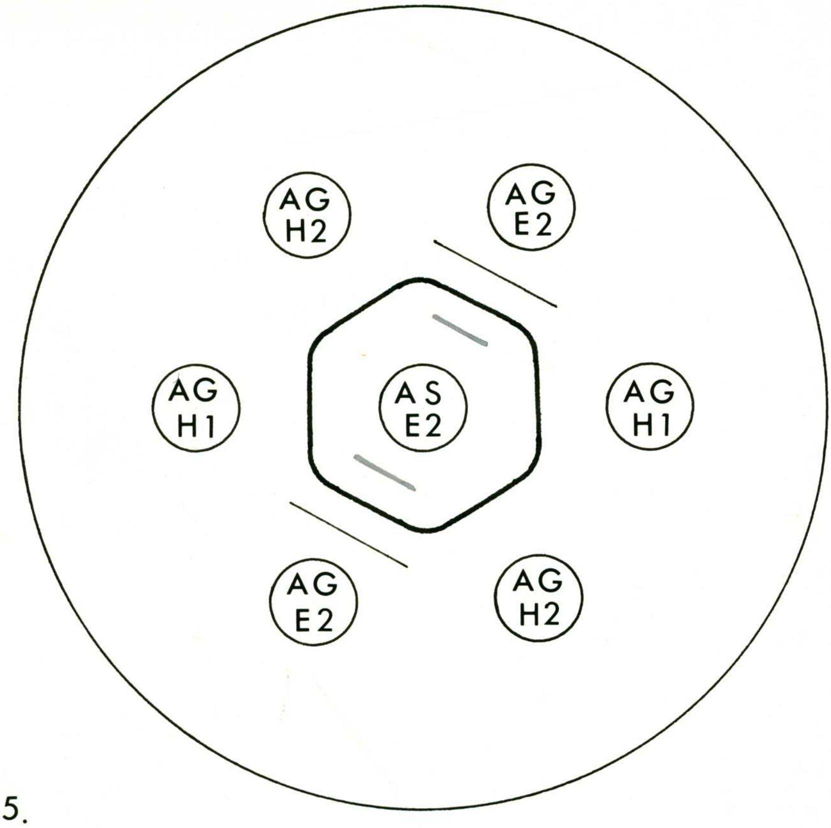

Fig. 5.

FIGURE 5: *Results of gel-diffusion test using antiserum E_2 against Histophilus ovis antigens E_2, H_1 and H_2*

EXPERIMENT 4.

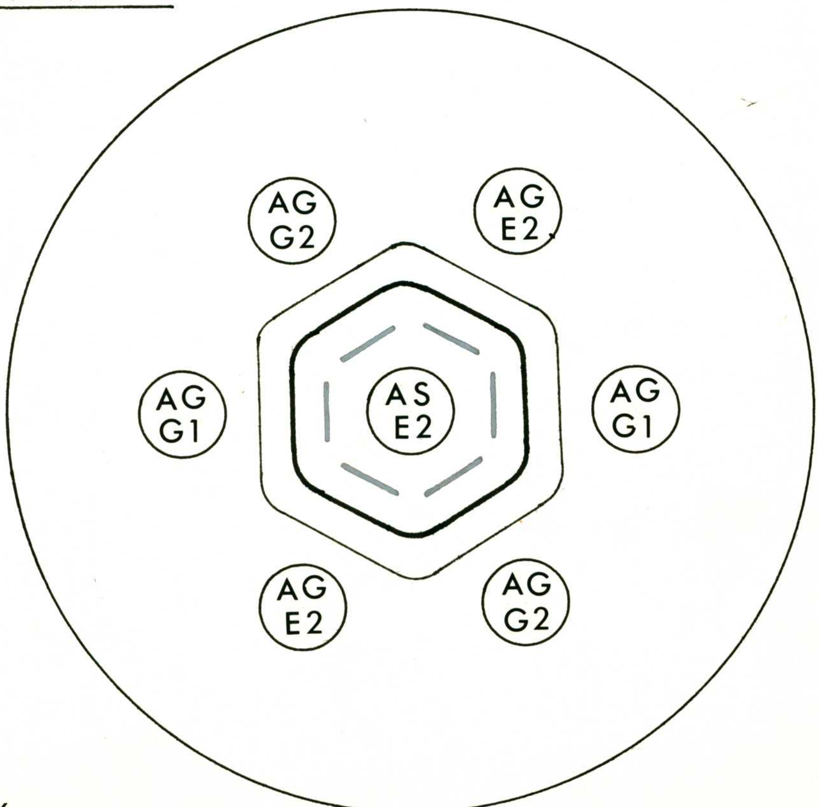

Fig. 6.

FIGURE 6: *Results of gel-diffusion test using antiserum E_2 against Histophilus ovis antigens E_2, G_1 and G_2*

showed the same response in eliciting the production of an identical antibody and that this was also identical to that elicited by two strains of an organism regarded as *Actinobacillus seminis*.

7.2.3 Complement Fixation Tests

The technique used for the Complement Fixation Tests was modelled on that described in the Public Health Monograph No. 74 (1965).

7.2.3.1 Diluent

The diluent used for the preparation of all reagents and for undertaking the various tests described below was Kolmer diluent.

Stock Solution

Calcium chloride ($CaCl_2 \cdot 2H_2O$)	24.5 g
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	101.6 g
Distilled water to 500 mls.	

This solution was Millipore filtered and stored at 4°C in the refrigerator in 20 ml volumes.

Preparation of Diluent

Sodium chloride (NaCl)	17 g
Stock calcium and magnesium solution	1.25 ml
Sodium azide (NaN_3), 10% solution	0.25 ml
Distilled water to 2 litres	
pH 7.4	

The diluent was stored at room temperature between tests but chilled to 4°C before use.

Initially barbital buffered solution, according to Kabat and Mayer (1961) was used but owing to the necessity of having to prepare it an consequently all reagents each day, Kolmer diluent was found to be more satisfactory. One experiment was done to show that no difference existed in the results obtained in tests using either diluent.

7.2.3.2 Red blood cells

Two sheep resident at the District Veterinary Laboratory, Armidale were selected whose erythrocytes, when sensitized with haemolysin, were known to be of a consistently satisfactory level of sensitivity to complement when used in complement fixation tests. Blood was collected from these sheep aseptically by cannulation of the jugular vein with a 13 gauge needle and bled into a sterile conical flask containing Alsever's solution.

Alsever's Solution

Glucose	20.5 g
Sodium chloride (NaCℓ)	4.2 g
Trisodium citrate ($Na_3C_6H_7 . 2H_2O$)	8.0 g
Citric acid	0.55 g
Distilled water to 1 litre	
pH	6.1

Sterilised at 121°C for 15 minutes in 200 ml volumes in 500 ml conical flasks with bleeding equipment attached. Stored in refrigerator at 4°C and used for up to 6 weeks after preparation. An equal volume of blood (i.e. 200 ml) was collected into each flask to give a final volume of 400 ml. The blood in Alsever's solution was then distributed into 1 oz universal containers and used from 1-6 weeks after collection.

To prepare an initial 4% red cell suspension, 8 containers of blood were taken and the cells washed by suspending in Kolmer diluent and centrifuging at 1,000 g for 20 minutes. The cells were washed three times, then pooled and re-suspended in an equal volume of diluent. The packed cell volume of this well mixed red cell suspension was determined by the microhaematocrit technique. The volume of diluent to be added to the concentrated suspension to give a 4% red cell suspension was then determined by:

$$\text{Volume of diluent} = \frac{(\text{PCV\%}-4)}{4} \times \text{Volume of concentrated red cells available.}$$

Example: If there was a 10 ml volume of washed red cells having a P.C.V. of 36% in Kolmer diluent, then the volume of additional diluent to be added would be $\frac{(36-4)}{4} \times 10 = 80$ ml, giving a final volume of 90 mls of a 4% suspension of red cells.

7.2.3.3 Haemolysin

Haemolysin was obtained in one ml ampoules from Commonwealth Serum Laboratories, Melbourne, in liquid form preserved with an equal quantity of glycerine. An initial 1:100 dilution was made in diluent and this was dispensed in 5 ml volumes and stored at -20°C prior to use. The haemolysin was titred each time a new ampoule was used and each time a new batch of red cells was brought into use. The method used for titrating the haemolysin was as described by the Australian Bureau of Animal Health (1975), and the steps taken were as follows:

1. Prepare the standardised erythrocyte suspension as already described.
2. From the 1:100 stock dilution of haemolysin prepare the following range of dilutions in Kolmer diluent: 1 in 500, 1000, 1500, 2000, 3000, 5000 and 10,000.

e.g. Add 1 ml 1:100 dilution of haemolysin to 4 ml diluent to give a 1:500 dilution

Add 1 ml 1:100 dilution of haemolysin to 9 ml diluent to give 1:1000 dilution

Add 2 ml 1:1000 dilution of haemolysin to 1 ml diluent to give 1:1500 dilution

Add 1 ml 1:1000 dilution of haemolysin to 1 ml diluent to give 1:2000 dilution

Add 1 ml 1:1000 dilution of haemolysin to 2 ml diluent to give 1:3000 dilution

Add 1 ml 1:1000 dilution of haemolysin to 4 ml diluent to give 1:5000 dilution

Add 1 ml 1:1000 dilution of haemolysin to 9 ml diluent to give 1:10,000 dilution.

3. Add 1 ml of each haemolysin dilution to 1 ml of standardised erythrocyte suspension while gently agitating the erythrocyte suspension, then leave the mixtures at 37°C for 15 minutes to allow sensitization of the erythrocytes to occur, agitating the tubes every five minutes.

   The rest of the procedure is done in duplicate to minimize pipetting errors, the tubes being kept cold, while the reagents are being dispensed, e.g. stood in a tray containing ice and water.

4. To each of a duplicate series of 7 tubes add 1.0 ml of diluent and 0.5 ml of complement diluted in such a way that it will produce approximately 70-80 per cent haemolysis with the more concentrated haemolysin dilutions (with good quality complements a 1:350 dilution is satisfactory).

5. From each of the haemolysin - erythrocyte mixtures in turn 0.5 ml is transferred to each of a pair of tubes containing complement and diluent and thoroughly mixed.

6. Incubate the tubes for half an hour in a water bath at 37°C, gently shaking after 15 minutes.

7. Remove the tubes from the water bath, add 2 ml of cold

diluent to each, then centrifuge the tubes to deposit any erythrocytes remaining unlysed.

8. Pour off the supernatant from each tube and read the optical densities in a photometer. This results in duplicate readings being obtained for each haemolysin dilution tested.

9. The optical density given by 100% haemolysis is the same as the target optical density used in preparing the standardized erythrocyte suspension.

10. Calculate the percentage haemolysis for each tube in the titration, e.g. supposing the optical density produced by 100% lysis is 0.5, a tube showing an optical density of 0.21 would have $\frac{0.21 \times 100}{0.5} = 42\%$ haemolysis.

11. Plot the per cent haemolysis given by each dilution on graph paper marked out as shown in Figure 7. To calibrate the abscissa, measure an arbitrary distance, say 10 scale divisions, from the left hand extremity and place a point here representing the 500 dilution; this is the extreme right hand end of the abscissa. Distances from the left hand end for the points representing the other dilutions are calculated by dividing the reciprocal of the dilution in 500, e.g. the point for the 1,000 dilution is placed 500/1000 or half way along the line, the 5,000 dilution 500/5000 or 1/10th of the way along, and so on.

    The percentage haemolysis is marked linearly along the ordinate, the points for percentage haemolysis are plotted for each haemolysin dilution and the line of best fit is drawn, ignoring outlying points.

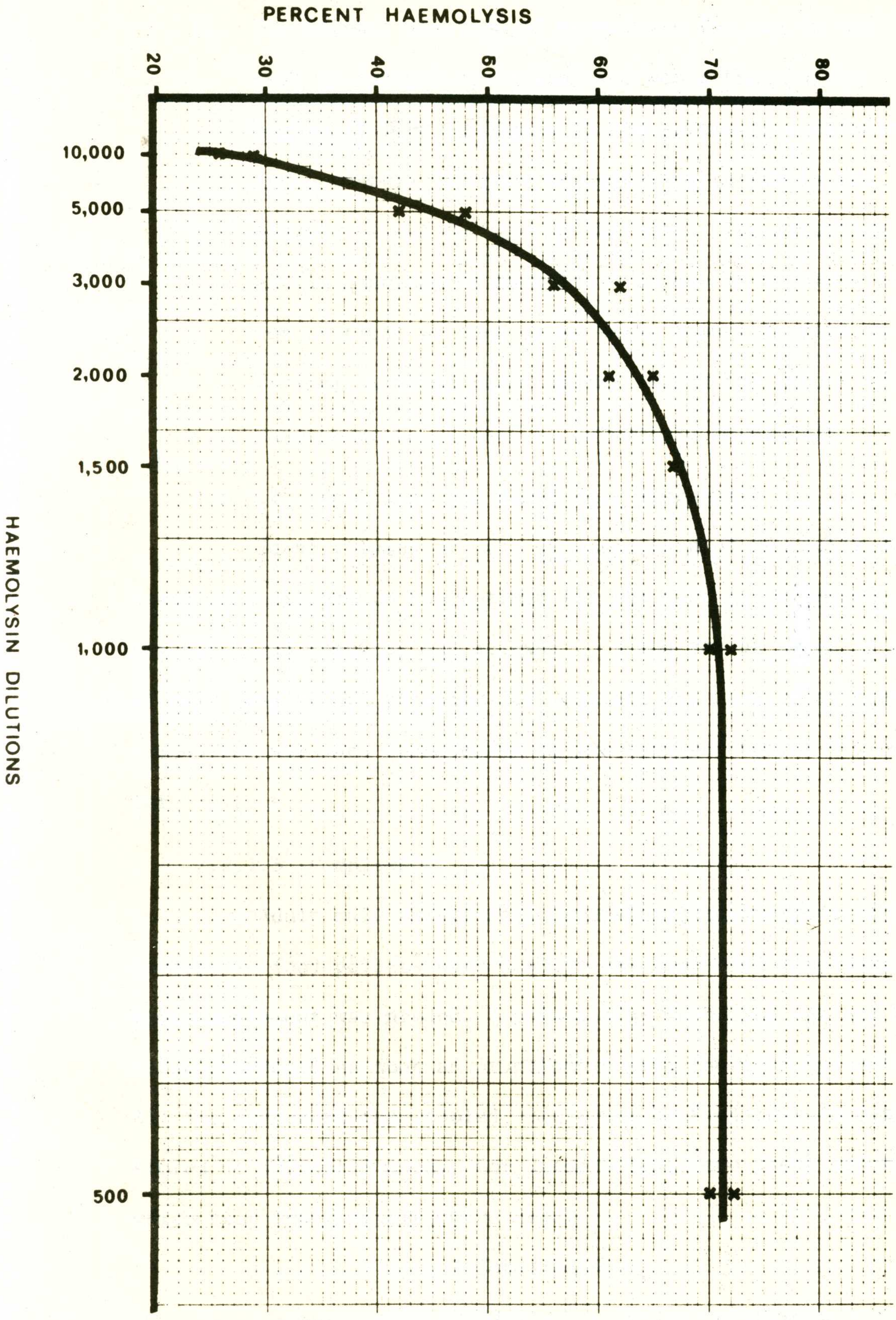

FIGURE 7: Graph for haemolysis titration (from Australian Bureau of Animal Health, 1975)

12. The optimal dilution of haemolysin for use in the test is decided by determining where the plateau begins (1:1000 in Figure 7) and selecting a dilution approximately 25% more concentrated (1:800 in the example given) for use in the test. The selection of the quantity of haemolysin to use in the test is not critical so long as an ample amount is used as it forms a fixed point against which the amount of complement to be used is determined accurately.

7.2.3.4 Sensitized red blood cell suspension

Equal volumes of (i) 4% suspension of red cells and (ii) haemolysin diluted to the optimum for the test as described, were rapidly mixed together and allowed to stand for at least 15 minutes at room temperature to allow for complete and even sensitization. The suspension was then transferred to the refrigerator at 4°C until used for the test.

7.2.3.5 Complement

Adult male guinea pigs receiving adequate green feed and commercial food pellets were used as the source of complement. Each time complement was needed, 8 guinea pigs were bled out and the serum separated as soon as practicable from the clot by centrifugation. All the sera were then pooled, preserved by Richardson's method and stored at 4°C until used. For preservation, two stock solutions, which keep indefinitely, were used:

Solution A

Boric acid (H_3BO_3)	0.93 g
Borax ($Na_2B_4H_7 \cdot 10H_2O$)	2.29 g
Sorbitol ($C_6H_{14}O_6 \cdot 1/2H_2O$)	11.74 g
Saturated NaCl solution to 100 ml	

Solution B

Borax	0.57 g
Sodium azide (NaN_3)	0.81 g
Saturated NaCℓ solution to 100 ml	

To preserve complement, 8 parts of guinea pig serum were mixed with 1 part of Solution B, followed by 1 part of Solution A. Before use it was necessary to restore tonicity by adding 1 part of preserved complement to 7 parts of distilled water. This gave a 1:10 dilution of complement.

The complement was titrated before using either a new batch of complement or a new batch of red cells. The quantity of complement required to lyse 50% of optimally sensitized erythrocytes was determined (1 $C'H_{50}$), and 5 $C'H_{50}$ were used in the test. A master dilution of complement was prepared being a 1:10 dilution of Richardson's preserved complement in cold distilled water. A small quantity of this master dilution was then further diluted in diluent to produce the titration dilution; with good quality complement the titration dilution was found to be 1:350 by trial. The procedure for the titration was as described by Australian Bureau of Animal Health (1975) and the steps taken were as follows:

1. Prepare sensitized erythrocytes by mixing equal volumes of standardised erythrocyte suspension and haemolysin diluted as determined in the previously described haemolysin titration. Allow the mixture to stand at 37°C for 15 minutes, mixing every 5 minutes.
2. Prepare master and titration dilutions of complement.
3. The test is done in twice the standard volume to minimise errors in pipetting and to produce a total

volume large enough to give a reading on the photometer. Arrange 6 suitable tubes in a rack and add the reagents in the quantities and the order shown in Table 8. After the diluent has been added the contents are mixed thoroughly and the tubes are placed in a water bath at 37°C for 30 minutes.

TABLE 8: Complement titration - tube arrangement for complement dilutions

	Tubes					
	1	2	3	4	5	6
Complement 1 in 350 (ml)	0.3	0.4	0.5	0.6	0.7	0.8
Diluent (ml)	1.2	1.1	1.0	0.9	0.8	0.7
Place in water bath at 37°C for 30 minutes then add						
Sensitized erythrocytes (ml)	0.5	0.5	0.5	0.5	0.5	0.5

4. After the addition of the sensitized erythrocytes the contents of each tube are mixed by gentle agitation then incubated in a water bath at 37°C for half an hour, during which time they are agitated at least once. After the incubation is completed the tubes are removed from the water bath, 2.0 ml of cold diluent is added to each tube and the tubes centrifuged to deposit the unlysed cells. The supernatants are then poured off and their optical densities determined in the photometer. Supposing the optical density corresponding to 100% lysis is 0.5, a reading of 0.2 would indicate -

$$\frac{0.2 \times 100}{0.5} = 40\% \text{ haemolysis.}$$

5. The dose of complement required to produce 50% haemolysis may now be determined graphically by plotting the degree of haemolysis for each dose of complement used in the titration on log/log paper, i.e. paper with log scales both vertically and horizontally (see Figure 8). The degree of haemolysis is calculated for each tube from the expression $\frac{y}{100-y}$ where y is the per cent haemolysis (only tubes showing between 10 and 90 per cent haemolysis are taken into consideration).

The calculation is made as in the example given in Table 9.

TABLE 9: Complement titration - example of results obtained

Dose of complement	Optical Density (OD)	Per cent Haemolysis (y)	$\frac{y}{100-y}$
0.3	0.06	13	$\frac{13}{87} = 0.15$
0.4	0.22	47	$\frac{47}{53} = 0.89$
0.5	0.34	72	$\frac{72}{28} = 2.6$
0.6	0.42	89	$\frac{89}{11} = 8.1$

6. The values for the expression $\frac{y}{100-y}$ are plotted for each dose of complement (see Figure 8) and a slope constructed by connecting the points as near as possible by a straight

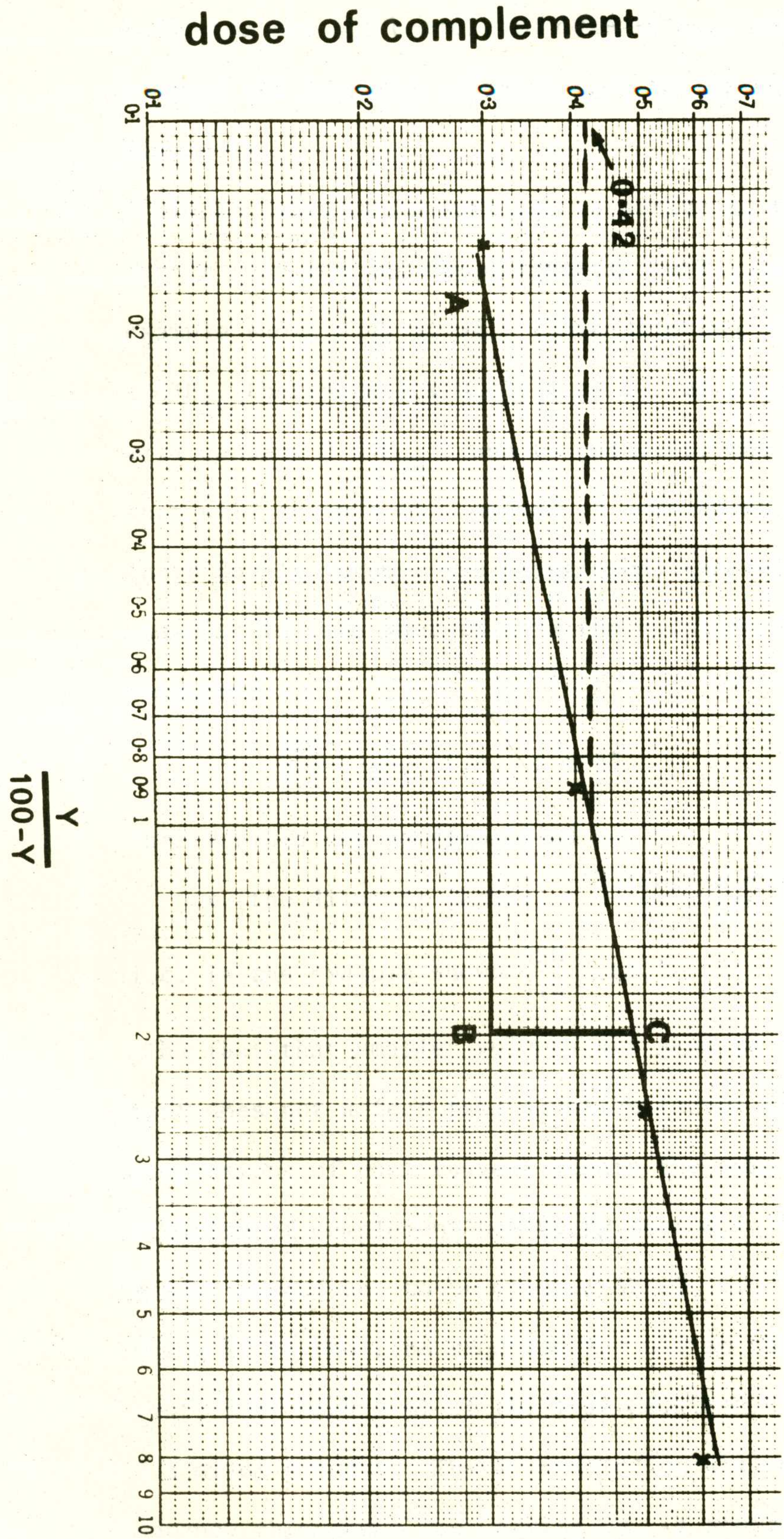

FIGURE 8: Graph for complement titration (from Australian Bureau of Animal Health, 1975)

line. The point where the slope crosses the "1" line indicates the 50% haemolytic dose ($C'H_{50}$) and this may be read off from the ordinate (see the broken line in Figure 8).

7. A simple calculation suffices to arrive at the dilution factor required for the diagnostic test. In the example shown 0.42 ml of a 1:350 dilution contains 1 $C'H_{50}$ therefore 5 x 0.42, i.e. 2.1 ml of this dilution will contain 5 $C'H_{50}$'s and to calculate the dilution that will contain this amount of complement in 0.5 ml (here double volumes are being used) the following equation is used wherein x is the dilution factor required:

$$\frac{350}{2.1} = \frac{x}{0.5} \quad \text{i.e.} \quad x = 83$$

This formula may be simplified to:

$$x \text{ (the dilution factor)} = \frac{\text{Titration dilution of complement}}{10 \times 50\% \text{ Haemolytic dose}}$$

i.e. in the example shown $\frac{350}{4.2} = 83$.

The fact that smaller volumes are used in the diagnostic test does not affect this dilution factor since the proportions of each reagent remain the same. The 1/10 dilution of complement now being stored in the refrigerator needs, therefore, to be diluted 83/10, i.e. 8.3 times, to bring it to the 1:83 required for use in the diagnostic test, i.e. each 1 ml of the 1:10 dilution is added to 7.3 ml of diluent. The 1:10 dilution will maintain its potency throughout the working day if stored as described.

7.2.3.6 Antigen

The antigen suspension was grown, harvested and preserved as described previously. The antigen was titrated against homologous hyperimmune serum produced in rabbits against it, using a "chess-board" technique to determine the optimum concentration of the antigen and the titre of the serum. Doubling dilutions of antigen and serum were made and 0.5 ml of each dilution was placed into complement fixation tubes arranged in an 8 x 8 pattern as shown in Tables 10-13, antigen dilutions in the columns and serum dilutions in the rows. 0.5 ml of titred complement was then added and the tubes

TABLE 10: Titration of fractionated antigen E_2 using hyperimmune serum E_2

		Dilutions of Fractionated Antigen E_2							
		1:5	10	20	40	80	160	320	Nil
Dilution of antiserum E_2	1:5	4	1	-	-	-	-	-	-
	10	4	1	-	-	-	-	-	-
	20	4	-	-	-	-	-	-	-
	40	4	-	-	-	-	-	-	-
	80	4	-	-	-	-	-	-	-
	160	3	-	-	-	-	-	-	-
	320	1	-	-	-	-	-	-	-
	Nil	-	-	-	-	-	-	-	-

incubated in a water bath at 37°C for 30 minutes. 0.5 ml of sensitized red cells were then added to each tube and the tubes replaced in the 37°C water bath for a further 30 minutes and then read for degree of fixation of complement according to the scale 4 (complete fixation or no lysis), 3, 2, 1 or 0 (no fixation, complete lysis).

a) The optimum dilution of antigen was set as that dilution which gave most fixation with the highest dilution of serum. This was then the dilution used in subsequent tests.

TABLE 11: Titration of unfractionated (whole-cell) antigen E_2 using hyperimmune serum E_2

Dilutions of antiserum E_2	Dilutions of whole-cell antigen E_2 1:5	10	20	40	80	160	320	Nil
1:5	4	4	4	4	4	3	-	-
10	4	4	4	4	4	2	-	-
20	4	4	4	4	4	1	-	-
40	4	4	4	4	4	1	-	-
80	4	4	4	4	4	1	-	-
160	4	4	4	4	4	-	-	-
320	4	4	4	4	4	-	-	-
Nil	1	-	-	-	-	-	-	-

b) The titre of the homologous serum was the highest dilution giving a reading of 3 or 4 with the optimal dilution of antigen.

Examples of the results obtained for antigen titrations in the presence of homologous antiserum and the titre of that serum are as outlined in Tables 10-13.

In the first experiment, antiserum E_2, produced in rabbits against whole-cell antigen *Histophilus ovis* E_2, was used to determine the titre of both fractionated antigen E_2 (Talbe 10) and whole-cell antigen E_2 (Table 11).

From the test using unfractionated antigen E_2 (Table 11), the titre of this antigen was shown to be 80 and the titre of the hyperimmune serum was 320. Using fractionated antigen (Table 10) its titre was shown to be 5 and the serum to be 160.

In the second experiment, antiserum L_2, produced in rabbits against whole-cell antigen *Histophilus ovis* L_2, was used to determine the titre of both fractionated antigen L_2 (Table 12) and unfractionated antigen L_2 (Table 13).

TABLE 12: Titration of fractionated antigen L_2 using hyperimmune serum L_2

	Dilution of fractionated antigen L_2							
Dilutions of antiserum L_2	1:5	10	20	40	80	160	320	Nil
1:5	4	4	3	1	-	-	-	-
10	4	4	3	-	-	-	-	-
20	4	4	1	-	-	-	-	-
40	4	4	-	-	-	-	-	-
80	4	3	-	-	-	-	-	-
160	4	-	-	-	-	-	-	-
320	-	-	-	-	-	-	-	-
Nil	-	-	-	-	-	-	-	-

From this test, the titre of unfractionated antigen L_2 was 80 (Table 13) and the serum had a titre of 160. Using fractionated antigen L_2 (Table 12), the antigen titre was 10 and the serum titre 80.

From the results of these antigen titration experiments, it was decided to use whole-cell unfractionated antigen for all comparative complement fixation tests.

7.2.3.7 Comparative complement fixation tests

1. Hyperimmune serum produced in rabbits against 8 strains of

TABLE 13: Titration of whole-cell (unfractionated) antigen L_2 using hyperimmune serum L_2

		Dilutions of whole-cell antigen L_2							
		1:5	10	20	40	80	160	320	Nil
Dilutions of antiserum L_2	1:5	4	4	4	4	4	4	2	-
	10	4	4	4	4	4	4	1	-
	20	4	4	4	4	4	4	-	-
	40	4	4	4	4	4	3	-	-
	80	4	4	4	4	4	2	-	-
	160	4	4	4	4	4	-	-	-
	320	4	4	4	3	-	-	-	-
	Nil	-	-	-	-	-	-	-	-

Histophilus ovis was tested against the antigen *Histophilus ovis* L_2 derived from a case of polyarthritis in lambs at Armidale. The 8 strains of *Histophilus ovis* originated from:

Histophilus ovis L_2 - polyarthritis in lambs at Armidale, New South Wales

Histophilus ovis T_1 - polyarthritis in lambs at Launceston, Tasmania

Histophilus ovis T_2 - polyarthritis in lambs at Launceston, Tasmania

Histophilus ovis E_2 - epididymitis in rams at Armidale, New South Wales

Histophilus ovis M_1 - mastitis in ewes at Armidale, New South Wales

Histophilus ovis A_1 - abortion in ewes at Armidale, New South Wales

Histophilus ovis H_1 - pyosepticaemia in lambs at Hamilton, Victoria

Histophilus ovis H_2 - pyosepticaemia in lambs at Hamilton, Victoria.

Doubling dilutions of serum from 1:5 to 1:320 were carried out using Kolmer diluent, leaving a final volume of 0.5 ml of each dilution. A 1:5 dilution of final volume 1.0 ml was used as the serum control for each sample. All diluted sera were inactivated in a water bath at 58°C for 30 minutes to remove any residual complement activity in the sera. After this time, the sera were placed at 4°C and on cooling, 0.5 ml of titred complement and 0.5 ml of *Histophilus ovis* antigen L_2, diluted 1:80 as described, were added. The tubes were then incubated in a water bath at 37°C for 30 minutes. Sensitized red cells were then added and the tubes reincubated for 30 minutes at 37°C. The titres of the 8 hyperimmune sera were then expressed as the reciprocal of the highest solution of serum at which maximal fixation of complement occurred. The results obtained are shown in Table 14.

TABLE 14: Complement fixing titres of hyperimmune sera produced in rabbits against 8 strains of Histophilus ovis, using Histophilus ovis L_2 *as the antigen for the test*

Serum	Titre
Anti-*Histophilus ovis* L_2	160
Anti-*Histophilus ovis* T_1	80
Anti-*Histophilus ovis* T_2	20
Anti-*Histophilus ovis* E_2	160
Anti-*Histophilus ovis* M_1	20
Anti-*Histophilus ovis* A_1	40
Anti-*Histophilus ovis* H_1	20
Anti-*Histophilus ovis* H_2	20

From this experiment it was deduced that the eight strains of *Histophilus ovis* derived from 4 pathological conditions and 3 different geographical areas were closely related as judged by their ability to elicit the production of complement fixing antibodies in rabbits against one strain of the organism used as antigen in the test.

2. The above test was repeated using a strain of *Actinobacillus seminis* obtained from the Veterinary Research Station, Glenfield as the antigen. The titres of the same eight sera were as shown in Table 15.

TABLE 15: Complement fixing titres of hyperimmune sera produced in rabbits against 8 strains of Histophilus ovis, using Actinobacillus seminis as the antigen for the test

Serum	Titre
Anti-*Histophilus ovis* L_2	20
Anti-*Histophilus ovis* T_1	20
Anti-*Histophilus ovis* T_2	40
Anti-*Histophilus ovis* E_2	40
Anti-*Histophilus ovis* M_1	20
Anti-*Histophilus ovis* A_1	40
Anti-*Histophilus ovis* H_1	20
Anti-*Histophilus ovis* H_2	40
Anti-*Actinobacillus seminis* (control)	40

From this test it was deduced that sera produced from all eight strains of *Histophilus ovis* fixed complement in the presence of *Actinobacillus seminis* antigen to an almost identical degree to that of the homologous serum produced against the antigen used. Thus the eight strains of *Histophilus ovis* could not be distinguished from *Actinobacillus seminis* using the complement fixation test.

3. Sera obtained from rams, ewes and lambs used in the experimentally reproduced diseases were tested against both antigens (*Histophilus ovis* L_2 and *Actinobacillus seminis*) but no significant serological results were obtained.

Chapter VIII

CONCLUSIONS

The estimation of lambing percentages by farmers is usually expressed as the percentage of lambs either marked or weaned over the number of ewes initially joined. An unsatisfactory lambing percentage is a significant cause of decreased income to many sheep farmers in Australia. A reduction of lambing percentage can be due to any factor which operates between the time of joining the rams and ewes and the time of either marking or weaning the lambs. As has been previously stated, infectious diseases operating in the various classes of sheep involved during this period (i.e. rams, ewes and lambs) are a significant cause of the reduction. Infection with *Histophilus ovis* has been shown to be one such disease affecting all three classes of sheep but giving rise to different disease syndromes in each class. To overcome the problem of reduced lambing percentages, it is essential that the correct treatment or control programmes are instituted and undertaken. Such programmes must be firmly based on an accurate and definitive diagnosis.

Rams affected with epididymitis have been shown to be a significant cause of reduced lambing percentages due to their partial or complete infertility. The contribution made by such rams to the problem is expressed as a failure of conception in ewes joined and is increased by either a low ram to ewe ratio or a restricted joining period. The diagnosis of epididymitis due to *Histophilus ovis* infection is dependent on its isolation from either the semen or pathological lesions of affected rams. Clinical examination of such rams will usually reveal the presence of the lesion but it is impossible to distinguish that due to *Histophilus ovis* from the other infectious causes of epididymitis.

Serological diagnosis has been attempted but the small number of affected rams tested does not allow a significant conclusion on its use to be made. The close antigenic similarity between *Histophilus ovis* and *Actinobacillus seminis* suggests that infection of rams with either organism could result in a positive complement fixation test result irrespective of which of the two organisms is used as antigen. Epididymitis due to *Histophilus ovis* has been shown to be sporadic in occurrence, involving only small numbers of rams in an affected flock. Infected rams usually have lesions in only one testicle so that fertility is reduced but not completely impaired. Reduced lambing percentages due to epididymitis caused by *Histophilus ovis* can thus be overcome by increasing the percentage of rams used or extending the joining period.

Following joining, abortion in pregnant ewes is the next chronological condition which contributes to depressed lambing percentages. The numerous infectious agents which have been reported as being of aetiological significance in this condition have been reviewed. Abortion in ewes due to *Histophilus ovis* was found to be of extremely rare occurrence but was of economic significance to the farmer involved. The condition was characterised by an acute haemorrhagic placentitis but no specific pathological changes were detected in the aborted foetus. Diagnosis of the condition was dependent upon the isolation of *Histophilus ovis* from the placenta. Serological diagnosis in ewes does not appear to be of practical significance. Treatment of affected ewes does not appear to be warranted as they do not show any clinical evidence of persistent infection. The epidemiology of abortions due to *Histophilus ovis* could not be determined in the one outbreak of the disease encountered.

Perinatal losses in lambs is the third condition which contributes to reduced lambing percentages. Numerous infectious agents have been incriminated by many authors as contributing to this syndrome and these have been reviewed. Infection of young, pre-marking lambs with *Histophilus ovis* has been shown to result in the development of a non-suppurative polyarthritis and polytenosynovitis. Workers in New Zealand have also described pyaemia in young lambs, in addition to synovitis, due to infection with an organism apparently the same as *Histophilus ovis*. Two isolates used in this study were obtained from R. S. Rahaley at Hamilton, Victoria. He advised that isolates of what appear to be *Histophilus ovis* had been isolated there from individual cases of bacterial septicaemia, miliary renal abscessation, meningitis and ependymitis, in addition to cases of polyarthritis in young lambs. It would thus appear that infection of lambs with *Histophilus ovis* results in initial bacteraemia, then subsequent septicaemia or localisation in a variety of sites, especially the synovial membranes of joints and tendons. Lamb survival is dependent upon which sites are colonised and the resulting pathological changes. In the cases of polyarthritis and polytenosynovitis described, satisfactory recovery was obtained where antibiotic therapy was instituted promptly. If this was withheld, lesions developed into a chronic form resulting in debilitation in affected lambs. Diagnosis of infection in young lambs due to *Histophilus ovis* is dependent upon its isolation in cultures prepared from the joints, tendon sheaths or other organs of bacterial localisation.

Acute mastitis in ewes leads to either death of the affected animal or, if they recover, a severe drop in milk production. Whatever the result of the infection in ewes, lamb losses occur from either death from starvation, or unthriftiness from enforced early weaning. Mastitis due to *Histophilus ovis* has been shown to be acute in nature but rare and sporadic in occurrence. Diagnosis of the condition depends upon the isolation of *Histophilus ovis* from either the milk or udder of affected ewes.

The bacteriological characteristics of *Histophilus ovis* are discussed in detail. From the outset of this project it was apparent that the organism was a member of the Family *Brucellaceae* as described in the seventh edition of Bergey's Manual of Determinative Bacteriology. Subsequently, the eighth edition of the Manual has dispensed with this family grouping. It has been shown that *Histophilus ovis* is most closely related to the genera *Haemophilus*, *Pasteurella* and *Actinobacillus* of the original Family *Brucellaceae* and these genera are now grouped amongst the "genera of uncertain affiliation", in Part 8 of the eighth edition of the Manual - the "Gram-negative facultatively anaerobic rods". Detailed comparisons of *Histophilus ovis* with the species described in these three genera are made and distinguishing features are high-lighted.

The relatively new technique of determining the Guanine + Cytosine (G+C) content of the bacterial DNA is finding increasing usage in comparing the relationships of bacterial genera. Although the G+C content of *Histophilus ovis* was not determined in this project, the three genera to which it is most closely related have similar G+C values as indicated by Bergey:

Haemophilus	38-42%
Pasteurella	36.5-43%
Actinobacillus	40.6-42%

It is thus felt that the determination of G+C content would have had little value in differentiating from these three genera but would be useful in differentiating from the other genera mentioned:

Moraxella	40-46%

Brucella	56-58%
Bordetella	61-66%

Histophilus ovis was found to be very fastidious in its requirements for growth, particularly on primary isolation. The addition of blood or serum was essential for growth and cultures had to be incubated under microaerophilic conditions. The time needed for the development of observable colonies and the morphological appearance of such colonies, were found to be useful factors for initial identification. Smears prepared from the colonies or from pathological tissues showed the organism to be an extremely pleomorphic Gram-negative rod. The results of numerous biochemical characterisation tests are described and the relevant ones for identification and differentiation are specified.

During the period of this project, as well as from information published, it became apparent that *Histophilus ovis* is closely related to the organism described as *Actinobacillus seminis*. The bacteriological differences between these two organisms have been described and these are based on growth requirements, colony morphology, catalase activity and indole production from tryptophane. Antigenic analyses of both organisms revealed that they were very closely related, if not identical. Strong, definite lines of identity were produced between the two species in gel diffusion precipitin tests and strong cross reactions were produced in comparative complement fixation tests. Pathologically, *Actinobacillus seminis* has been described from naturally occurring outbreaks of epididymitis in rams and polyarthritis in lambs and caused mastitis in ewes by experimental inoculation. It thus seems highly probable that these two species may in fact be the same organism and the problem then arises as to what the correct name should be. The name *Actinobacillus seminis* suggested by Baynes and Simmons (1960) appears unacceptable by virtue of Phillips (1974) stating that it does not satisfy the criteria for inclusion in that genus.

Bacteriological findings from this project reveal that *Histophilus ovis* cannot be unequivocably differentiated from the two previously described species *Haemophilus ovis* and *Haemophilus agni*. Furthermore, evidence is given why these latter two species do not satisfy the requirements of the genus *Haemophilus*.

Differentiation of *Histophilus ovis* from the genus *Pasteurella* is mainly on the basis of colony morphology and growth requirements. These factors can be quite variable and differences between laboratories using the same type of media are often greater than the differences in organisms. *Histophilus ovis* is shown to be closely related to the previously described species *Pasteurella mastitidis*, differing only in the latter's ability to grow more abundantly and ferment a small number of extra sugars. The specified name *Pasteurella mastitidis* has been refuted by different workers, some regarding it as a strain of *Pasteurella haemolytica*, while others regard it as indistinguishable from *Actinobacillus lignieresi*.

In conclusion, the correct classification and naming of the Gram-negative pleomorphic organism described in this thesis is open to question. Due to the stated differences of it from the three most closely related genera, the creation of a new genus for it may be justified. The suggested name of *Histophilus* appears to have chronological precedence having been suggested in 1956 but the studies presented here do not indicate the total dependence for growth on tissue, or more specifically meat particles, that was initially stated by Roberts (1956). However, disregarding the latin meaning of the name, it is felt that this generic name should be maintained. If, as antigenic studies indicate, the organism named *Actinobacillus seminis* by Baynes and Simmons (1960) is the same as *Histophilus ovis*, then it is felt that that name is incorrect as it was suggested later, has subsequently not been regarded as an *Actinobacillus*, and has furthermore been isolated from more pathological conditions than the semen of rams with epididymitis. The specific name "*ovis*" appears acceptable as it has only been isolated from disease syndromes in sheep.

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