THE DISTRIBUTION AND LOCATION OF *MYCOPLASMA OVIPNEUMONIAE* IN THE LUNGS OF PNEUMONIC SHEEP

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ABBREVIATIONS USED IN TEXT

c.f.u.	Colony forming units
D.A.B.	Diaminobenzidine
F.A.T.	Fluorescent antibody technique
F.C.A.	Freundt's complete adjuvant
FITC	Fluorescein isothiocyanate
HRP	Horseradish peroxidase
Ig	Immunoglobulin
i/v	Intravenous
NRS	Normal rabbit serum
PBS	Phosphate buffered saline
P.E.	Prolife rative exudative (pneumonia)
P.P.L.O.	Pleuropneumonia-like organism
SAR	Sheep anti-rabbit (FITC)
SPA	Sheep pulmonary adenomatosis
SPF	Specific pathogen free
SWAR	Swine anti-rabbit (FITC)
w/v	Weight per volume

ABSTRACT

The localisation and distribution of Mycoplasma ovipneumoniae was demonstrated using both immunofluorescent and immunoperoxidase techniques.Paraffin-wax sections were prepared by the "Sainte-Marie" (1962) method and cut to a thickness of 6 u.An indirect fluorescent antibody technique (F.A.T.) was employed using rabbit anti-M.ovipneumoniae antiserum and swine anti-rabbit antiserum labelled with fluorescein isothiocyanate (SWAR/FITC).A triple step "sandwich" immunoperoxidase technique was used by treating sections with rabbit anti-M.ovipneumoniae antiserum,sheep anti-rabbit IgG and rabbit anti-sheep conjugated to horseradish peroxidase (HRP).Destruction of endogenous peroxidase was by sodium borohydride in disodium hydrogen phosphate,and SPF sheep serum was applied to sections to reduce non-specific staining.An indirect F.A.T. was used to demonstrate Immunoglobulin G (IgG) and Immunoglobulin A (IgA) in sheep lungs.Rabbit anti-sheep IgG (IgA) and then SWAR/FITC were applied to sections of normal and pneumonic lungs.

Positively stained mycoplasma were demonstrated lining ciliated bronchial epithelium.Bright fluorescence was seen slightly away from the cell surface,and particulate peroxidase stain in the tips of cilia.No staining was seen in non-ciliated bronchi or alveolar lining. Particulate stain was seen in the bronchial and alveolar exudate. F.A.T. revealed coating of a proportion of alveolar macrophages with a layer of fluorescence.Peibronchial and perivascular cuffs fluoresced brightly when treated with rabbit anti-sheep IgG,as did the intra-alveolar exudate and some alveolar macrophages.Cells stained for IgA were found largely in the interstitium of the lungs,and fluorescence of exudate and alveolar macrophages appeared as for

IgG.

Thus,M.ovipneumoniae was demonstrated lining bronchi in close association with cilia,and possibly within the intraluminar exudate.There was an increase in both IgG and IgA in pneumonic lungs compared to normal lungs,but relative importance of each immunoglobulin was not determined.

INTRODUCTION

Atypical pneumonia of sheep has been recognised for many years (Stamp & Nisbet,1963) but the exact aetiology of the disease remains in doubt.A mycoplasma would appear to be involved in the aetiology of ovine atypical pneumonia in similarity with chronic pneumonias of other animals and man.Mycoplasma ovipneumoniae and Mycoplasma arginini are the two mycoplasma species commonly isolated from the respiratory tract of sheep,but M.ovipneumoniae alone would appear to be capable of primary colonisation of the lungs (Jones,1978). Pasteurella haemolytica has frequently been associated with chronic pneumonia of sheep,but experimental evidence would suggest a role of secondary invader in the course of the disease (Gilmour,Jones & Rae,1979).

Thus,M.ovipneumoniae is the organism most frequently isolated from sheep lungs with atypical pneumonia,and has been demonstrated as capable of producing lesions,similar to the naturally occurring disease,experimentally (Glimour et al,1979).However,the pathogenic mechanisms and predilection sites for multiplication of this mycoplasma within ovine lungs has not been demonstrated.Mycoplasmas responsible for producing chronic pneumonia in other species have been demonstrated by immunomorphological staining techniques,and their location and distribution within pneumonic lungs defined. thus,the aim of this study was to demonstrate M.ovipneumoniae in sheep respiratory tract by these methods in order to give some insight in to the role of the mycoplasma in the pathogenisis of atypical pneumonia of sheep.

CHAPTER 1

LITERATURE REVIEW

Introduction.

The pathology of pneumonia of sheep has been described by Stamp & Nisbet(1963).They listed the respiratory diseases of sheep in Britain as "atypical"pneumonia,"enzootic"pneumonia,parasitic pneumonia and sheep pulmonary adenomatosis (S.P.A.) or jaagsiekte.

Primary mycoplasmal involvement has been suggested for both atypical and enzootic pneumonia,but the aetiology of both diseases is still uncertain.Mycoplasmas have also been isolated from cases of S.P.A.,but they are not recognised as primary agents of the disease (Jones,1978).

Atypical pneumonia.

The term "atypical pneumonia" was put forward by Stamp & Nisbet(1963) for a chronic pneumonia of sheep in Britain which they considered had caused significant losses over a number of years.On the other hand,it has been statedthat the disease has little clinical effect on lambs unless subsequent bacterial infection intervenes(Gilmour & Brotherston,1963;Sullivan,St.George & Horsfall,1972b;Jones,1978). Friis,Palsson & Petursson(1976),however,describe a chronic pneumonia of Icelandic lambs called "kregda",which is similar pathologically to atypical pneumonia,but which manifests itself as a clinical syndrome in which there is considerable wasting.

Atypical pneumonia usually affects lambs between three and ten months of age,although evidence of infection is present earlier than this(Sullivan,St.George & Horsfall,1973a;Jones,Buxton & Harker,1979.).The clinical signs as recorded by Gilmour & Brotherston/

Brotherston (1963) are,occasional coughing,high temperature and fast respiratory rates.They did not record dullness,inappetance or dyspnoea, and there was no appreciable change in the general condition of the sheep.

Sullivan et al (1973a) investigated a flock of pneumonic sheep in Australia.These authors found in the lambs that there was an initial period of mild clinical signs,where the only evidence of pneumonia was dry inspiratory rales.This was followed by the sudden onset of clinical pneumonia in five to ten week old lambs, characterised by moist coughing,sneezing and a copious,clear,mucoid nasal discharge.Loud moist inspiratory rales could be heard on auscultation.A third stage in the clinical course of this outbreak of pneumonia was remission of acute signs,but clinical evidence of persistent lung lesions.

Jones et al (1979) similarly observed mild respiratory disease in housed lambs up to fifty-five days old.Diagnosis was based on occasional coughing,nasal discharge and abnormal auscultatory sounds. However,moderate to severe signs of respiratory disease were recorded in lambs between ninety-nine and one hundred and fourteen days of age.They did not examine the dams closely,but occasional coughing and nasal discharge were noted.

Pathology.

The macroscopic areas of consolidation affect mainly the right apical and cardiac,and left cardiac lobes,and occasionally the anterior borders of the diaphragmatic lobes (Stamp & Nisbet,1963;Sullivan et al, 1973a).The lesions consist of red or grey to pink areas of consolidation and dull red areas of collapse.Pleural adhesions are often present.The lungs were described as tough on cutting,and no pus exuded from the cut surface of lesions unless abscesses were

present.Air passages contained small amounts of tenacious mucus and were surrounded by grey-coloured cuffs.In some cases,narrow branching areas of collapse,confined to apical or cardiac lobes,were the only lesions seen (Stamp & Nisbet,1963).Sullivan et al (1973a) and St.George,Sullivan,Love & Horsfall (1971) described the lungs of infected sheep as having a greyish appearance overall,and areas of lobar consolidation.

Stamp & Nisbet (1963) divided the microscopical lesions into two types;lymphoid hyperplasia and interstitial pneumonia (with or without lymphoid hyperplasia).Lesions of the former type alone were comparatively rare.

The lymphoid hyperplasia consisted of peribronchial and peribronchiolar lymphocytic cuffing,confined mainly to the apical lobes when present alone.There was also some infiltration of macrophages into the surrounding alveoli,and lymphocytes,plasma cells and reticuloendothelial cells were commonly observed.

Interstitial pneumonia was the most common finding in pneumonic lungs.In early lesions,there was infiltration of the interalveolar septa by lymphocytes and macrophages leading to a reduction in alveolar space,and large macrophages could be found in surrounding alveoli.Fibrous tissue ingrowths,or,in more advanced cases,hyaline scars could be seen resulting from damage to bronchi and bronchioles.This was a consistent finding by Stamp & Nisbet (1963), but Stevenson (1969),and Sullivan et al (1973b) found little evidence of hyaline membrane formation.In advanced cases, peribronchial and perivascular lymphocytic cuffing was a constant feature,and many well developed lesions showed focal epithelialisation of alveoli.

The characteristic features of the disease described by

St.George & Carmichael(1975) were thickening of alveolar septa due to proliferative changes in the alveolar wall,hyperplasia of bronchiolar epithelium and alveolar atelectasis with mild lymphoid hyperplasia or exudative changes.Intra-luminal aggregations of neutrophils and marked proliferative epithelial dysplasia were also recorded.

Mycoplasmas.

Introduction.

Mycoplasmas are the smallest and simplest self-replicating procaryotes(Razin,1978).Most species of mycoplasma are,as far as is known,host-specific or shared by closely-related animal species, although many "aberrant" isolations have been made.A few species, notably Mycoplasma bovigenitalium and Mycoplasma arginini are able to infect a wide range of hosts (Boughton & Thorns,1978). Mycoplasmas have a predilection for cell surfaces especially moist mucosa;thus the sites most commonly colonised are the respiratory and urogenital tracts,eyes and synovial membranes,.

The first description of mycoplasmas,termed by the authors as "pleuropneumonia organisms" was in 1898 by Nocard and Roux.in their study into the aetiology of contagious bovine pleuropneumonia. Subsequently many observations of similar such organisms,termed "pleuropneumonia-like organisms" (PPLO) were made in a variety of hosts.In 1956, Edward and Freundt proposed a system of nomenclature and classification for PPLO:the organisms were placed in a single family,the Mycoplasmataceae,and contained a single genus Mycoplasma.In 1964,the first cultivation of a mycoplasma on artificial medium was achieved,the organism being Mycoplasma pneumonia ("Eaton's Agent"),the primary agent of human atypical pneumonia

(Chanock,Hayflick & Barile,1962).Since that time many new species of mycoplasma have been isolated and described.The nomenclature and taxonomy have been expanded and amended accordingly to accommodate the sixty-four species at present recognised within the class Mollicutes which have been isolated from man,animals,plants, insects and inanimate matter.

Classification and taxonomy.

The class Mollicutes (Edward & Freundt,1967) contains one order, the Mycoplasmatales (Freundt,1955),andthe description of the order is therefore the same as the class.

The accepted classification at present is,according to Freundt & Edward (1979),as follows:

Class: Mollicutes.

Order: Mycoplasmatales

Family I Mycoplasmataceae

1.Sterol required for growth

2.Genome size about 5.0×10^8 daltons

3.NADH oxidase localised in cytoplasm.

Genus I Mycoplasma (do not hydrolyse urea).

GenusII Ureaplasma (hydrolyse urea).

FamilyII Acholeplasma

1.Sterol not required for growth

2.Genome size about 1.0×10^9 daltons

3.NADH oxidase localised in membrane.

Genus I Acholeplasma

FamilyIIISpiroplasmataceae

1.Helical organisms during some phase of growth

2.Sterol required for growth

3.Genome size about 1.0×10^9 daltons

4. NADH oxidase localised in cytoplasm.

Genus I Spiroplasma

There are also two genera of uncertain taxonomic position,namely Thermoplasma and Anaeroplasma.

Members of the genus Mycoplasma are distinguished to some extent by their biochemical activity,but final definition of the various species depends on serological tests (Howard & Gourlay,1978).

Characteristics of mycoplasmas.

The members of the genus Mycoplasma are described by Freundt in Bergey's Manual of Determinative Bacteriology (1974) as highly pleomorphic,varying from spherical or slightly ovoid to slender branched filaments.They lack a true cell wall,but are bounded by a single,triple-layered membrane,and are gram-negative.The typical colony is biphasic,with a"fried-egg" appearance.

Most species utilise either glucose or arginine as a major source of energy.Urea is not hydrolysed,but,2,3,5-triphenyltetrazolium chloride is frequently reduced under anaerobic conditions.
All species require cholesterol for growth.

Boatman (1970) considers three basic morphological forms; coccoidal cells,diploforms and filaments.These forms arise from cell division,which can occur by binary fission,fragmentation of filaments (formed where cytoplasmic division lags behind genome replication) and by a budding process.Very small bodies (0.1 um in diameter) have previously been considered in an alternative method of reproduction,but Boatman concludes that these are degenerate particles,and not viable.The smallest size attainable by mycoplasmas is considered by Maniloff (1972) to be approximately 330 nm.

Electron microscopy reveals a trilaminar cell membrane,and, in certain species,the presence of a microcapsule.The internal

ultrastructure of mycoplasmas generally lacks organisation,and consists of strands of DNA and ribosomes scattered randomly through the cytoplasm.However,specialised structures and organelles occur in a few species such as M.pneumoniae and Mycoplasma gallisepticum.

Pathogenic mechanisms of mycoplasmas.

Most mycoplasma pathogens are not highly invasive,but confine themselves to epithelial surfaces,and produce mild localised infections without penetrating the deeper tissues or disseminating to other organs(Barile,1979).Fernald(1969) described respiratory changes following infection with Mycoplasma pulmonis as a marshalling of a local immune system in response tothe superficial infection within the bronchial tree,and not part of an invasive or destructive process. Fernald(1975) further suggested that pneumonic disease due to M.pneumoniae was an expression of increasing host response to recurrent infection.

Ciliated epithelium is thought to be the target cell for mycoplasma infections,therefore tracheal organ cultures have been used to demonstrate the course of localised mucosal infection and resulting cellular damage,the ability of mycoplasma to bind to receptor sites,the role and function of "virulence factors",such as haemolysins(peroxidase and others) and other products,in producing ciliary damage,and the effect of mycoplasmas on the metabolism and function of infected tracheal epithelium (Barile,1979).

Some mycoplasmas initiate infection by attaching to ciliated bronchial tissues;this is followed by cell damage.Attachment allows the mycoplasma to resist the natural "flushing" action of the respiratory tract,and allows the release of harmful substances, such as haemolysins,proteolytic enzymes,nucleases and toxic substances close to the infected cell(Barile,1979).Specialised

terminal structures for attachment to host cells have been demonstrated for M.gallisepticum (Uppal & Chu,1977),M.pneumoniae (Collier,1972) and M.pulmonis(Organick,Siegesmund & Lutsky,1966; Richter,1970).Mycoplasma dispar has been shown capable of stopping ciliary action,and destroying the ciliated epithelium in vitro (Thomas & Howard,1974),and large numbers of mycoplasmas were found attached to the ciliated epithelium.

Certain mycoplasmas release haemolysins(hydrogen peroxide and others)and enzymes(Whittlestone,1972a).Although association of these products with pathogenicity of the mycoplasma is not always present, hydrogen peroxide has been demonstrated as important in the pathogenisis of certain species,Mycoplasma mycoides subspecies capri for example(Aluotto,Wittler,Williams & Faber,1970).
Hydrogen peroxide has been demonstrated in Mycoplasma ovipneumoniae, but this may be irrelevant to its mode of pathogenisis,especially as it possesses no specialised attachment structures(Jones,1978), and no report of its physical attachment to,or penetration of,cells has been found.

Mycoplasma neurolyticum,M.gallisepticum,M.pulmonis and Mycoplasma arthritidis exert toxic effects,but in only M.neurolyticum has a soluble exotoxin been demonstrated(Whittlestone, 1972a).

The mycoplasmas of sheep and goats.

The first reported isolation of mycoplasma from the sheep respiratory tract was by Greig(1955) in Canada.The isolations of mycoplasmas from sheep and goats have been reviewed by Cottew & Leach(1969),Nayil(1973) and Jones(1978).
The species of mycoplasma occuring in sheep and goats as listed by Cottew(1979) are as follows:

1. Mycoplasma agalactiae
2. Mycoplasma arginini

1.*Mycoplasma agalactiae*

2.*Mycoplasma arginini*

3.*Mycoplasma capricolum*

4.*Mycoplasma conjunctivae*

5.*Mycoplasma mycoides* subspecies *mycoides*
subspecies *capri*

6.*Mycoplasma ovipneumoniae*

7.*Mycoplasma putrefactiens*

8.*Acholeplasma granularum*

9.*Acholeplasma laidlawii*

10.*Acholeplasma oculi*

11.*Ureaplasmas*.

Various other strains,notably the F38 strain(MacOwan,1976),and the EHM and QEW strains(Cottew,1974),have been isolated but remain as yet unnamed.

M.agalactiae causes contagious agalactia of sheep and goats, an important cause of economic loss in some countries.The organism may also occasionally cause keratoconjunctivitis,but has only once been implicated in pleurisy and pneumonia(Cottew & Lloyd,1965).

M.conjunctivae is considered to be an aetiological agent of ovine and caprine infectios keratoconjunctivitis,and is not recognised as a pathogen of the respiratory tract(Jones,Foggie, Sutherland & Harker,1976;Cottew,1979).

A.oculi has only rarely been isolated from sheep and goats, once from an outbreak of caprine keratoconjunctivitis(Al-Aubaidi, Dardiri,Muscoplatt & McCauley,1973),and once from the urogenital tract(Kumar & Pathak,1978).

M.putrefaciens in sheep and goats and *A.granularum* are uncommon isolates(Tully,Barile,Edward,Theodore & Ernø,1974;Ernø cited by

Cottew,1979;Freundt cited by Cottew,1979;Ernø cited by Cottew,1979).

M.ovipneumoniae and M.arginini have regularly been recovered from the respiratory tract of sheep(Cottew,1979),and will be considered further.

A.laidlawii and ureaplasmas have been isolated from the respiratory tract,but lung recoveries of the organisms have not been associated with characteristic macro- or micro-scopic lesions of pneumonia(Jones,1978).

Mycoplasma arginini.

M.argininiwas characterised and named by Barile,Del Giudice,Carski, Gibbs & Morris(1968).It produces typical centred colonies on solid media,is non-haemolytic and hydrolyses arginine.The organism has been isolated from many mammalian species(Cottew,1979). M.arginini has frequently been isolated from the respiratory tract of sheep(Leach,1970;Al-Aubaidi,Taylor,Bubash & Dardini,1972; Carmichael,St.George,Sullivan & Horsfall,1973;St.George & Carmichael,1975;Alley,Quinlan & Clarke,1975;Leach,Cottew,Andrews & Powell,1976;Jones et al,1979).These authors consistently report recovery of the organism from the upper respiratory tract,but reports of lower respiratory tract isolations vary.Alley et al(1975) and Jones et al(1979) isolated M.arginini from pneumonic and normal sheep lungs.However,there was no relation shown between isolation of the organism and any specific pathological changes.Others were unable to recover the organism from the lungs of normal sheep(Leach, 1970;Carmichael et al,1973;Leach et al,1976).

Experiments to investigate the pathogenicity of M.arginini have been performed by Foggie & Angus(1972) and Jones(1978). Following endobronchial inoculation,M.arginini was recovered from tonsillar tissue at post mortem,but lung infection was not

established.Significant antibody production against the mycoplasma was demonstrated in four out of six animals(Jones,1978).Jones suggested that M.arginini was a secondary invader from the upper respiratory tract,and not a primary pathogen in sheep pneumonia.

Mycoplasma ovipneumoniae.

M.ovipneumoniae was characterised,and the name proposed by Carmichael et al(1972).They isolated mycoplasmas from a pneumonic flock of sheep in southern Queensland,and a healthy flock near Brisbane, Australia.Two lung isolates were made,one being identified as M.arginini.The other isolate was characterised by colony morphology (slightly raised,granular and somewhat circular,centreless colony), its ability to ferment glucose with acid production,haemolysis of sheep erythrocytes,and distinct serotyping as shown by metabolic and growth inhibition tests.

Mycoplasma showing these characteristics had previously been isolated by MacKay,Nisbet & Foggie(1963);Cottew(1971) and St.George et al(1971).

M.ovipneumoniae has subsequently been shown to have a world-wide distribution,with isolations from sheep in Australia (Carmichael et al,1972;Sullivan et al,1973;Furlong & Cottew,1973), New Zealand(Clarke,Brown & Alley,1974),United States of America (St.George & Carmichael,1975),Hungary(Stipkovits,Belak,Palfi & Tury,1975),United Kingdom(Jones,Foggie,Mould & Livitt,1976;Leach et al,1976),Iceland(Friis et al,1976),Iraq(Al-Sultan & Zubaidy,1978), Sudan(Mahi & Nayil,1978),Switzerland(Nicolet,Tontis,Giger, Schällibaum,Wüthrich-Parvarien,Krawinkler,Paroz,Bestetti & Boss, 1979) and goats in Sudan(Ali,1977) and U.S.A.(Livingston & Gauer, 1979).

Major,Clarke and Alley(1979) examined the morphology of

M.ovipneumoniae by electron microscopy,and demonstrated the mycoplasma to be "typically roughly spherical in shape".The general size range was between 400 and 900 nm in diameter,but cells up to 1500 nm in diameter were recorded.Dumb-bell forms were common,and this was taken as indicative of binary fission as a mode of replication.Budding or filamentous forms were not observed.

Distribution in the respiratory tract.

Carmichael et al(1972) compared mycoplasma isolates from normal and pneumonic sheep by swabbing the nasal sinus and pharynx. Thirty-four out of thiry-eight pneumonic animals were infected with M.ovipneumoniae whereas only two out of thirty-three healthy animals were infected.

The levels of infection with M.arginini were approximately the same in the two flocks.However,on resampling two months later, significantly higher recovery rates for M.arginini,while reduced recovery rates were recorded for M.ovipneumoniae.

Alley et al(1975) found that nasal carriage of M.ovipneumoniae and M.arginini was ubiquitous in both normal and pneumonic animals.Isolation of both organisms was more common in pneumonic than healthy sheep,and M.ovipneumoniae was more common than M.arginini. No other mycoplasmas were isolated.However,the recovery rates and titres of M.ovipneumoniae were significantly higher in pneumonic than in normal lungs,thus suggesting a role for M.ovipneumoniae in the pathogenisis of chronic pneumonia of sheep.

Jones et al(1979)also found that M.ovipneumoniae and M.arginini were frequent inhabitants of the respiratory tract of a pneumonic flock of sheep.These authors found a rapid increase in nasal isolations of mycoplasmas as the lambs aged,but that the colonisation

of the lower respiratory tract was significantly lower in 71-80 day old lambs compared to older lambs.Isolation of M.ovipneumoniae was highest from animals with proliferative exudative (P.E.) pneumonia,but M.arginini could not be associated with any specific lung changes.Pasteurella haemolytica and M.ovipneumoniae were isolated from all cases of P.E. pneumonia.

A number of ewes examined at necropsy revealed lung infection with M.ovipneumoniae,M.arginini and P.haemolytica,but P.E. pneumonia was not recorded in any of these animals.

Pathogenicity of Mycoplasma ovipneumoniae and the aetiology of atypical pneumonia.

The first pathogenicity experiments using M.ovipneumoniae were by St.George et al (1971).They isolated a glycolytic mycoplasma from a lamb with chronic pneumonia, and showed it to have the same characteristics as later described by Carmichael et al (1972). Infection was attempted by intra-tracheal injection,aerosol and in-contact.A proliferative interstitial pneumonia developed,and clinical pneumonia was evident,but bacterial infection was also present.Mycoplasmas were recovered from the infected lambs,and the control lambs showed no sign of pneumonia.

Sullivan et al (1973b) reproduced proliferative interstitial pneumonia by intravenous,aerosol and in-contact infection,but found little clinical evidence of respiratory disease apart from a dry,high-pitched,harsh inspiratory rale heard on auscultation. Pathological changes were similar to those seen in naturally infected sheep (Sullivan et al,1973a;St.George et al,1971).Those consisted of proliferation of alveolar septal cells and bronchiolar epithelium, and alveolar atelectasis.Mycoplasmas were not recovered from the

pneumonic lungs,and so specificity of infection was not proved. However,no viruses and only limited bacteria (coliforms) were isolated.

Foggie,Jones & Buxton (1976) found the effect of M.ovipneumoniae in specific pathogen free (SPF) lambs to be considerably milder than that described by Sullivan et al (1973b).Clinical signs were minor or inapparent,and mild lesions were found in only three out of six lambs.However,mycoplasmas were recovered from five out of those six lambs.In-contact lambs became infected,but lesions were absent,and no mycoplasmas were recovered from the lungs.Electron microscopy revealed mycoplasma in close association with the remaining cilia.

Jones,Gilmour & Rae (1978) reproduced lung lesions indistinguishable from naturally occuring cases of atypical pneumonia by the endobronchial inoculation of two suspensions containing M.ovipneumoniae, M.arginini and P.haemolytica.One suspension comprised a homogenate of lung lesions obtained from naturally occuring cases of pneumonia;the other contained cloned cultures of the three microorganisms isolated from the lung homogenate. M.ovipneumoniae was recovered from all experimentally infected cases,while the recovery of P.haemolytica and M.arginini was less constant. Samples taken prior to inoculation showed the presence of all three organisms in low numbers in some of the animals,but the control animals did not develop signs of respiratory disease.

In further studies,M.arginini was omitted from the inoculum with no apparent effect (Gilmour,Jones & Rae,1979).Inoculation of P.haemolytica together with M.ovipneumoniae into conventionally reared sheep consistently produced a proliferative exudative pneumonia. M.ovipneumoniae was always recovered from the lungs, and P.haemolytica from five out of seven animals.When

M.ovipneumoniae was inoculated alone,four out of seven animals developed proliferative exudative pneumonia,and three developed interstitial changes alone. P.haemolytica was recovered from one case when inoculated alone,and only interstitial changes were seen. When SPF lambs were challenged with the same organisms,only mild pneumonia developed,with a noticeable lack of lymphoid hyperplasia. However,high numbers of mycoplasma were recovered from the lungs. The authors therefore concluded that proliferative exudative pneumonia could be reproduced using P.haemolytica and M.ovipneumoniae,and also possibly M.ovipneumoniae alone. Inclusion of P.haemolytica appeared to increase the severity of the lung changes.

Alley & Clarke (1979) conducted similar experiments to Jones et al (1978),and also found that,while pneumonic lung homogenates caused lesions indistinguishable from the natural disease, M.ovipneumoniae inoculates produced only mild lesions,but did colonise the sheep lungs.They thus concluded that the whole-lung material contained other substances necessary in the pathogenisis of chronic pneumonia.

The presence of M.ovipneumoniae and certain bacteria (namely P.haemolytica,Neisseria catarrhalis and Staphylococcus aureus) were examined quantitatively by Alley & Clarke (1977).They collected sixty affected lungs from sheep with chronic or sub-acute pneumonia from an abattoir.These were examined histologically and micro-biologically,and divided into four groups depending on the presence of high or low titres of M.ovipneumoniae and bacteria. An association between M.ovipneumoniae and chronic proliferative changes,mainly peribronchiolar fibrosis and alveolar interstitial thickening,was evident.The authors suggested that their results

indicated an active role for both bacteria and M.ovipneumoniae in the development of chronic and sub-acute pneumonia in lambs. They also suggested that the bronchial tree may be the favoured site for mycoplasma multiplication,and cited the work of Organick et al (1966) and Baskerville & Wright (1973) as further evidence of this.

Alley & Clarke (1977) were also of the opinion that viruses did not play a major role in the aetiology of chronic pneumonia, but in a more recent study (Alley & Clarke,1980),they state that the relevance of virus infection,especially Parainfluenza type 3 virus,is not yet clear and merits investigation.In this later work,the effects of various chemotherapeutic agents on the transmission of chronic non-progressive pneumonia in lambs indicate again that bacteria as well as mycoplasmas are involved in the aetiology of the disease.

Atypical mycoplasmas and chronic pneumonia of other animals.

M.ovipneumoniae has frequently been described as "atypical"due to certain unusual biological characteristics.Several other mycoplasmas have been shown to share some of these unusual features.

M.dispar was first described by Gourlay (1969),then characterised and named by Gourlay and Leach (1970).The organism has been shown to be capable of prod-ucing pneumonia in calves. (Gourlay & Thomas,1969).

This mycoplasma displays several of the features found in M.ovipneumoniae;

1. Glycolysis
2. Fastidious nutritional requirements
3. It is larger than mycoplasmas in general

(500-1500 nm in diameter).

4. Atypical colonies on solid media

-slow growing

-centreless colonies

-granular or lacy appearance.

Thomas & Smith (1972) examined the distribution of mycoplasmas in apparently normal bovine lungs.Mycoplasmas were found in much higher numbers throughout the respiratory tract of calves aged 3-4 months,while younger calves,yearlings and adults showed only few organisms present,mainly in the upper respiratory tract.

This agreed with the studies on sub-clinical pneumonia by St.George,Horsfall & Sullivan (1973).They found proliferative interstitial pneumonia in calves aged 1-6 weeks;slightly older calves exhibited a proliferative exudative pneumonia,and lesions in much older calves showed evidence of regression.Clinical disease was never apparent.

Pirie & Allan (1975) demonstrated that M.dispar and ureaplasma species were significantly associated with the presence of "cuffing pneumonia".

Gourlay,Howard,Thomas & Stott (1976) produced pneumonia by endobronchial inoculation of pneuminic lung homogenate.Development of pneumonic lesions was dependant on ampicillin-resistant and tylosin tartrate-sensitive microorganisms presumed to be mycoplasmas. M.dispar and ureaplasmas were the only mycoplasmas consistently isolated from the original lung pool.

Further pathogenicity experiments showed M.dispar and ureaplasmas,either singly or together,to be capable of producing a sub-clinical proliferative interstitial pneumonia in gnotobiotic calves (Howard,Gourlay,Thomas & Stott,1976;Gourlay,Howard,Thomas & Wyld,1979).

M.dispar was examined by electron microscopy by Allan & Pirie (1977).The organisms were found in close association with bronchial epithelium,on or between cilia.Few mycoplasmas were seen in alveoli in close contact with leucocytes,occasionally within intracytoplasmic vacuoles.They were never seen in epithelial cell cytoplasm,and no cellular connections were observed.

Pathological lesions present were cilia loss,and destruction of the regular contour of the epithelial surface.Intracytoplasmic vacuolation and activated mitochondria could be seen in epithelial cells.

This work agrees with previous electron microscopical studies by Organick et al (1966) and Kohn (1971) on M.pulmonis infection in mice and rats respectively,and Baskerville (1972) on Mycoplasma hyorhinis infection in pigs.

Mycoplasma hyopneumoniae,considered to be the primary aetiological agent of enzootic pneumonia of pigs (Whittlestone,1973), also shows some biological features in common with M.ovipneumoniae. It was described by Goodwin,Pomeroy & Whittlestone (1965),and Mare & Switzer (1965),who proposed the names Mycoplasma suipneumoniae and Mycoplasma hyopneumoniae respectively.Their description was of a glycolytic mycoplasma with fastidious growth requirements, which produced a centreless colony on solid media.

The pathological study using both conventional light and electron microscopy by Baskerville (1972) shows the salient features of porcine enzootic pneumonia to be very similar to those of ovine atypical pneumonia.

Livingston,Stair,Underdahl & Mebus (1972),using electron microscopy,observed the mycoplasmas in close association with cilia and plasma membrane of epithelial cells of the bronchioles and

bronchi and not within epithelial cells.

Mebus & Underdahl (1977) described extensive loss of cilia associated with M.hyopneumoniae.The organisms were seen to accumulate over the remaining ciliated cells.

Demonstration of mycoplasmas and immunoglobulin by immunomorphological staining.

1. Immunofluorescence

Coons,Creech,Jones & Berliner (1942) first introduced the fluorescent antibody test (F.A.T.) for the localisation of pneumococcal antigens in tissue.The fluorescent antibodt test has been widely used for the identification of mycoplasma colonies grown on solid media (L'Ecuyer & Boulanger.1970;Bass & Jasper,1970;Schuller,lehmkuhl & Switzer,1976).

The test has further been used to demonstrate various mycoplasma species in lesions.Noel,DeVolt & Faber (1964) described the identification of M.gallisepticum in fowl tissue and exudate by the use of direct and indirect methods.

M.pulmonis was demonstrated in the bronchi of infected gnotobiotic mice by immunofluorescence (Organick & Lutsky,1968). They used an indirect technique,and clearly demonstrated the presence of mycoplasmas forming an almost continuous blanket on the surface of the columnar bronchial epithelium.This was readily comparable with electron microscopical studies by the same authors (Organick et al,1966).

M.hyopneumoniae and M.hyorhinis have been demonstrated in porcine pneumonic lung tissue by L'Ecuyer & Boulanger (1970) and Meyling (1971).Potgieter & Ross (1970) also demonstrated M.hyorhinis and Mycoplasma hyosynoviae in porcine synovial

membrane.These authors used the direct method on frozen tissue.

Meyling (1971) found specific fluorescence lining,either completely or in-part,the bronchial and bronchiolar epithelium. He also compared the presence and distribution of M.hyopneumoniae, M.hyorhinis and M.hyosynoviae.

M.hyorhinis was demonstrated in pig lungs by direct and indirect fluorescent antibody techniques on paraffin embedded tissue by Pospisil,Gois,Cerny & Mensik (1971).They examined lung sections from gnotobiotic piglets infected intranasally with M.hyorhinis and found a bright line of specific,yellow-green fluorescence at the bronchial and bronchiolar epithelial surface,frequently in a continuous layer.Some amorphous fluorescent clumps were demonstrated in the lumena of some bronchi.

Alley,Wells,Smith & Gardiner (1979) demonstrated the distribution of Immunoglobulin G (IgG),Immunoglobulin M (IgM) and Immunoglobulin A (IgA) in sheep respiratory tract by indirect immunofluorescence and indirect immunoperoxidase. These authors found that the numbers of all three classes of immunoglobulin-containing cells increased in sheep with chronic respiratory disease.Although the IgA:IgG ratio was reduced,IgA remained the major locally secreted immunoglobulin.

2. Immunoperoxidase

This test has been used for the identification of mycoplasma colonies (Polak-Vogelzand & Hagenaars,1976;Polak-Vogelzand, Hagenaars & nagel,1978).

Bruggman,Engberg & Ehrensperger (1977) used the enzyme-linked immunoperoxidase technique to demonstrate M.hyopneumoniae in porcine lungs by a direct staining method on frozen tissue sections and bronchial smears.M.hyopneumoniae was demonstrated

bordering the bronchial epithelium.Similar methods were used by Hill (1978) to demonstrate M.pulmonis in the lungs of rats with chronic pneumonia.

Conclusions from the literature review.

Atypical pneumonia of sheep,in common with "atypical" chronic pneumonias of several other species,appears to involve a mycoplasma, namely M.ovipneumoniae.However,the pathogenicity of M.ovipneumoniae remains in doubt as high numbers can be recovered from normal lumgs. M.ovipneumoniae has been suggested as the primary agent of a chronic pneumonia of sheep,but bacteria are thought necessary for the full expression of the disease.In this case,what does the mycoplasma do to potentiate the bacterial infection,and where does it do it?

Mycoplasmas have been shown to persist in the lung,despite high humoral antibody levels developing (Jones,personal communication). Thus,what immunological responses are mounted by the host?

Mycoplasmas in general have been shown to cause superficial infection within the bronchial tree,but the distribution and pathogenic mechanisms of M.ovipneumoniae are still in doubt. The purpose of this study is to demonstrate the location and distribution of,and the host response to,M.ovipneumoniae in sheep lungs by immunomorphological methods in the same manner as has been described by various authors for other mycoplasma species.

CHAPTER 2

MATERIALS AND METHODS

Reagents and stock solutions.

The following reagents and stock solutions were prepared:

1. Buffered glycerol.pH 8.0.
2. Calcium chloride (B.D.H. Chemicals Ltd.).
3. Celloidin (B.D.H. Chemicals Ltd.).0.3% celloidin in ether.
4. D.A.B. solution.4 mg of 3,3-diaminobenzidine tetrachloride (B.D.H. Chemicals Ltd.) was dissolved in 10 ml 0.1M Tris/HCl buffer,pH 7.6. 0.1 ml of 1% hydrogen peroxide was added just before use.
5. Egg albumen (slides).5% egg albumen in distilled water.
6. Egg albumen (washing).78 gm egg albumen in 3900 ml of 0.01M PBS. Adjust pH to 7.5 with M NaOH.
7. Eriochrome Black (Sigma Chemical Company).Diluted to 1 in 60(w/v) in distilled water.
8. Periodic acid (B.D.H. Chemicals Ltd.). 1 gm in 100 ml of distilled water.
9. Phosphate buffered saline (PBS).Prepared at Moredun Institute.

NaCl	400 gm
KCl	10 gm
Na_2HPO_4	57.5 gm
KH_2PO_4	10 gm

Add distilled water to 5,000 ml.Dilute 1 in 10 for use.

10. Sheep liver powders.Prepared at the Institute.
11. Sodium borohydride ($NaBH_4$)(B.D.H.Chemicals Ltd.),in disodium hydrogen phosphate (Na_2HPO_4)(B.D.H.Chemicals Ltd.).
    0.2 gm $NaBH_4$ and 1 gm Na_2HPO_4 in 100 ml distilled water.
12. Starch adhesive.1 gm in 100 ml water + 20 ml boiling water +

2 drops 1N HCl.Boil for 5mins.Dilute 1:3 with distilled water to use.

13. Tris hydrochloric acid (HCl buffer.2400 ml of 0.05M Tris buffer;add 0,1N HCl to final pH 7.6 (approximately 600-800 ml).

14. Trypsin (Griffin and George (Sales) Ltd.).

15. Distilled water.Prepared at the Institute.

Sera.

1. Rabbit anti-M.ovipneumoniae.Prepared at the Institute by slight modification of the method used by Morton & Roberts (1967). Antigen suspension containing 2 mg/ml of protein in distilled water was emulsified with an equal volume of Freundt's Complete Adjuvant (F.C.A.).

   Inoculation schedule:

   a) 0.1 ml emulsion to each footpad
      0.1 ml emulsion to eight sites along flank
      0.5 ml emulsion to 2 sites on each shoulder.
   b) 3 weeks later,
      1 ml emulsion to each hind leg.
   c) 4-5 weeks later,
      0.5 ml of antigen suspension (2 mg/ml),i/v.
   d) 1 week later,
      1 ml antigen suspension i/v.
   e) Bleed out 5-7 days after final inoculation.

2. Normal rabbit serum (N.R.S.).Prepared at the Institute.

3. Swine IgG anti-rabbit IgG fluorescein isothiocyanate (SWAR/FITC) (Nordic Immunological Laboratories).

4. Sheep anti-rabbit Ig fluorescein isothiocyanate (SAR/FITC) (Wellcome Laboratories).

5. Sheep anti-rabbit IgG.Provided by the Institute.Prepared by intravenous injection of rabbit IgG into a sheep.Tested for

specificity by immuno-electrophoresis.

6. Rabbit anti-sheep conjugated to Horseradish peroxidase (HRP). Prepared at the Institute.Normal rabbit serum was conjugated to HRP (Sigma Chemical Company),as described by Avrameas (1969).

7. Rabbit anti-sheep IgA.Prepared at the Institute as described by Smith,Dawson,Wells & Burrells (1975).

8. Rabbit anti-sheep IgG.Prepared at the Institute as described by Smith et al (1975).

9. Sheep $F(ab^1)_2$ anti-rabbit F(ab) conjugated to HRP,by papaine digestion of ShaR IgG followed by conjugation with HRP as described by Nakane & Kawaoi (1974).Prepared at the Institute.

Sheep respiratory tract.

The source of lung material was the following:

1. Sheep used in experiments being carried out by Dr.G.E.Jones & Mr.J.S.Gilmour of the Moredun Research Institute.

   a) Six month old conventionally reared Cheviot sheep infected by the endobronchial route with pneumonic lung homogenate containing M.ovipneumoniae (1.65×10^6 c.f.u./ml) and P.haemolytica (1.55×10^3 organisms/ml),as described by Jones,Gilmour & Rae (1978).

   b) Similar sheep infected by in-contact exposure to group(a), and some previously inoculated with sterilised lung homogenate.

   c) Sixty day old,specific pathogen free (SPF) sheep infected with a series of media,

      i.Sterile lung homogenate + ampicillin.

      ii.Lung homogenate + Tylan 200 (Elanco).

      iii.Sterile lung homogenate + M.ovipneumoniae, (2×10^7 c.f.u./ml).

iv. Lung homogenate + ampicillin.

The lung homogenate contained M. ovipneumoniae (6.5×10^5 c.f.u./ml) and P. haemolytica (8.75×10^2 organisms/ml). The animals were killed 14 days after inoculation.

2. Ovine lungs obtained from the abattoir, some exhibiting gross lesions of atypical pneumonia, and some apparently healthy.

Sites of sampling.

1. Samples of trachea from mid-cervical region(T_1) and 1cm above the bifurcation of the bronchi(T_2).
2. Portion of right apical lobe.
3. Portion of right cardiac lobe.
4. Portion of right diaphragmatic lobe.

Samples were taken from areas exhibiting gross lesions of pneumonia, and also from apparently healthy regions. In later experiments, samples were taken only from the right apical lobe.

Treatment of Samples.

1. One sample (approximately 4mm x 4mm x 2mm) from each site was immersed in cold 95% alcohol for 24 hours, and later processed through to paraffin by the method employed by Sainte-Marie (1962). The blocks were then stored at +4°Centigrade(C). Sections 6 u thick were cut on a microtome and placed on egg-albumen coated slides.
2. A second sample was snap frozen in petroleum ether by immersion in a mixture of acetone and solid carbon dioxide. The frozen tissues were stored at -70°C.
3. The lungs were examined for the presence of mycoplasmas by Dr. G.E. Jones of the Microbiology Department, Moredun Research

Institute.Bronchial swabs and lung homogenates were used to inoculate mycoplasma media routinely used for the isolation of glycolytic and arginine-reducing mycoplasmas and ureaplasmas;titres of M.ovipneumoniae,M.arginini and ureaplasmas were thus obtained for each sample.

Routine bacteriological examination for P.haemolytica was performed by Dr.N.J.L.Gilmour of the Bacteriology Department, Moredun Research Institute.

Staining procedures.

A. Fluorescent antibody staining of M.ovipneumoniae.

The staining schedule was as follows:

1. Sections were taken to water and placed in PBS.Smears were air dried and fixed with acetone,and then placed in PBS.
2. Apply rabbit anti-M.ovipneumoniae antiserum for one hour.
3. Rinse in PBS,3 x 10 minutes.
4. Apply swine(sheep) anti-rabbit IgG(Ig)FITC for 30 minutes.
5. Rinse in PBS,3 x 10 minutes.
6. Mount in buffered glycerol.

Procedures were carried out at room temperature,and all antibody incubations were in a humidity chamber.The sections were examined by incident light and Ploëm system on a Leitz Orthoplan microscope,fitted with a mercury vapour bulb (Wotass HB0200),a BG38 red and heat absorption filter,a CB16.5 excitation filter,and a K510 barrier filter.

i. The method was first applied to smears of M.ovipneumoniae and M.arginini to show specifity of fluorescence and optimal dilutions.

The following incubations were carried out:

M.ovipneumoniae + rabbit anti-M.ovipneumoniae

M.ovipneumoniae + NRS

M.arginini + rabbit anti-M.ovipneumoniae

M.arginini + NRS.

ii. The fluorescent antibody technique was then applied to the lung sections, again using normal rabbit serum as the control.

Problems were encountered at this stage with specific yellow-green fluorescence being masked by non-specific background fluorescence. The dilutions of antiserum and FITC conjugates, and the duration of their application to the tissues were therefore varied, and swine anti-rabbit IgG FITC was used instead of sheep anti-rabbit Ig FITC to eliminate non-specific reaction with sheep tissue.

Methods for reducing background fluorescence:

1. Application of SPF sheep serum, diluted 1 in 10 with sterile PBS, to the sections before the specific antiserum.
2. Absorption of antisera with sheep liver powder to remove non-specific immunoglobulins.
3. Trypsin pretreatment of sections to uncover specific binding sites, thus enhancing specific fluorescence (Huang, Minassian & More, 1976; Qualman & Keren, 1979; Radaszkiewiez, Dragosics, Abdelfattahgad & Denk, 1979; Swoveland & Johnson, 1979.). Dilutions of trypsin, ranging from 0.1-2% in PBS were applied for times varying from 5 minutes to 1 hour. Calcium chloride (CaCl) was added to a final concentration of 0.1% and pH adjusted to 7.8 with 0.1N Sodium hydroxide (NaOH). In all cases, sections were incubated at room temperature in coplin jars, and washed in PBS before applying specific antisera. Control sections were

incubated in 0.1% $CaCl_2$ in PBS without trypsin.

In an attempt to improve the adhesion of lung sections two alternate methods were attempted;

a) use of starch adhesive instead of egg albumen, and incubation at 60°C for one hour,

b) covering the slide with a layer of celloidin prior to trypsin pretreatment(Radaszkiewicz et al,1979).

4.Counterstaining with Eriochrome Black after fluorescent antibody staining (Fey,1972).Eriochrome Black was dissolved 1 in 60 (w/v) in deionised water.The slide was flooded for ten seconds,and then washed with PBS.

5.Preparation of an IgG fraction of rabbit anti-M.ovipneumoniae antiserum by absorption on Protein A sepharose (Goding,1976).

B. Immunoperoxidase staining of M.ovipneumoniae.

a)Peroxidase labelled antibody('indirect technique').

The staining procedure was as follows:

1.Sections were taken to water and then placed in buffer.Smears were air dried,fixed and placed in PBS.

2.Apply rabbit anti-M.ovipneumoniae antiserum at optimal dilution for $1\frac{1}{2}$ hours.

3.Wash in PBS,3 x 10 minutes.

4.Apply sheep $F(ab^1)_2$ anti-rabbit F(ab) HRP(diluted 1 in 20) for 1 hour.

5.Wash in PBS,3 x 10 minutes.

6.Stain with D.A.B. solution for 20 minutes or until required colour intensity achieved.

7.Wash in distilled water.

8.Dehydrate and mount in Harleco mounting fluid.

All procedures were carried out at room temperature,and all antibody incubations were in a humidity chamber.Control slides were treated with normal rabbit serum at a 1 in 20 dilution in PBS.The slides were then examined under a conventional transmitted light microscope.

b)Peroxidase labelled antibody('sandwich technique').

The staining technique was as follows:

1.Sections were taken to water and then placed in buffer.Smears were air dried,fixed and placed in PBS.

2.Apply rabbit anti-M.ovipneumoniae antiserum at optimal dilution for 1½ hours.

3.Wash in egg albumen,15 minutes.

4.Apply sheep anti-rabbit IgG antiserum (1 in 20 dilution),45 minutes.

5.Wash in egg albumen,15 minutes.

6.Apply rabbit anti-sheep conjugated with HRP (1 in 20 dilution), 45 minutes.

7.Wash in egg albumen,15 minutes.

8.Wash in Tris/HCl buffer,2 x 10 minutes.

9.Stain with D.A.B. solution,10 minutes or until required colour intensity achieved.

10.Wash in running tap water,10 minutes.

*11.Counterstain with haematoxylin,10 seconds.

*12."Blue up" in Scott's tapwater substitute.

13.Dehydrate,clear and mount in Harleco mounting fluid.

*Both of these steps were omitted in some cases.

All procedures were carried out at room temperature,and all antibody incubations were in a humidity chamber.Control slides were treated with normal rabbit serum at a 1 in 20 dilution in

PBS in step 2, or PBS alone in step 4. The slides were then examined under a conventional transmitted light microscope.

Both methods were first applied to mycoplasma smears as for F.A.T. to demonstrate specificity of staining and optimal dilutions.

All the following procedures were applied to both techniques in an attempt to develop a satiafactory working technique for specific staining of mycoplasmas in lung tissue.

Destruction of endogenous tissue peroxidase activity prior to treating with specific antisera was by either:

a) immersion of sections in 0.5% H_2O_2 in methanol for 30 minutes. Rinse in PBS for 15 minutes (2 changes) (Streefkerk, 1972).

or b) i. immerse in periodic acid, 10 minutes

ii. Wash in running tapwater, 5 minutes.

iii. Immerse in $NaBH_4/NaHPO_4$, 2 minutes (Jobst & Horvath, 1961).

iv. Rinse in egg albumen for 30 minutes (2 changes).

Reduction of background staining was attempted by several methods previously described for the F.A. technique:

a) Trypsin digestion.

b) Addition of sheep liver powders to sera.

c) Application of SPF lamb serum prior to specific staining.

d) Variable time exposure of sections to reacting sera of varying dilutions, and to buffer washings.

e) Protein A sepharose fractionation of anti-M. ovipneumoniae antiserum.

The method ultimately chosen and used to stain the available tissues was:

1.Pretreatment with sodium borohydride for destruction of endogenous peroxidase.

2.Pretreatment with 1 in 10 SPF sheep serum for 15 minutes.

3."Sandwich technique"using dilutions of 1 in 40 and 1 in 80 of anti-M.ovipneumoniae antiserum.

C. Immunofluorescent demonstration of immunoglobulins in lung tissue.

An indirect F.A. technique was used to demonstrate the distribution of immunoglobulins (IgG and IgA) in the lungs of sheep.Lungs from SPF lambs either inoculated endobronchially with a pneumonic lung homogenate,containing M.ovipneumoniae and P.haemolytica,or a similar,but sterilised,lung homogenate were studied.

The staining procedure was as follows:

1.Take sections to water and then PBS.

2.Apply either rabbit anti-sheep IgG or rabbit anti-sheep IgA antiserum,diluted 1 in 20,for 30 minutes.

3.Wash in PBS,3 x 10 minutes.

4.Apply sheep anti-rabbit Ig FITC diluted 1 in 30,for 30 minutes.

5.Wash in PBS,3 x 10 minutes.

6.Mount in buffered glycerol(pH 8.0).

Control sections were prepared in which the first antiserum (step 2) was replaced by PBS.These were examined by the system previously described in staining procedure A.

CHAPTER 3

RESULTS

THE IMMUNOFLUORESCENT DEMONSTRATION OF M.OVIPNEUMONIAE.

Treatment of broth culture smears with rabbit anti-M.ovipneumoniae antiserum in the F.A. technique described in chapter 2 resulted in a yellow-green fluorescent staining of the mycoplasma.A smear of the same culture showed no fluorescence when treated with normal rabbit serum (N.R.S.) instead of specific antiserum.No fluorescence was seen when culture smears of M.arginini were treated with either specific antiserum or N.R.S.

When applied to paraffin-wax sections of pneumonic lung tissue however,the above technique produced an unacceptably high level of non-specific background fluorescence with both specific antiserum and N.R.S..Although strong specific fluorescence could be identified by intensity and location,low levels of specific staining would be completely masked by this background fluorescence.

Methods of reducing non-specific background fluorescence.

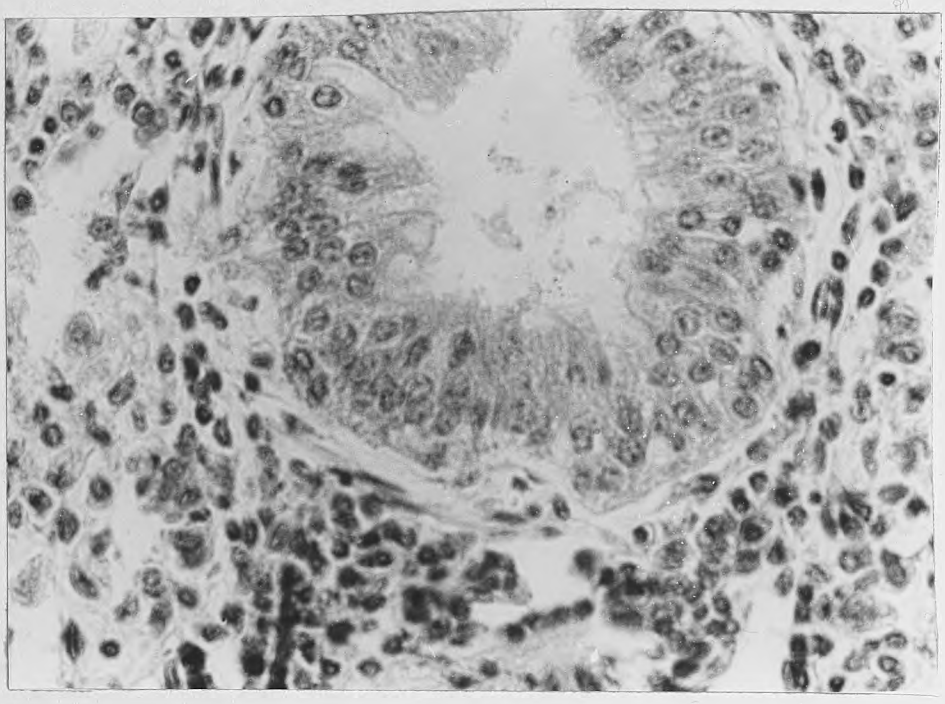
Reducing the concentration of the specific antiserum and the F.I.T.C. conjugated serum,and the time of their exposure on the sections gave inconsistent results,but,in general,the level of background fluorescence was slightly reduced with no discernable effect on presumptive specific fluorescence.Trypsin pretreatment of sections and application of sheep liver powders to both antisera gave similar results with little reduction of non-specific fluorescence.Sections exposed to trypsin digestion had to be coated with celloidin as the adhesion of sections to egg albumen and starch adhesive coated slides was poor,and the tissue tended to break up and/or wash off the slide.Counterstaining with

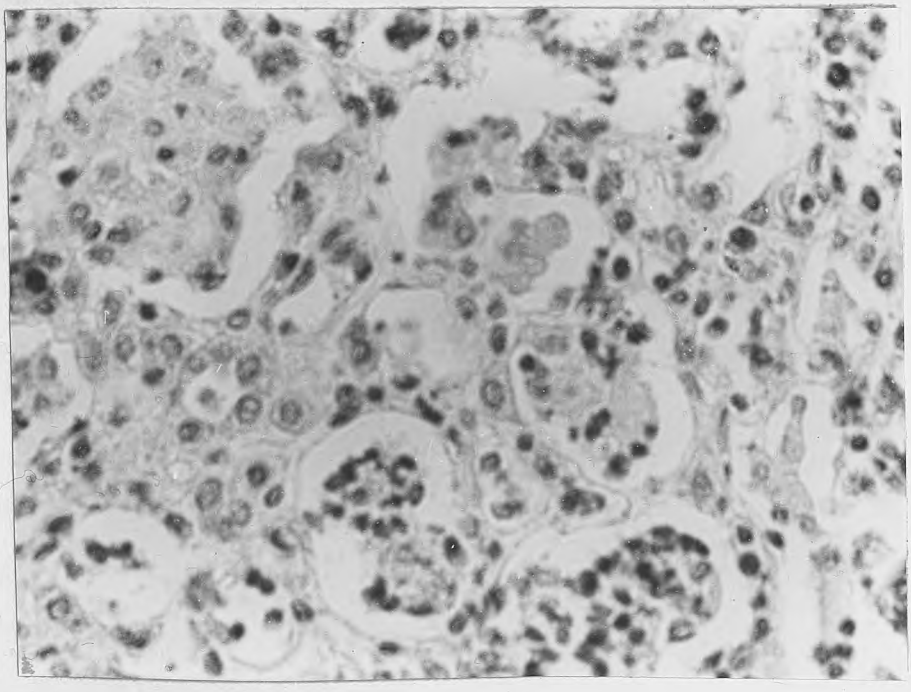
Plate 1. (a)upper,(b)lower.

(a) Lymphocytic accumulation associated with bronchus, x 252

(b) Alveolar epithelialisation and intra-alveolar exudate, x 310

Haematoxylin





Eriochrome Black resulted in abolishment of all fluorescence.

Sections pretreated with SPF sheep serum showed a significant reduction in non-specific background fluorescence with only a mild reduction in specific fluorescence.When the rabbit anti-M.ovipneumoniae Ig fraction was used instead of whole antiserum,little or no bright fluorescence could be seen.

The distribution of specific fluorescence.

The identification of specific fluorescence was most easily made following SPF sheep serum pretreatment of lung sections,and then staining with 1 in 20 rabbit anti-M.ovipneumoniae antiserum and 1 in 20 S.W.A.R./F.I.T.C..It was from sections stained in this manner that the following observations were made.

Specific fluorescence was seen as a layer of bright yellow-green fluorescence lining the surface of bronchial and bronchiolar epithelium in pneumonic lung tissue.In some areas the fluorescence was continuous,but particulate fluorescence was also recorded. Specific fluorescence was seen to be associated with ciliated epithelium, and was present amongst the tips of the cilia a small distance away from the epithelial surface.No fluorescence was seen in association with non-ciliated bronchial/bronchiolar epithelium or alveolar linings.Fluorescence of exudate present in the bronchial lumen was marked in some cases,and intense free particulate fluorescence was often seen within the exudate.Sections of pneumonic lung treated with N.R.S. showed no fluorescent lining of the bronchial epithelium,but intraluminar exudate,including free particulate fluorescence was retained to some extent.

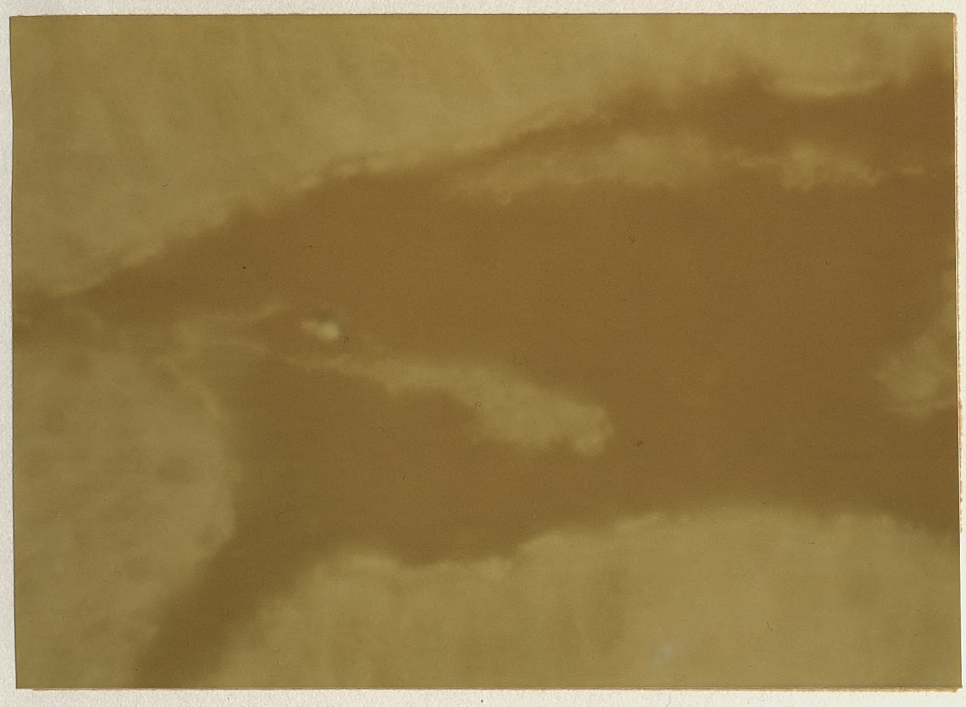
Non-specific or autofluorescence was observed in a number of leucocytes,and was seen as bright particulate intracellular fluorescence.However,specific staining was recorded in occasional alveolar macrophages;these cells had a thin layer of bright yellow-

Plate 2. (a)upper,(b)lower.

(a) Bronchus of conventional lamb with fluorescence of M.ovipneumoniae along epithelial surface, x 63

(b) Bronchus of conventional lamb with no fluorescence adjacent to epithelial surface.Use of NRS instead of specific antiserum, x 63

Immunofluorescent staining



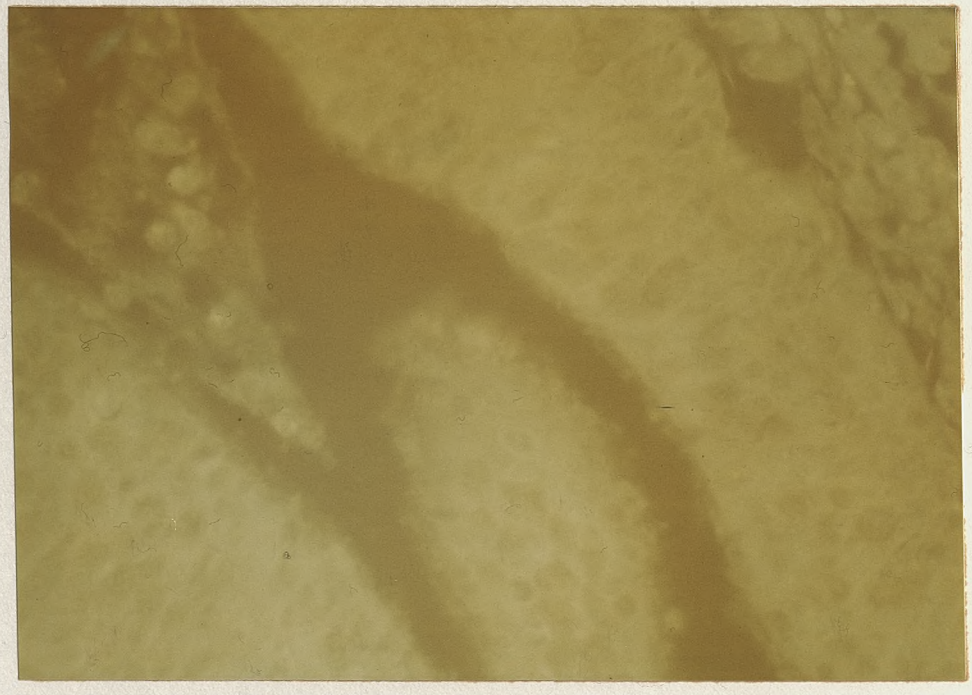


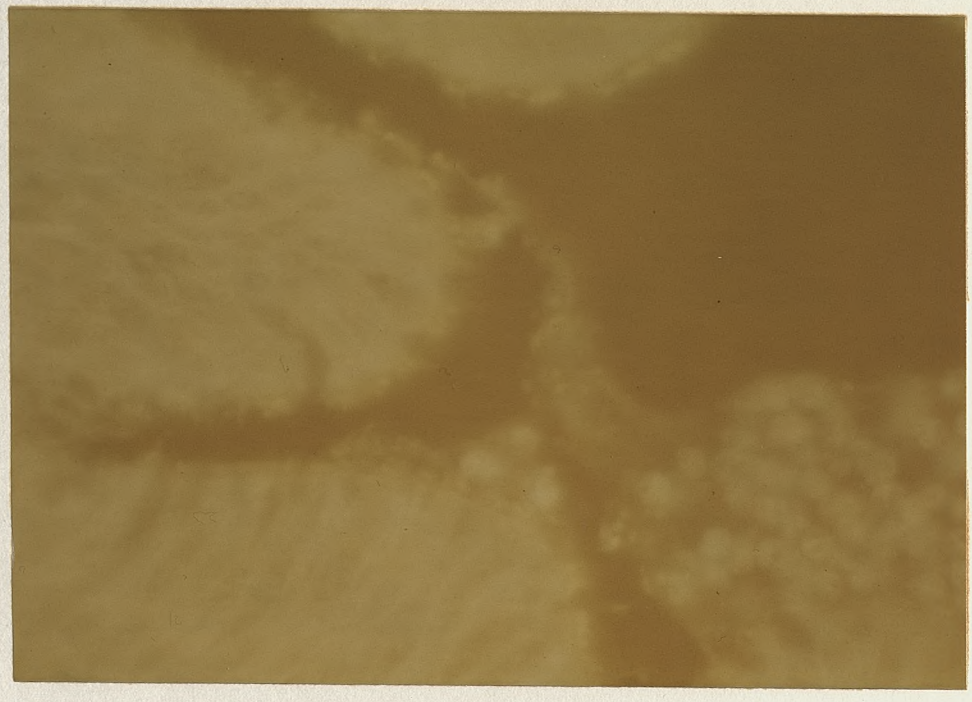
Plate 3. (a)upper, (b)lower.

Positive fluorescence of M. ovipneumoniae along bronchial epithelium

(a) x 310

(b) x 630

Immunofluorescent staining



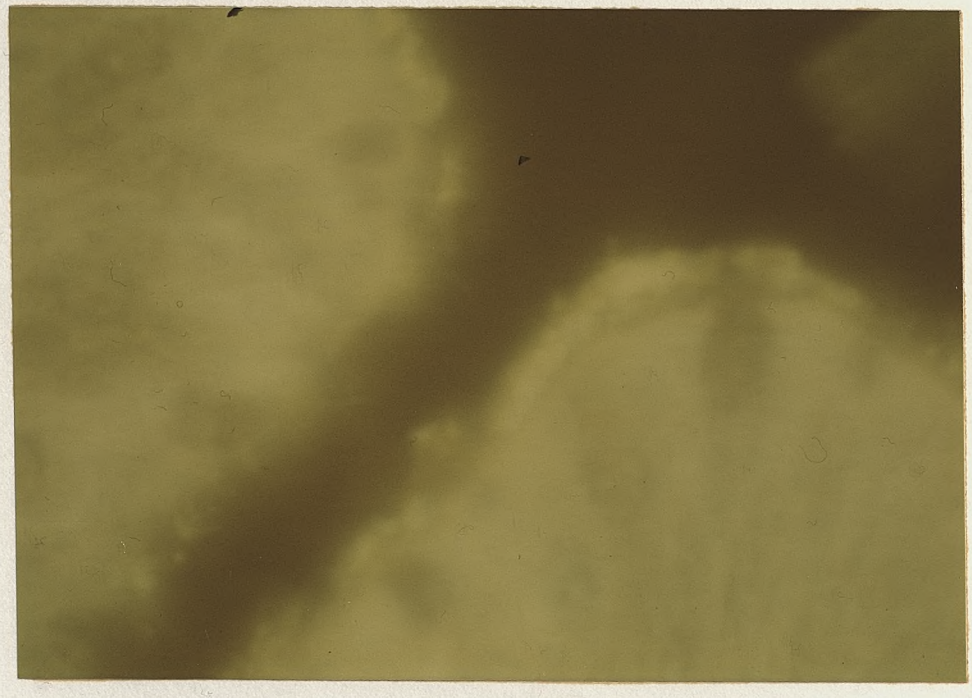
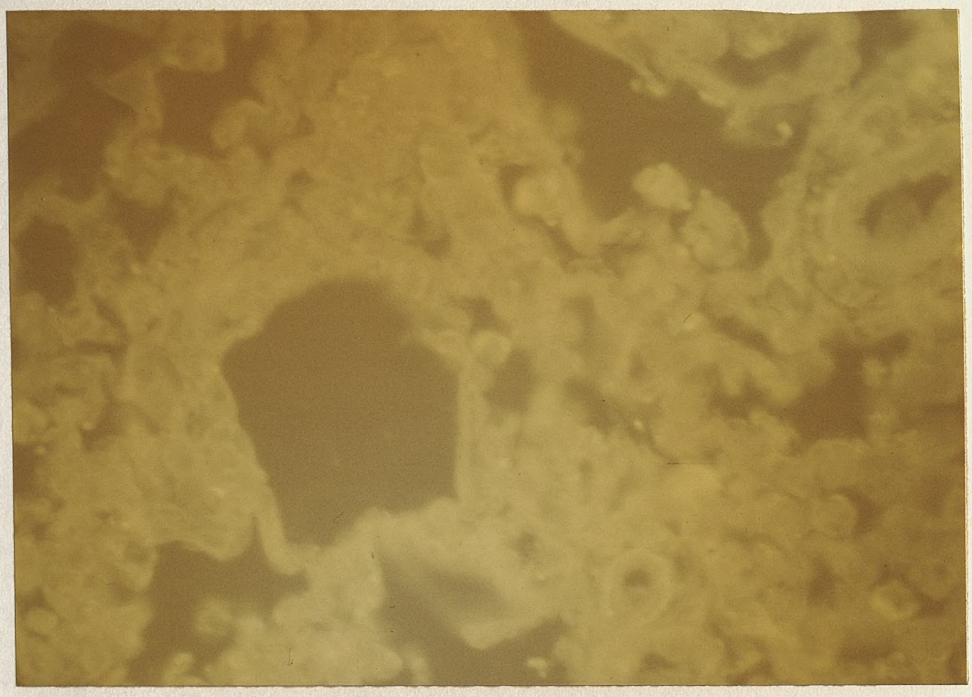
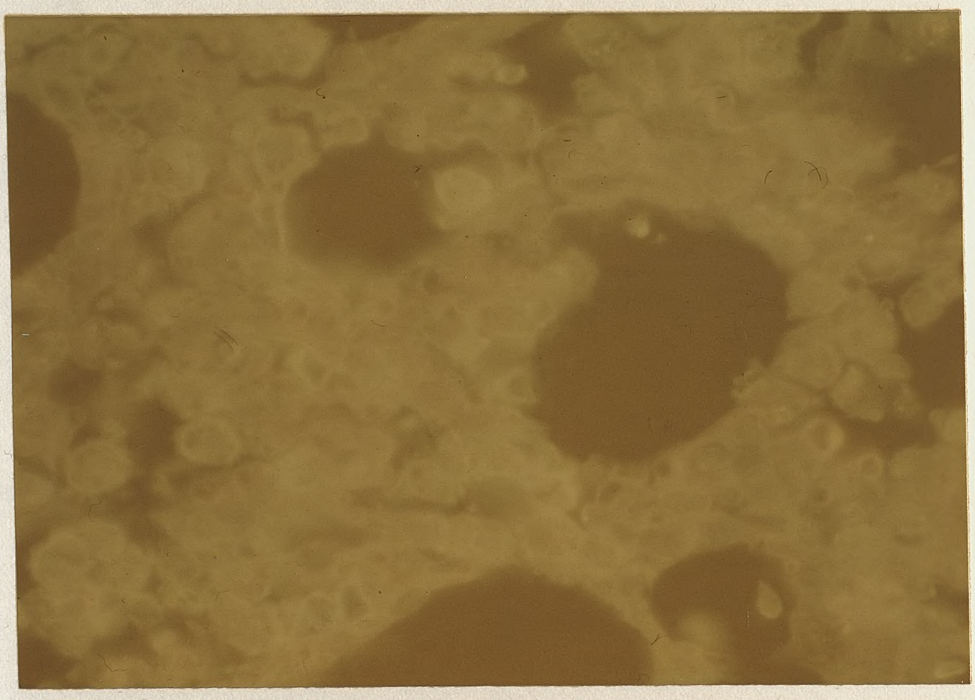


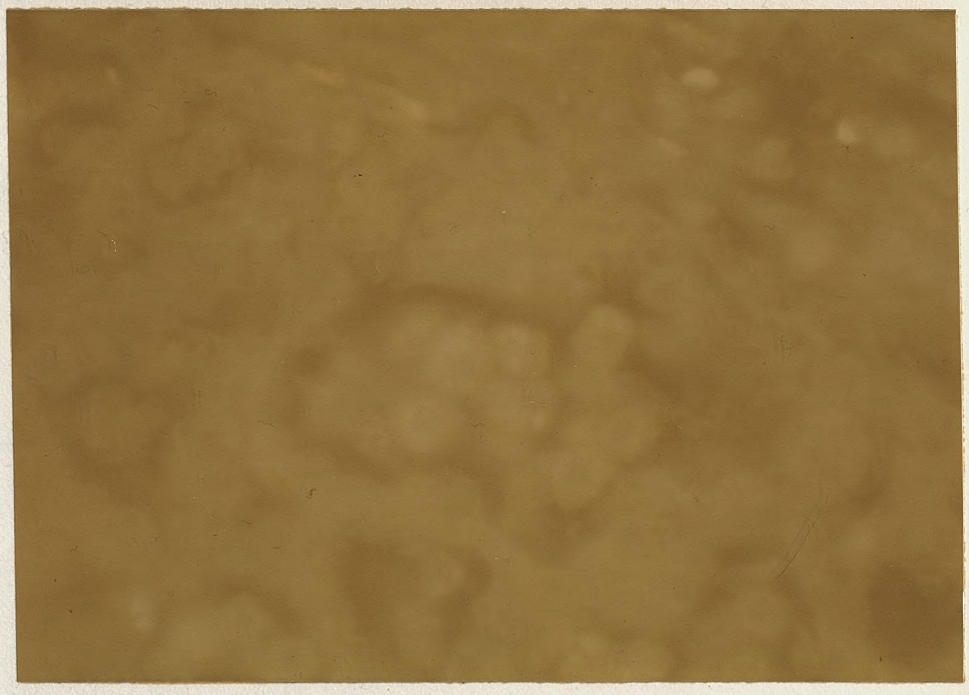
Plate 4. (a)upper,(b)middle,(c)lower,

(a) & (b) Specific fluorescence around alveolar macrophages and occasional particulate fluorescence in alveolar space, x 310

(c) Non-specific or autofluorescence of alveolar macrophages, x 630







-green fluorescence apparently coating the cell,occasionally associated with particulate fluorescence which seemed to be present on the cell surface.Bright free particulate fluorescence was occasionally recorded within the intra-alveolar exudate.Pneumonic lung sections treated with N.R.S. also exhibited non-specific staining of leucocytes,and,to a lesser extent,intra-alveolar exudate.The fluorescent "coating" of macrophages was not recorded in these cases.

Examination of normal lung tissue showed some degree of non-specific background fluorescence and non-specific or autofluorescence of some leucocytes,but no "specific" fluorescence was recorded.

THE IMMUNOPEROXIDASE DEMONSTRATION OF M.OVIPNEUMONIAE.

M.ovipneumoniae was demonstrated as brown particles either singly or in clumps following both the"indirect" and "sandwich" immunoperoxidase techniques using rabbit anti-M.ovipneumoniae antiserum on broth culture smears.No staining was observed when the rabbit anti-M.ovipneumoniae antiserum was replaced by N.R.S. in both methods,or when the sheep anti-rabbit antiserum was replaced by PBS in the sandwich technique.Also M.arginini was not stained by either method using rabbit anti-M.ovipneumoniae antiserum.Both immunoperoxidase techniques without pretreatments resulted in diffuse brown colouration of the lung sections.

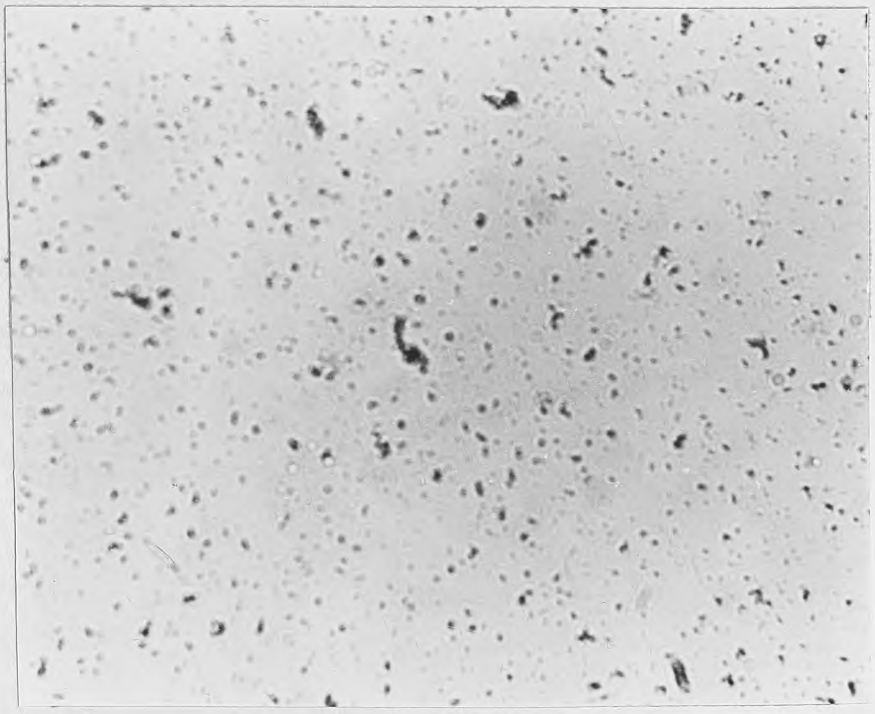
Reduction of non-specific staining and endogenous peroxidase activity in paraffin-wax sections of lung tissue.

a) Indirect immunoperoxidase technique:

Pretreatment with H_2O_2/methanol or sodium borohydride both gave very little reduction in background staining in sections treated with rabbit anti-M.ovipneumoniae or N.R.S..Thus identification of positively stained mycoplasmas remained difficult.

Plate 5.

Positive staining of M. ovipneumoniae smear by immunoperoxidase staining, x 630



Pretreatment with trypsin and use of liver powders as described for the F.A.T. had no apparent effect on staining characteristics. By varying time of staining and diluting the sera and D.A.B. solution,some reduction in the general staining intensity was achieved,but this failed to enhance positive staining.This was also true when sections were pretreated with SPF sheep serum.

b) "Sandwich" immunoperoxidase technique:

When sections were treated with PBS in place of Sha-Rab IgG only a trace of background staining remained.Sections treated with N.R.S. followed by Sha-Rab IgG showed more background staining and those treated with rabbit anti-M.ovipneumoniae antiserum more again.The H_2O_2/methanol method also proved effective,but background levels were significantly higher than with the borohydride method.Thus,non-specific staining of sections treated with rabbit anti-M.ovipneumoniae antiserum or N.R.S. still remained high,making identification of positive brown staining somewhat uncertain.

Pretreatment of sections with trypsin and the use of liver powders on sera again proved ineffective.However,pretreatment of sections with SPF sheep serum resulted in a reduction of non-specific background staining.Alterations in the dilution of the rabbit anti-M.ovipneumoniae antiserum gave further reduction in non-specific background staining while appearing to retain maximum positive staining.Dilutions used in examining the ovine lung tissue were 1 in 40,and 1 in 80. Further optimal reduction of non-specific staining was achieved when exposure of the sections to D.A.B. solution was reduced to four minutes.These modifications finally resulted in a slightly less intense brown colouration of M.ovipneumoniae,but greatly reduced non-specific staining.

The distribution of positive stain.

The identification of positive brown staining was most easily made following sodium borohydride and 1 in 10 SPF sheep serum pretreatment of lung sections and staining by the sandwich method using 1 in 40 and 1 in 80 dilutions of rabbit anti-M.ovipneumoniae antiserum.D.A.B.solution was applied for four minutes.

Positive brown staining was associated with ciliated bronchial and bronchiolar epithelium in pneumonic lungs.In many areas,brown-staining particulate material was seen in close association with the tips of the cilia,but not adjacent to the epithelial cell surface.The positive staining was occasionally seen as a continuous layer along the ciliated border in which individual particles could not be identified.No brown staining around the bronchi was observed in pneumonic sections treated with N.R.S.,or in M.ovipneumoniae-free lungs (as determined by cultural techniques) treated with rabbit anti-M.ovipneumoniae antiserum.

Type II pneumocytes and alveolar macrophages free in the alveolar space occasionally exhibited positive brown staining.This consisted of a diffuse light brown colouration of the cell with occasional darker brown particulate staining within the cell.The majority of these cells however,showed no positive staining.Some positive staining of these cells was also seen in the same pneumonic sections treated with N.R.S. instead of specific antiserum,but the number of type II pneumocytes or alveolar macrophages exhibiting positive staining appeared higher in sections treated with the specific antiserum.Rarely,positively-stained cells were observed in the alveolar septa in sections treated with specific antiserum and N.R.S..

Fewer alveolar macrophages and type II pneumocytes were present in sections from normal lungs,but occasional positive staining was

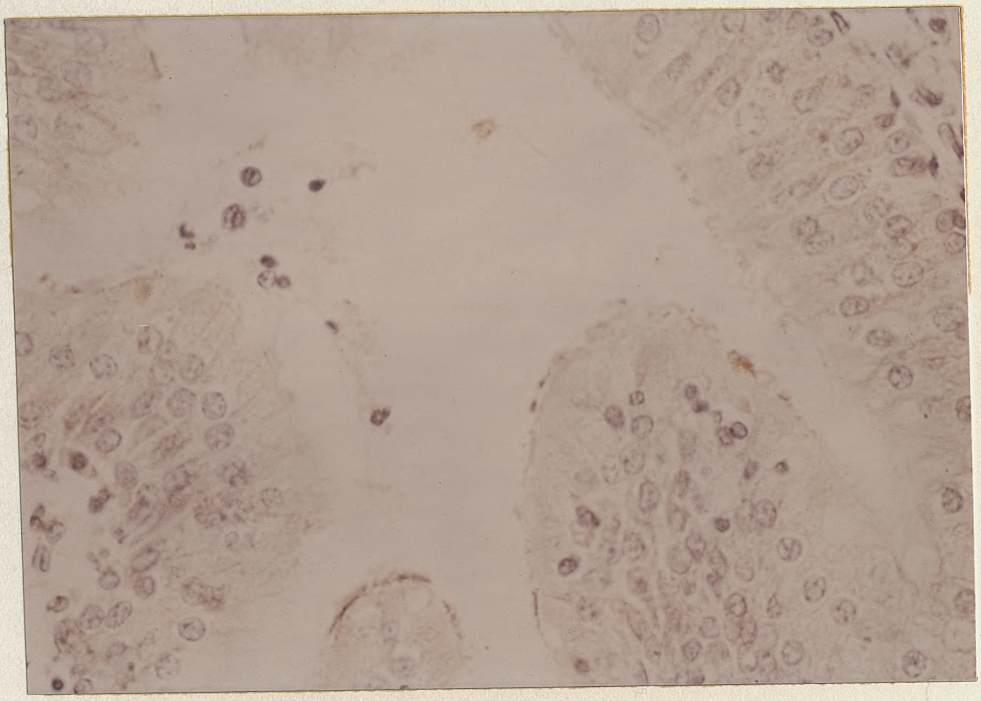
Plate 6. (a)upper,(b)lower.

Bronchus of SPF lamb showing positive staining of M.ovipneumoniae along ciliated epithelium.

(a) x 63

(b) x 252

Immunoperoxidase staining and Haematoxylin



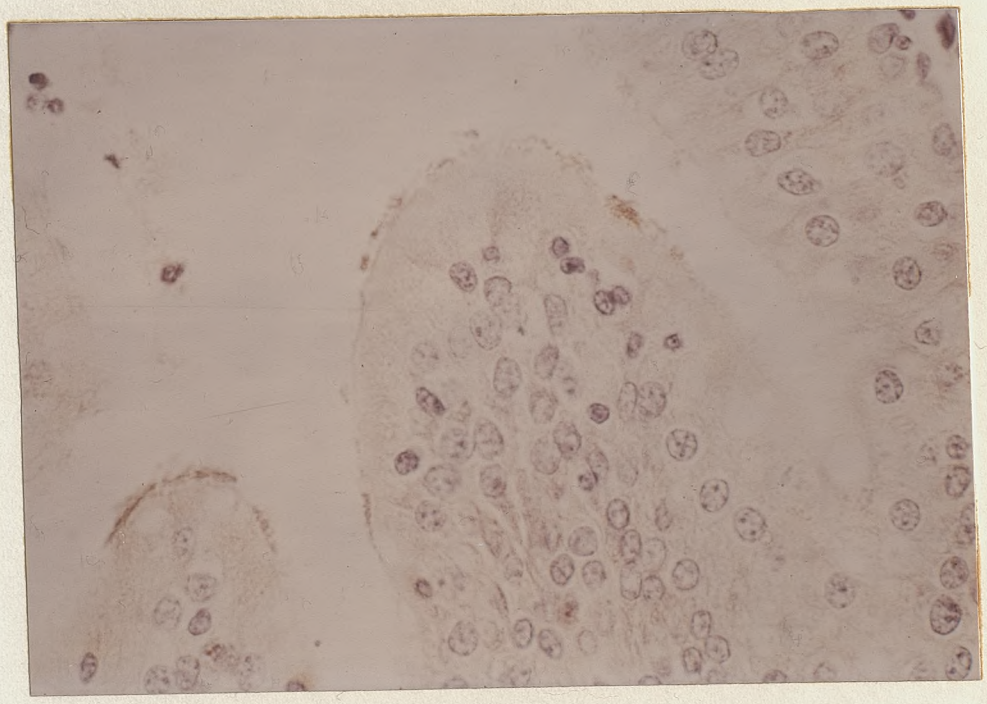


Plate 7. (a)upper,(b)middle,(c)lower.

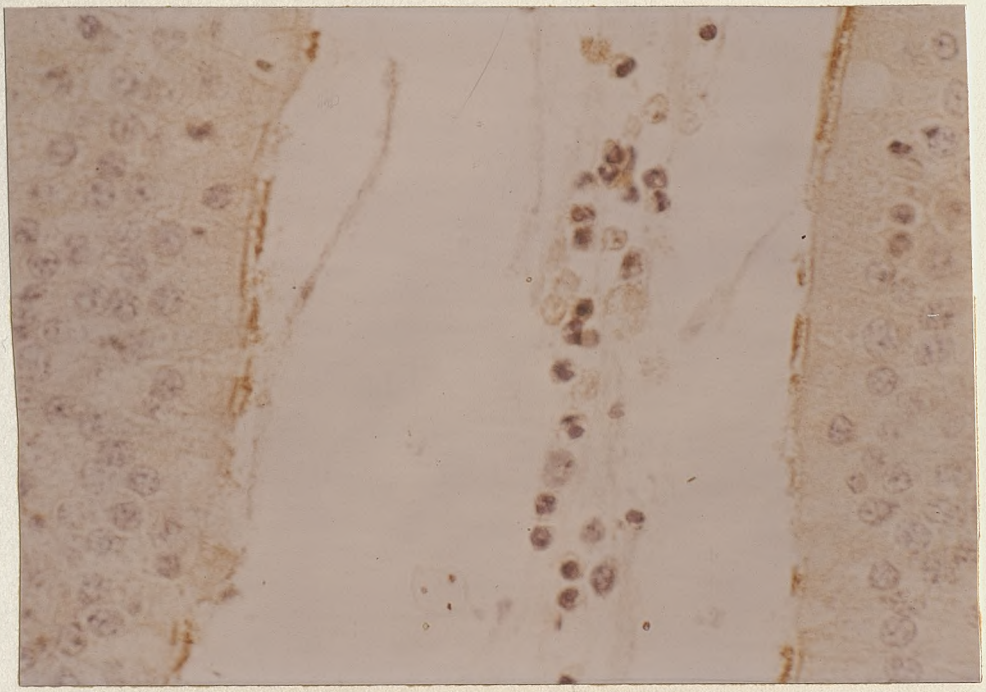
Bronchus of SPF lamb.

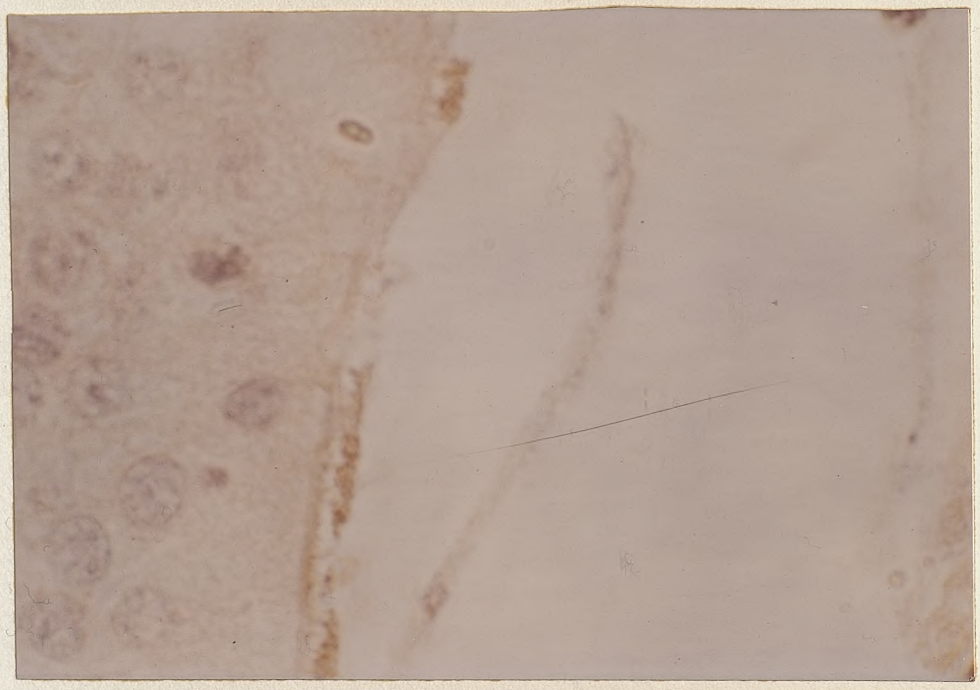
(a) M.ovipneumoniae demonstrated along bronchial epithelium and positive particulate stain in the exudate, x 310

(b) M.ovipneumoniae demonstrated along ciliated bronchial epithelium, x 630

(c) Bronchial epithelium showing no positive stain.Use of NRS instead of specific antiserum, x 310

Immunoperoxidase staining and Haematoxylin





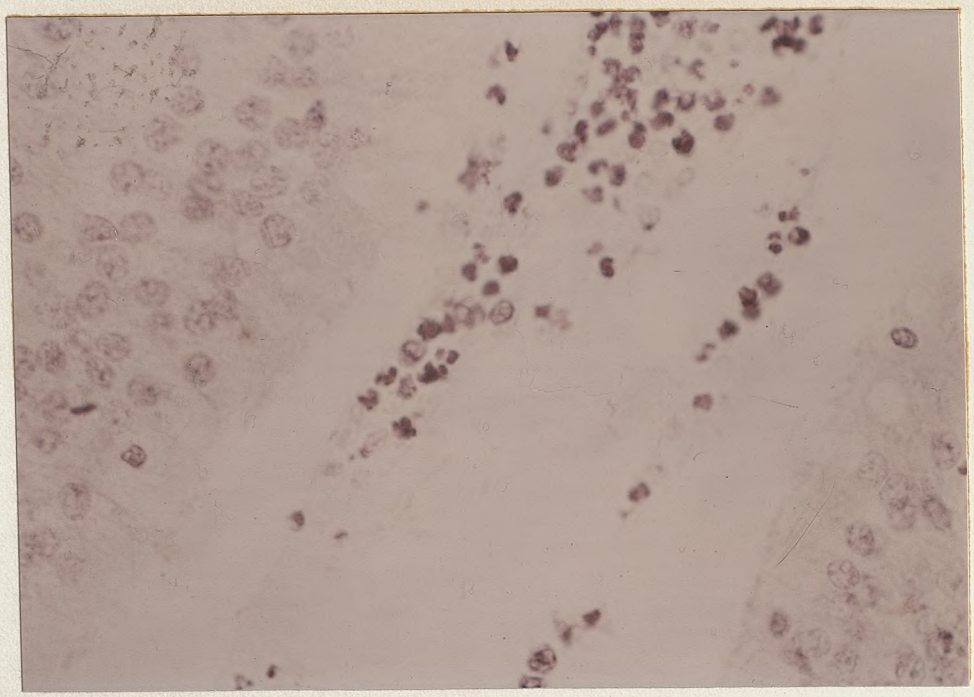


Plate 8. (a)upper,(b)middle,(c)lower.

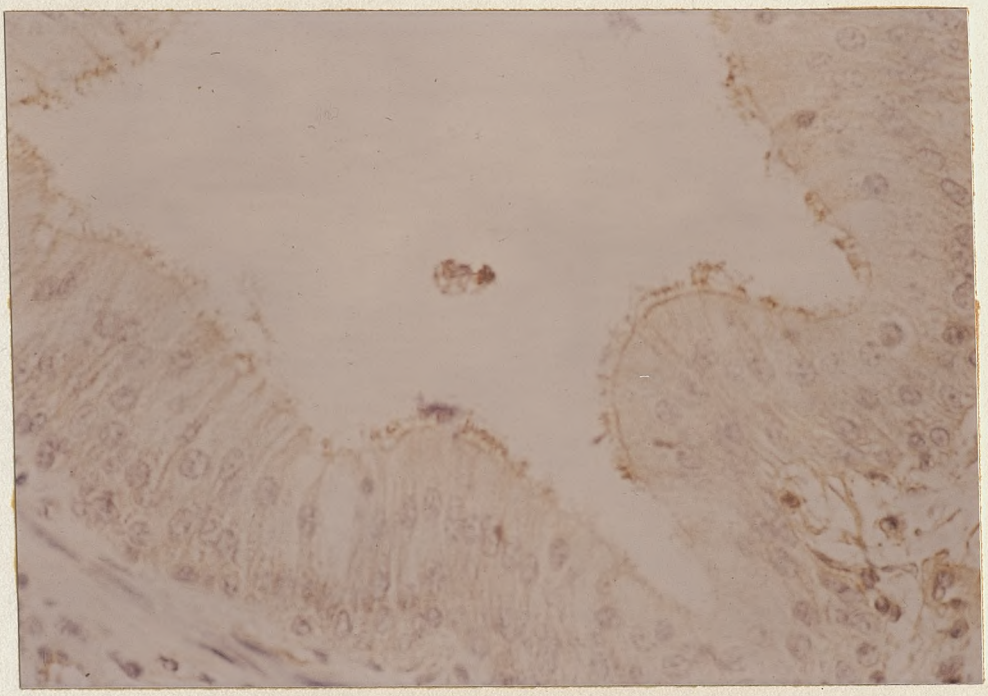
Bronchus of conventional lamb.

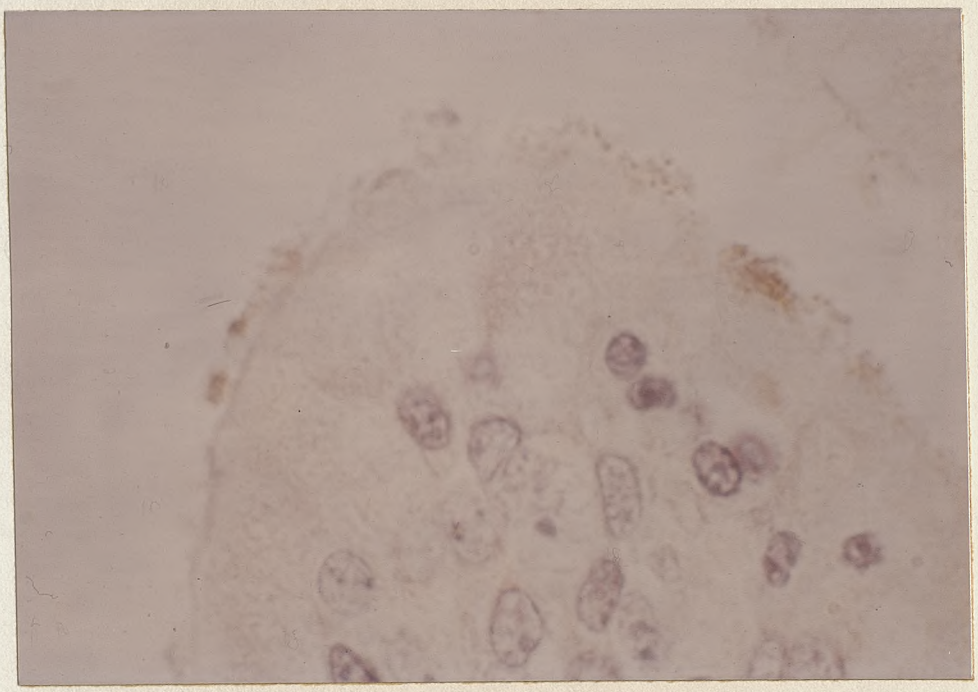
(a) Continuous particulate staining of M.ovipneumoniae around bronchial epithelium, x 63

(b) M.ovipneumoniae present amongst cilia, x 630

(c) Bronchial epithelium showing no positive stain.Use of NRS instead of specific antiserum, x 630

Immunoperoxidase staining and Haematoxylin





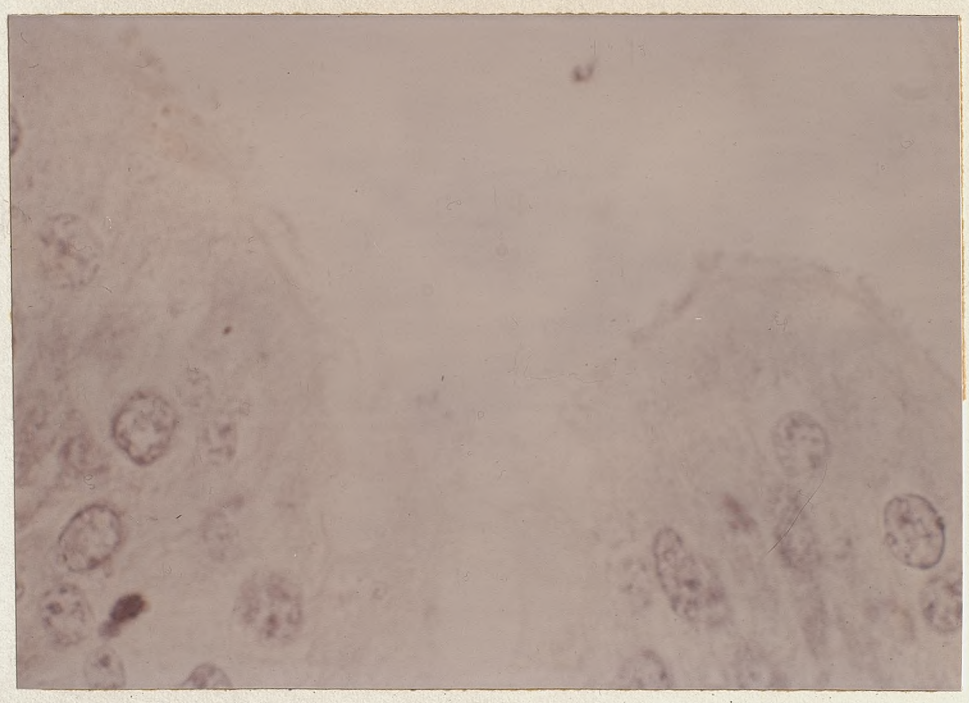


Plate 9. (a)upper,(b)lower

Bronchus of SPF lamb showing positive staining of M.ovipneumoniae. Note difference in background staining due to reduction in D.A.B.solution application to section.

(a) x 252

(b) x 630

Immunoperoxidase staining

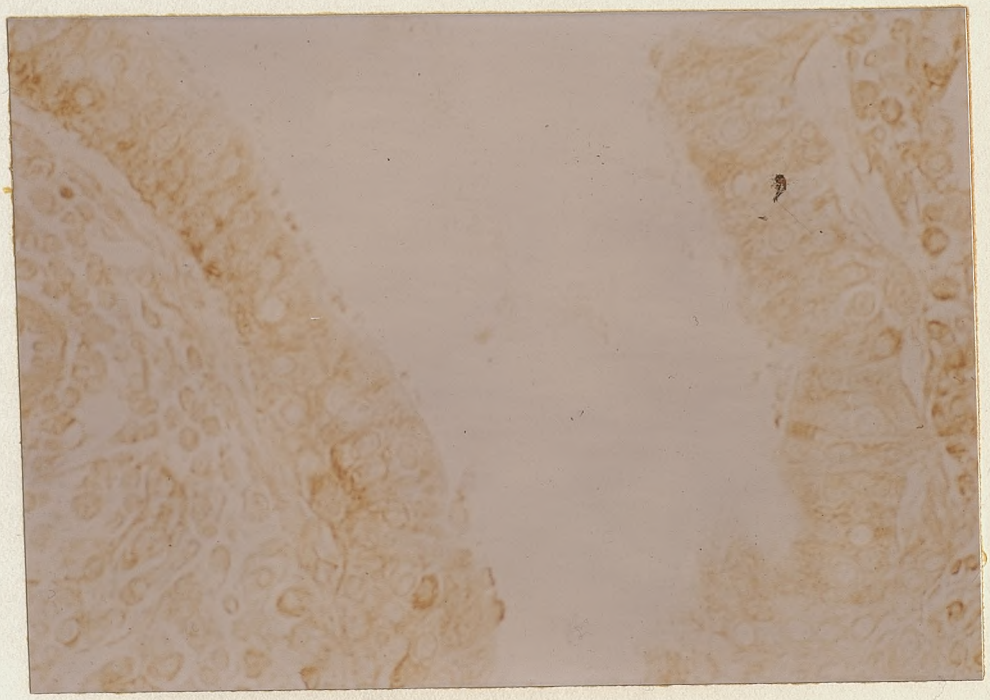


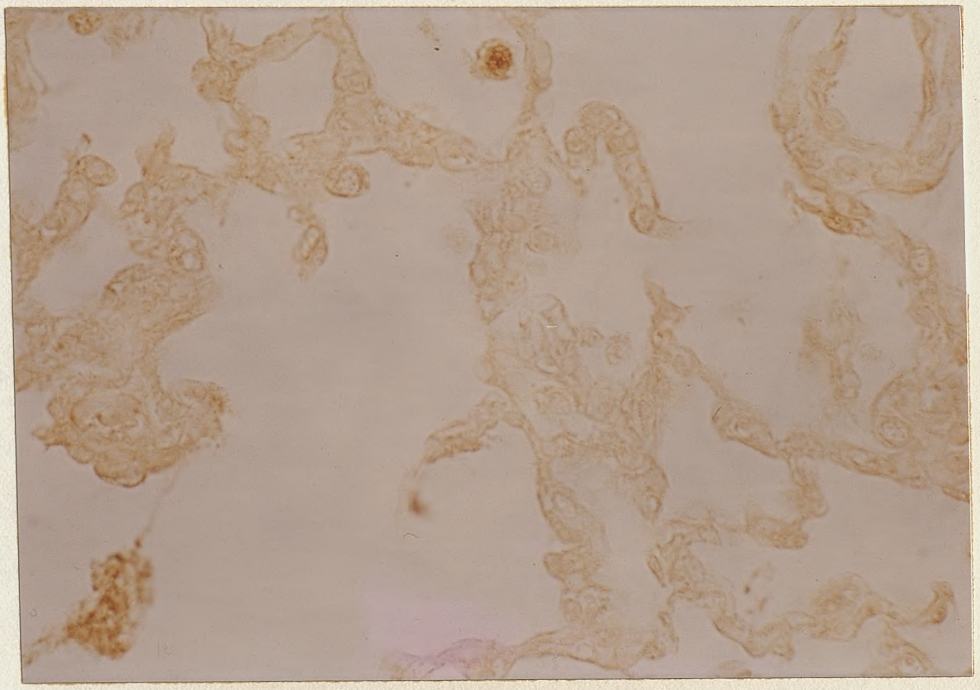
Plate 10. (a)upper,(b)lower.

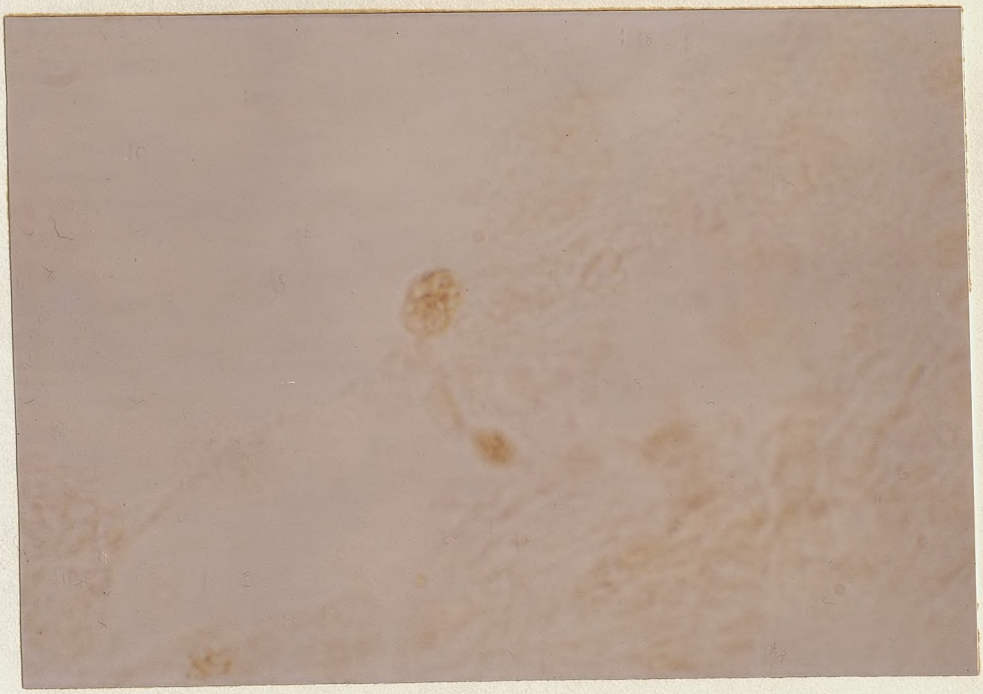
Positive staining of alveolar macrophages,and alveolar exudate (exudate (a) only).

(a) x 310

(b) x 630

Immunoperoxidase staining





again recorded.

The SPF lambs used in experiments on "atypical pneumonia were examined by the "sandwich" technique.They were grouped according to the inoculum administered,and only right apical lobes were examined.The results are summarised in Table I.This shows that M. M.ovipneumoniae was demonstrated in a proportion of animals in groups 2,3, & 4,but not in group 1.Demonstration of M.ovipneumoniae was more frequently made in lungs bearing gross lesions of atypical pneumonia than apparently normal lungs.

Specific staining was also demonstrated in lung sections from conventionally-reared sheep infected by endobronchial inoculation or in-contact with the former group.The results are summarised in Table II.This further shows that the demonstration of M.ovipneumoniae was made in a higher proportion of lungs with gross lesions than apparently normal lungs.There was positive isolation by cultural methods from all lung samples.

M.ovipneumoniae was isolated by cultural methods from lung samples of eight "abattoir sheep" of unknown origin with grossly and histologically normal lungs.However,microscopic examination using the "sandwich" immunoperoxidase technique failed to demonstrate the organism in sections of the right apical lobe or trachea.

Cryostat sections of conventionally-reared sheep lungs were made and treated with the F.A. technique and immunoperoxidase methods. Specific staining was seen,although non-specific background fluorescence was also present.The general morphology of the lung sections was very poor,and thus it was decided not to use cryostat sections for the study of M.ovipneumoniae described here.

Table I.

Demonstration of *M.ovipneumoniae* in the lungs of SPF sheep.

Group	No.in group	Inoculum*	No.of lungs from which: *M.ovipneumoniae* recovered	Gross lesion recorded**	*M.ovipneumoniae* demonstrated.
1	7	A	0	0	0
2	7	B	6	2	3
3	4	C	4	2	3
4	7	D	7	4	5

* A - sterile lung homogenate + ampicillin

B - lung homogenate + Tylan 200

C - sterile lung homogenate + *M.ovipneumoniae* (2×10^7 c.f.u./ml)

D - lung homogenate + ampicillin.

** Recovery titre of *M.ovipneumoniae* 10^6 c.f.u./ml.

Table II.

Demonstration of M.ovipneumoniae in the lung from conventionally-reared sheep.

Lung Pathology	Number of lungs showing: Positive demonstration of M.ovipneumoniae	No demonstration of M.ovipneumoniae
Gross lesions	4	2
Apparently normal	1	3

THE IMMUNOFLUORESCENT DEMONSTRATION OF IgG AND IgA

Bright yellow-green fluorescence was limited to certain specific sites of lung tissue,and the general background fluorescence was quite low.Thus no pretreatments were employed.

Distribution of specific fluorescence.

The lymphocytes involved in the peri-bronchial/bronchiolar cuffing in lung sections from sheep with atypical pneumonia showed bright yellow-green fluorescence when treated with rabbit anti-sheep IgG antiserum.Occasional cells scattered in the interstitium also exhibited the fluorescence.Bright fluorescence was also observed from exudate and alveolar macrophages present in the lumen of the alveoli.The staining of the alveolar macrophages was seen as a layer of fluorescence around the periphery of the cell.Except for occasional stained cells in the interstitium,little fluorescence was seen in lungs with little or no microscopical pathology.

A similar distribution of specific fluorescence was seen with sections treated with rabbit anti-sheep IgA,but a higher proportion of interstitial cells in the alveolar septa of pneumonic lungs exhibited fluorescence,and fluorescence in the peribronchial region was restricted to much fewer cells.

Sections treated with PBS instead of specific antiserum showed no fluorescence except for the non-specific or autofluorescence seen in leucocytes,observed in all preparations.

Plate 11.

Bright fluorescence of peribronchial lymphocyte cuff following staining for IgG, x 252

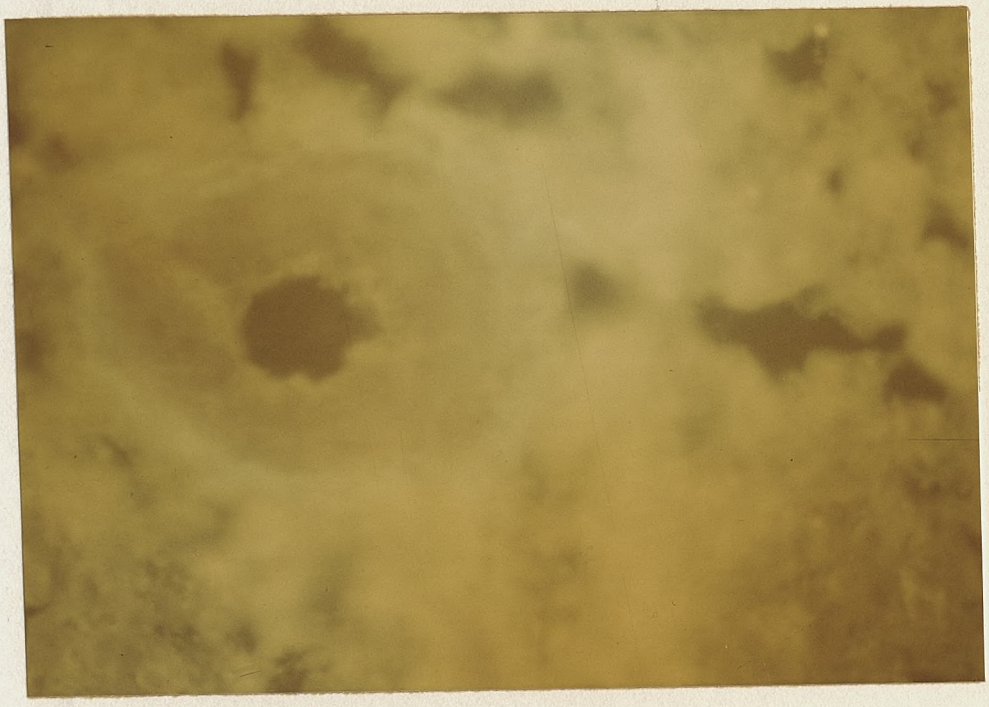
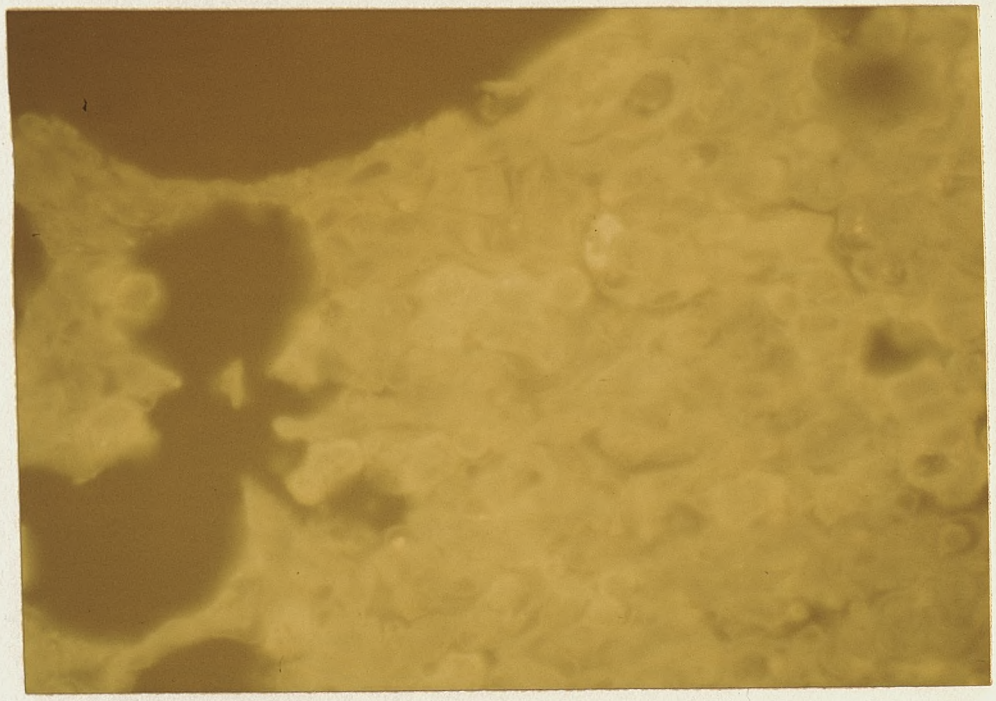


Plate 12. (a)upper,(b)lower.

(a) Demonstration of specific fluorescence of alveolar macrophages and alveolar exudate following stains for IgA, x 252

(b) No fluorescence present following PBS treatment instead of specific antiserum, x 252



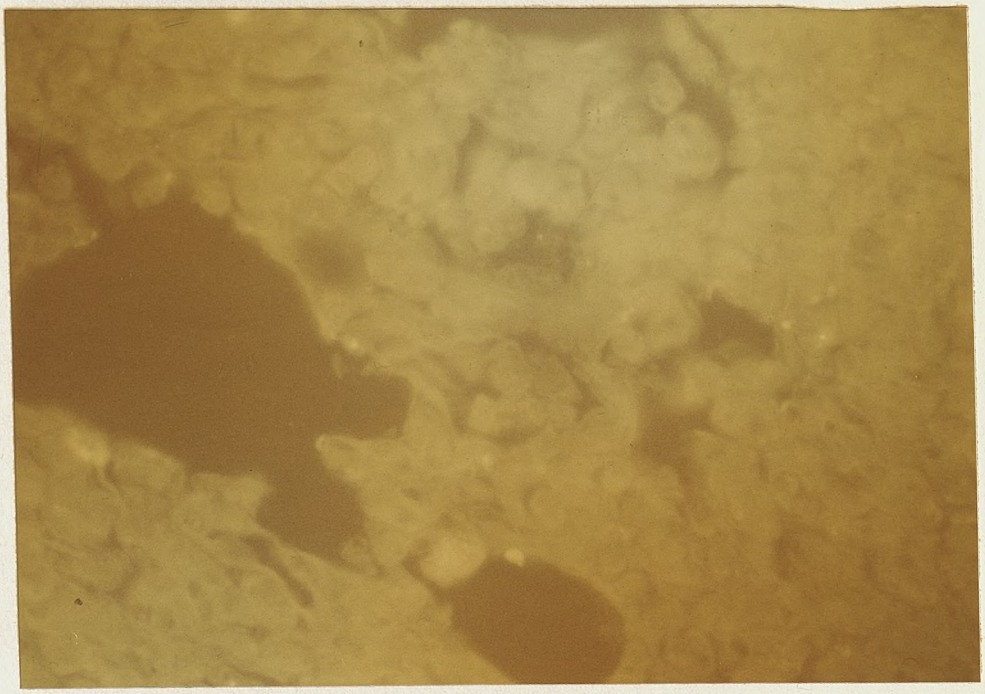
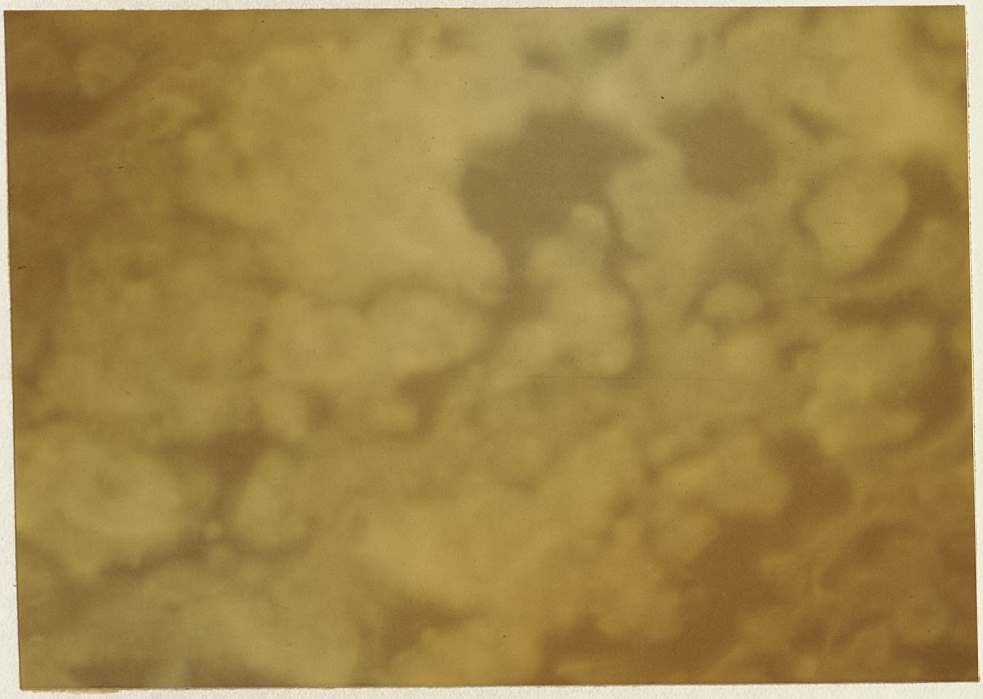
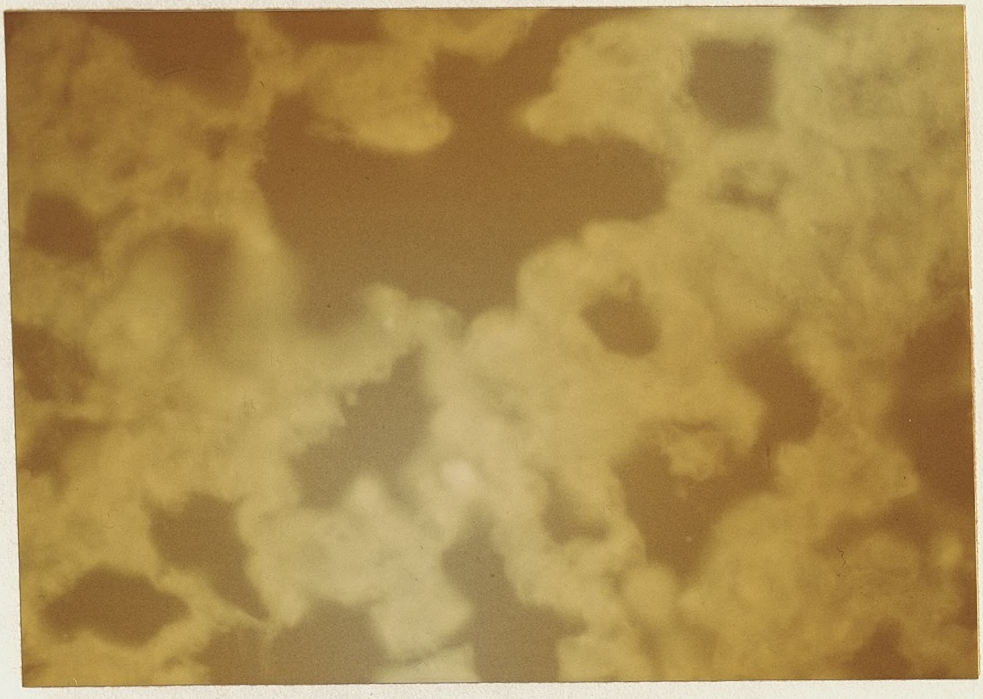


Plate 13. (a)upper,(b)lower.

(a) Specific fluorescence of alveolar macrophages and alveolar exudate, x 630

(b) No specific fluorescence following PBS treatment, x 310





CHAPTER 4

DISCUSSION

The aims of this study were to demonstrate M.ovipneumoniae in ovine lung tissue by means of immunofluorescence and/or immunoperoxidase staining,and to use these techniques to determine the distribution of M.ovipneumoniae withon the lungs of pneumonic and healthy sheep. Determination of the conditions for optimal staining in each case proved to be a major task in itself,as indicated by the number of different treatments and their effects described in chapters 2 & 3.

Reduction of non-specific staining.

a) Immunofluorescence:

The demonstration of immunofluorescent staining of mycoplasma species in cryostat sections of lung tissue has been previously described (Organick & Lutsky,1968;L'Ecuyer & boulanger,1970; Meyling,1971).and in acetone-fixed paraffin-wax sections(Pospisil et al,1970).Potgieter & Ross (1972) used the F.A.technique on cryostat sections of synovial membranes to demonstrate mycoplasmas. The use of cryostat sections gave problems of non-specific fluorescence,which the authors tackled in a number of ways;ion exchange chromatography,adsorption of sera with liver powders, normal serum or leucocytes,azo-dye counterstaining,and pretreatment of sections with non-immune serum.Potgieter & Ross (1972) described only slight to moderate reduction of non-specific fluorescence following adsorption of conjugates with swine liver powders, swine serum and swine leucocytes.Organick & Lutsky (1968), however,routinely used adsorption with mouse liver powders and guinea pig serum to improve their results.L'Ecuyer & Boulanger (1970) used repeated adsorption with liver powders to reduce n

non-specific fluorescence but this was accompanied by a reduction in intensity of specific fluorescence.Slight reduction of non-specific fluorescence was demonstrated following pretreatment of tissues with concentrated non-immune serum (Potgieter & Ross, 1972).Counterstaining with azo-dyes has been used successfully to mask non-specific fluorescence (Noel et al,1964;Fey,1972), but specific staining may also be reduced or lost (Fey,1972; Potgieter & Ross,1972).Ion-exchange chromatography of FITC conjugates has also been shown to be very effective in reducing non-specific fluorescence (Meyling,1971;Fey,1972;Potgieter & Ross, 1972),but again they reported that any increase in specificity was at the expense of a certain loss of brilliancy.

The results of the present study agree with these findings, ,except that pretreatment of sections with SPF sheep serum gave reasonable reduction in non-specific staining.The commercially purchased fluorescent conjugates had been purified by the manufacturers using ion-exchange chromatography and insoluble immunosorbents to remove all but correctly FITC labelled immunoglobulin,and appeared to be effective here.Trypsin pretreatment of sections reported as enhancing specific staining by uncovering binding sites on antigens (Huang et al,1976;Qualman & Keren,1979;Radaszkiewicz et al,1979;Swoveland & Johnson,1979) was found to have little or no effect in this study.

The preparation of an IgG fraction of the rabbit anti-M. ovipneumoniae antiserum considerably reduced all fluorescence, specific and non-specific.It was possible that the final protein concentration of the fraction (which was not estimated) was too low,and that insufficient immunoglobulin was present for the F.A. technique.Some loss of specific immunoglobulin was expected,but

no enhancement in fluorescent staining was observed using the fraction undiluted rather than at a 1 in 20 dilution.Time restrictions did not allow the testing of further fractionations of rabbit anti-M.ovipneumoniae antiserum,but it is suggested that reduction of non-specific fluorescence by this method is worthy of further investigation.

Pospisil et al (1970) demonstrated mycoplasma in lung tissue using acetone-fixed paraffin-wax sections,but describes no interference by non-specific fluorescence,contrary to the findings of this study,and others described above.Thus acetone-fixation of sections may be worth investigation.

b) Immunoperoxidase:

The immunoperoxidase techniques used by Bruggman et al (1977) and Hill (1978),on pig and rat lung respectively,clearly demonstrated the presence of mycoplasma in the bronchi of these animals.Bruggman (1977) employed "frozen tissue sections" while Hill (1978) used the"Sainte-Marie method" of paraffin embedding tissue.In both studies,hydrogen peroxide in methanol was used to destroy endogenous peroxidase activity.In this study,hydrogen peroxide in methanol was effective,but less so than sodium borohydride in the reduction of endogenous peroxidase activity.

Bruggman et al (1977) and Hill (1978) used indirect and direct immunoperoxidase methods respectively,and neither author describes problems of non-specific staining as encountered in this study.Burns (1975) however,describes non-specific background staining following highly sensitive immunoperoxidase methods,and he experimented with block titration,filtering of sera and buffers,and variable time of exposure of sections to sera and buffer washings as methods of reducing this.Burns (1975) found

that pretreatment of sections with normal non-immune,non-conjugated serum and the use of optimally diluted first antiserum were the best methods for reducing non-specific staining.He stated that doubling dilutions of the first antiserum resulted in a significant reduction in non-specific background fluorescence before any reduction in specific staining was observed.

The "indirect" method employed in this study resulted in retention of a moderate degree of non-specific staining following SPF sheep serum pretreatment,but the "sandwich"technique produced very little background staining.

Distribution of specific stain.

The localisation of specific stain in the bronchi/bronchioles of pneumonic lung sections treated with rabbit anti-M.ovipneumoniae antiserum was the same by both techniques (immunofluorescence and "sandwich"immunoperoxidase),and agreed with the findings of other authors.

Specific staining (whether bright yellow-green fluorescence or brown stain) was seen as a layer on the surface of bronchial and bronchiolar epithelium.This layer appeared mostly homogenous,but occasionally particulate when fluorescent staining was used,but more often particulate in peroxidase stained preparations.The specific fluorescence appeared to be dissociated from the columnar cell surface,and the immunoperoxidase stain was seen to be amongst the tips of cilia.Positive staining was not recorded in non-ciliated bronchi/bronchioles.These results would seem to agree with electron microscopical studies which have previously demonstrated M.ovipneumoniae in close association with cilia (Foggie et al,1976). In the same pneumonic lung tissue,bronchi and bronchioles which lacked cilia showed no evidence of positive staining,as did

non-ciliated terminal bronchioles.The association of M. ovipneumoniae with cilia may represent specific localisation or merely physical entrapment.In either case,the presence of the organism could be contributory to the observed loss of cilia.An examination,by immunomorphological and electron microscopical methods,of lungs of infected animals killed at intervals after infection might reveal progressive destruction of cilia when mycoplasma organisms are present.

Bright fluorescent particles both discrete and in clumps were occasionally observed in the bronchial lumen and alveolar spaces in this study.This has previously been described by Organick & Lutsky (1968) and L'Ecuyer & Boulanger (1970) who suggested that this was positive staining of mycoplasmas.However,Organick & Lutsky (1968) state that they were unable to confirm the presence of mycoplasma and that this feature of the staining was not consistent.Meyling (1971) described the presence of specific fluorescence in and amongst the cells of the bronchial exudate,and sometimes in alveolar tissue,and took this as indicative of the presence of M.hyopneumoniae.

In this study,bright particulate intracellular fluorescence was seen in a proportion of alveolar macrophages.In peroxidase stained sections a proportion of such cells had diffuse brown stain together with darkly stained particles in the cytoplasm.Although this staining occurred in sections of both pneumonic and normal lungs, and also with both rabbit anti-M.ovipneumoniae and N.R.S.,it appeared to be more abundant when the specific antiserum was used.There may thus be some specific staining which is being largely obscured by the non-specific uptake of serum or other components during the staining procedures.Structures less dense but of similar size to mycoplasmas have been demonstrated in alveoli by electron microscopy. These were seen both free and in the process of phagocytosis by

alveolar macrophages,but no positive identification was possible(Foggie et al,1976).Reduction of non-specific leucocyte fluorescence has been achieved by liver powder adsorption (Potgieter & Ross,1972) and this technique may help to answer the question of whether the positive stain seen in intra-alveolar cells is specific or non-specific for the presence of M.ovipneumoniae antigen.

In the fluorescent antibody technique occasional alveolar macrophages appeared to have taken up stain on their surface.A thin layer of bright yellow-green fluorescence could be seen around these cells,and occasionally particulate fluorescence was recorded on the cell surface.Mycoplasmas have been shown,by electron microscopy, to adhere to,and be phagocytosed by,neutrophils and eosinophils; mycoplasmas also adhere to mononuclear leucocytes and to a small percentage of lymphocytes.(Zucker-Franklin,Davidson & Thomas,1966a &b). Thus the fluorescent staining observed may have been specific fluorescence of mycoplasma antigen bound to the cell surface. Further reduction of non-specific background fluorescence may make the identification of positively stained cells easier and their significance clearer.

In the SPF sheep lungs examined,M.ovipneumoniae was identified by the immunoperoxidase "sandwich" technique in most but not all lungs from which M.ovipneumoniae was isolated in culture.Positive identification was made in a higher proportion of lungs bearing gross lesions of atypical pneumonia than in those appearing normal.This may be a reflection of the recovery titres of M.ovipneumoniae, which were significantly higher from lesion of pneumonia.Meyling (1971) reported a discrepancy between the number of cases positive for M.hyorhinis by cultivation and F.A.T. and suggested that the mycoplasmas might already be coated with antibody and thus inaccessible

to the antiserum used in the staining technique.Local antibody may also be responsible for weak or negative staining results in chronic cases of mycoplasma infection (Meyling,1971),and this might explain the lack of positive staining of M.ovipneumoniae in lungs from the"abattoir sheep".Although no titrations were made of M.ovipneumoniae recovered from these lungs,the lack of gross or microscopical lesions would indicate that only low levels of infection existed in these sheep.

During disease,host cellular components could adsorb onto mycoplasma,forming a coat which could protect the pathogen from host defences (Barile,1979).Such a protein coat might also interfere with the binding of specific antiserum used in the F.A.T. and immunoperoxidase staining of mycoplasma.

The immunofluorescent demonstration of IgG and IgA.

The F.A.T. revealed that when compared to normal lungs,pneumonic lungs had increased amounts of both types of immunoglobulin (IgA and IgG) and increased numbers of Ig-containing cells,some of which may be responsible for their production.IgG-producing cells were demonstrated mainly in the peribronchial and perivascular lymphocytic cuffs.A few IgA-producing cells were also seen in the peribronchial and peirvascular cuffs,but were principally found in the interstitium of the alveolar septa.Positive IgG and IgA staining of intra-alveolar exudate was seen,and some alveolar macrophages appeared to be coated with these stains.The fluorescence around alveolar macrophages may represent a coating of mycoplasma-specific opsonising antibodies. However,this may also be due to positively stained exudate concentrating around these cells.

Thus the sites of production and action of the immunoglobulins was demonstrated,and a relative increase of both IgG and IgA

in pneumonic lungs was shown,but the relative proportions of IgG- and IgA-producing cells in pneumonic and normal lung tissue was not estimated in this study.Further sections of both pneumonic and normal lungs should be examined to estimate the relative importance of each immunoglobulin in the host response to M.ovipneumoniae infection.However,it should be noted that this IgG and IgA demonstration was not specific for M.ovipneumoniae.

In conclusion,M.ovipneumoniae has been demonstrated in pneumonic lung tissue of sheep by both immunofluorescence and immunoperoxidase staining techniques.M.ovipneumoniae was shown to be associated with ciliated bronchial and bronchiolar epithelium. It was possibly demonstrated in alveoli,both free and associated with macrophages,but further studies are required to provide evidence of specific identification.An increase of immunoglobulins and the cells responsible for their production was also demonstrated, but it would be interesting to examine more lungs to determine the relative importance of IgG and IgA production,and their relative importance in resistance to infection.

This demonstration of M.ovipneumoniae in pneumonic ovine lung tissue and its distribution suggest that the organism is not highly invasive,but is confined to the epithelial surface.This agrees with Fernald(1969) who stated that "the changes observed are not part of an invasive or destructive process,but rather the marshalling of a local immune defence system in response to the superficial infection within the bronchial tree".

REFERENCES

AL-AUBAIDI,J.M., DARDIRI,A.H., MUSCOPLATT,C.C. & MCCAULEY,E.H.(1973). Identification and characterisation of Acholeplasma oculusi spec.nov from the eyes of goats with keratoconjunctivitis. Cornell Veterinarian,63, 117-129.

AL-AUBAIDI,J.M., TAYLOR,W.D., BUBASH,G.R. & DARDIRI,A.H.(1972). Identification and characterisation of Mycoplasma arginini from Bighorn sheep (Ovis canadensis) and goats. American Journal of Veterinary Research,33,87-90.

AL-SULTAN,I.I., ZUBAIDY,A.J. (1978).Chronic ovine pneumonia associated with mycoplasma infection.Veterinary Pathology,15,682.

ALI,M.S.MOHAMED (1977).Isolation and identification of Mycoplasma ovipneumoniae from Sudanese goats. Bulletin of Animal Health and Production in Africa,25,91-95.

ALLAN,E.M. & PIRIE,H.M. (1977).Electronmicroscopical observations on mycoplasmas in pneumonic calves. Journal of Medical Microbiology,10,469-472.

ALLEY,M.R. & CLARKE,J.K. (1977).The influence of microorganisms on the severity of lesions in chronic ovine pneumonia. New Zealand Veterinary Journal,25,200-202.

ALLEY,M.R. & CLARKE,J.K. (1979).The experimental transmission of ovine chronic non-progressive pneumonia. New Zealand Veterinary Journal,27,217-220.

ALLEY,M.R. & CLARKE,J.K. (1980).The effect of chemotherapeutic agents on the transmission of ovine chronic non-progressive pneumonia. New Zealand Veterinary Journal,28,77-80.

ALLEY,M.R.,QUINLAN,J.R. & CLARKE,J.K. (1975).The prevalence of Mycoplasma ovipneumoniae and Mycoplasma arginini in the respiratory tract of sheep. New Zealand Veterinary Journal,23,137-141.

ALLEY,M.R., WELLS,P.W., SMITH,W.D. & GARDINER,A.C. (1980).The distribution of immunoglobulins in the respiratory tract of sheep. Veterinary Pathology,17,372-380.

ALUOTTA,B.B., WITTLER,R.G., WILLIAMS,C.O. & FABER,J.E. (1970). Standardized bacteriological techniques for the characterisation of Mycoplasma species. International Journal of Systematic Bacteriology, 20,35-58.

AVRAMEAS,S. (1969). Coupling of enzymes to proteins with gluteraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry,6,43-52.

BARILE,M.F. (1979). Mycoplasma-tissue cell interactions.In:The Mycoplasmas II Human and Animal Mycoplasmas,pp425-464. Ed. Tully,J.G. &Whitcomb,R.F. Academic Press.

BARILE,M.F., DEL GIUDICE,R.A., CARSKI,T.R., GIBBS,C.J. & MORRIS,J.A. (1968). Isolation and characterisation of Mycoplasma arginini spec.nov. Proceedings of the Society for Experimental Biology and Medicine, 129,489-494.

BASKERVILLE,A. (1972). Development of the early lesions in experimental enzootic pneumonia of pigs:an ultrastructural and histological study. Research in Veterinary Science,13,570-578.

BASKERVILLE,A. & WRIGHT,C.L. (1973). Ultrastructural changes in experimental enzootic pneumonia of pigs. Research in Veterinary Science,14,155-160.

BASS,E.J. & JASPER,D.E. (1972). Agar block technique for identification of mycoplasmas by use of fluorescent antibody. Applied Microbiology,23,1097-1100.

BOATMAN,E.S. (1979).Morphology and ultrastructure of the Mycoplasmatales. In:The Mycoplasmas I Cell Biology,pp63-99. Ed. Barile,M.F. & Razin,S. Academic Press.

BOUGHTON,E. & THORNS,C.J. (1978). An introduction to mycoplasmas. Laboratory Practice,27,1052-1055.

BURNS,J. (1975). Background staining and sensitivity of the unlabelled antibody-enzyme (P.A.P.) method.Comparison with the peroxidase labelled antibody sandwich method using formalin fixed paraffin embedded material. Histochemistry,43,291-294.

BRUGGMAN,S., ENGBERG,B. & EHRENSPERGER,F. (1977). Demonstration of M.suipneumoniae in pig lungs by enzyme-linked immunoperoxidase technique. Veterinary Record, 101,137.

CARMICHAEL,L.E., ST.GEORGE,T.D., SULLIVAN,N.D. & HORSFALL,N. (1972). Isolation,propagation and characterisation studies of an ovine mycoplasma responsible for proliferative interstitial pneumonia. Cornell Veterinarian, 62,654-679.

CHANOCK,R.M., HAYFLICK,L. & BARILE,M.F. (1962). Growth on abnormal medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proceedings of the National Academy of Sciences,U.S.A. 48,41-48.

CLARKE,J.K., BROWN,V.G. & ALLEY,M.R. (1974). Isolation and identification of mycoplasmas from the respiratory tract of sheep in New Zealand. New Zealand Veterinary Journal,22,117-121.

COLLIER,A.M. (1972).Pathogenisis of Mycoplasma pneumoniae infection as studied in the human foetal trachea in organ culture. In: Pathogenic Mycoplasmas,pp307-320. A Ciba Foundation Symposium,Associated Scientific Publishers,Amsterdam.

COONS,A.H., CREECH,H.J., JONES,R.N. & BERLINER,E. (1942). The demonstration of pneumococcal antigens in tissues by the use of fluorescent antibody. Journal of Immunology, 45,157-170.

COTTEW,G.S. (1971). Characterisation of mycoplasmas isolated from sheep with pneumonia. Australian Veterinary Journal,47,591-596.

COTTEW,G.S. (1974). The mycoplasmas of sheep and goats. INSERM 33, 357-362.

COTTEW,G.S. (1979). Caprine-ovine mycoplasmas. In: The Mycoplasmas II Human and Animal Mycoplasmas,pp103-130. Ed. Tully,J.G. & Whitcomb,R.F. Academic Press.

COTTEW,G.S. & LEACH,R.H. (1969). Mycoplasmas of cattle,sheep and goats. In:Mycoplasmatales and the L-phase of bacteria,pp527-570. Edited by Leonard Hayflick, Appleton-Century-Crofts,New York.

COTTEW,G.S. & LLOYD,L.C. (1965). An outbreak of pleurisy and pneumonia in goats in Australia attributed to a Mycoplasma species. Journal of Comparative Pathology,75,363-374.

EDWARD,D.G.ff & FREUNDT,E.A. (1956). The classification and nomenclature of organisms of the pleuropneumonia group. Journal of General Microbiology,14,197-207.

EDWARD,D.G.ff & FREUNDT,E.A. (1967). Proposal for Mollicutes as a name of the class established for the order Mycoplasmatales. International Journal of Systematic Bacteriology,17,267-268.

FERNALD,G.W. (1969).Immunologic aspects of experimental Mycoplasma pneumoniae infection. Infectious Diseases,119, 255-266.

FERNALD,G.W., COLLIER,A.M. & CLYDE,W.A.Jr. (1975). Respiratory infections due to Mycoplasma pneumoniae in infants and children. Pediatrics,55,327-335.

FEY,H. (1972). Eriochrome Black,a means of reduction of non-specificity in immunofluorescence. Pathologia et Microbiologia, 38,271-277.

FOGGIE,A. & ANGUS,K.W. (1972). observations on the distribution of Mycoplasma arginini as a respiratory tract infection in sheep,and its pathogenicity for specific pathogen free lambs. Veterinary Record, 90,312-313.

FOGGIE,A., JONES,G.E. & BUXTON,D. (1976). The experimental infection of specific-pathogen-free lambs with Mycoplasma ovipneumoniae. Research in Veterinary Science, 21,28-35.

FREUNDT,E.A. (1955). The classification of the pleuropneumonia group of organisms (Borrelomycetales). International Bulletin of Bacteriological Nomenclature and Taxonomy,5,67-78.

FREUNDT,E.A. (1974). The Mycoplasmatales. In:Bergey's Manual of Determinative Bacteriology,8th Edition,pp929-955. Eds. Buchanan,R.F. & Gibbons,N.E.,The Williams & Wilkin Co.,Baltimore.

FREUNDT,E.A. & EDWARD,D.G.ff (1979). Classification and taxonomy. In:The Mycoplasmas I Cell Biology,pp1-37. Ed. Barlie,M.F. & Razin,S.,Academic Press.

FRIIS,N.F., PALSSON,P.A. & PETURSSON,G. (1976). Mycoplasma ovipneumoniae demonstrated in Icelandic sheep. Acta Veterinaria Scandinavica,17,255-257.

FURLONG,S.L. & COTTEW,G.S. (1973). Identity of mycoplasmas from sheep pneumonias in Queensland and Victoria. Australian Veterinary Journal,49,215-216.

GILMOUR,J.S., JONES,G.E. & RAE,A.G. (1979). Experimental studies of chronic pneumonia in sheep. Comparative Immunology,Microbiology & Infectious Diseases, 1,285-293.

GILMOUR,N.J.L. & BROTHERSTON,J.G. (1963). Clinical and pathological findings in an outbreak of pneumonia in sheep. Journal of Comparative Pathology and Therapeutics, 73,329-335.

GODING,J.W. (1976).Rabbit IgG fraction prepared using Protein A sepharose. Journal of Immunological Methods,13, 215-226.

GOODWIN,R.F.W., POMEROY,A.P. & WHITTLESTONE,P. (1975). Production of enzootic pneumonia in pigs with a mycoplasma. Veterinary Record,77,1247-1249.

GOURLAY,R.N. (1969). Isolation of a mycoplasma-like organism from calf lungs.(letter). Veterinary Record,84,229.

GOURLAY,R.N., HOWARD,C.J., THOMAS,L.H. & STOTT,E.J. (1976). Experimentally produced calf pneumonia. Research in Veterinary Science,20,167-173.

GOURLAY,R.N., HOWARD,C.J., THOMAS,L.H. & WYLD,S.G. (1979). Pathogenicity of some mycoplasma and acholeplasma species in the lungs of gnotobiotic calves. Research in Veterinary Science,27,233-237.

GOURLAY,R.N. & LEACH,R.H. (1970). A new mycoplasma species isolated from pneumonic lungs of calves (Mycoplasma dispar sp.nov.). Journal of Medical Microbiology,3,111-123.

GOURLAY,R.N. & THOMAS,L.H. (1969). Experimental pneumonia in calves produced by inoculation of mycoplasmas. (letter). Veterinary Record,85,583.

GREIG,A.S. (1955). The isolation of pleuropneumonia-like organisms from the respiratory tracts of sheep. Canadian Journal of Comparative Medicine,19,265-271.

HILL,A.C. (1978). Demonstration of mycoplasmas in tissue by the immunoperoxidase technique. Journal of Infectious Diseases,137,152-154.

HOWARD,C.J. & GOURLAY,R.N. (1978). Mycoplasmas of animals. Scientific Progress,65,313-329.

HOWARD,C.J. GOURLAY,R.N., THOMAS,L.H. & STOTT,E.J. (1976). Induction of pneumonia in gnotobiotic calves following inoculation of Mycoplasma dispar and ureaplasmas (T-mycoplasmas). Research in Veterinary Science,21,227-231.

HUANG,S., MINASSIAN,H. & MORE,J.D.(1976). Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. Laboratory Investigation,35,383-390.

JOBST, & HORVATH, (1961). Contributory data on the reduction of oxo-groups in lipids. Journal of Histochemistry & Cytochemistry,9,711.

JONES,G.E. (1978). Studies on mycoplasmas of the respiratory tract of sheep. Ph.D. Thesis,University of Edinburgh.

JONES,G.E., BUXTON,D. & HARKER,D.B. (1979). Respiratory infections in housed sheep with particular reference to mycoplasmas. Veterinary Microbiology,4,47-59.

JONES,G.E., FOGGIE,A., MOULD,D.L. & LIVITT,S. (1976). The comparison and characterisation of glycolytic mycoplasmas isolated from the respiratory tract of sheep. Journal of Medical Microbiology,9,39-52.

JONES,G.E., FOGGIE,A., SUTHERLAND,A. & HARKER,D.B. (1976). Mycoplasmas and ovine keratoconjunctivitis. Veterinary Record,99,137-141.

JONES,G.E., GILMOUR,J.S. & RAE,A. (1978). Endobronchial inoculation of sheep with pneumonic lung-tissue suspensions, and with the bacteria and mycoplasmas isolated from them. Journal of Comparative Pathology,88, 85-96.

KOHN,D.F. (1971). Bronchiectasis in rats infected with Mycoplasma pulmonis:an electron microscopy study. Laboratory Animal Science,21,856.

KUMAR,A. & PATHAK,R.C. (1978). Isolation and characterisation of some members of mycoplasma and acholeplasma from uterus of sheep and goats. Indian Journal Experimental Biology,16,1011-1013.

LEACH,R.H. (1970). The occurence of Mycoplasma arginini in several animal hosts. Veterinary Record,87,319-320.

LEACH,R.H., COTTEW,G.S., ANDREWS,B.E. & POWELL,D.G. (1976). Atypical mycoplasma from sheep in Great Britain and Australia identified as Mycoplasma ovipneumoniae. Veterinary Record,98,377-379.

L'ECUYER,C. & BOULANGER,P. (1970). Enzootic pneumonia of pigs: identification of a causative mycoplasma in infected pigs and in cultures by immunofluorescent staining. Canadian Journal of Comparative Medicine,34,38-46.

LIVINGSTON,C.W.Jr. & GAUER,B.B. (1979). Isolation of Mycoplasma ovipneumoniae from Spanish and Angora goats. American Journal of Veterinary Research,40,407-408.

LIVINGSTON,C.W., STAIR,E.L., UNDERDAHL,N.R. & MEBUS,C.A. (1972). Pathogenisis of mycoplasmal pneumonia in swine. American Journal of Veterinary Research,33,2249-2258.

MACKAY,J.M.K., NISBET,D.I. & FOGGIE,A. (1963). Isolation of pleuro-pneumonia-like organisms (genus Mycoplasma) from cases of sheep pulmonary adenomatosis (SPA). Veterinary Record,75,550-551.

MACOWAN,K.J. (1976). A mycoplasma from chronic caprine pleuropneumonia in Kenya. Tropical Animal Health and Production, 8,28-36.

MAHI,M.M.EL & NAYIL,A.A. (1978). I.Isolation of mycoplasmas from pneumonic sheep lungs in the Sudan. II.The serological reactions of mycoplasma isolated from pneumonic sheep lungs in the Sudan. Research in Veterinary Science,24,314-321.

MAJOR,R.M., CLARKE,J.K. & ALLEY,M.R. (1979). An in vitro study of the replication of Mycoplasma ovipneumoniae.
*Erratum page 63. New Zealand Veterinary Journal,27,58-61.

MARE,C.J. & SWITZER,W.P. (1965). New species - Mycoplasma hyopneumoniae, a causative agent of virus pig pneumonia. Veterinary Medicine,60,841-846.

MEBUS,C.A. & UNDERDAHL,N.R. (1977). Scanning electron microscopy of trachea and bronchi from gnotobiotic pigs inoculated with Mycoplasma hyopneumoniae. American Journal of Veterinary Research,38,1249-1254.

MEYLING,A. (1971). Mycoplasma suipneumoniae and Mycoplasma hyorhinis demonstrated in pneumonic pig lung by the fluorescent antibody technique. Acta Veterinaria Scandinavica,12,137-141.

MORTON,H.E. & ROBERTS,R.J. (1967). Production of antimycoplasma (PPLO) antibodies in rabbits. Proceedings of the Society for Experimental Biology and Medicine,
**Erratum page63. 125,538-543.

NAYIL,A.A. (1973). Mycoplasmas isolated from the pneumonic lungs of sheep. Index to Theses accepted for Higher Degrees,21,218.

NICOLET,J., TONTIS,A., GIGER,T., SCHALLIBAUM,M., WÜTHRICH-PRAVIAINEN,P., KRAWINKLER,M., PAROZ,P., BESTETTI,G. & BOSS,P.H. (1979). Mycoplasma ovipneumoniae in enzootic,proliferative interstitial pneumonia in sheep. Schweizer Archiv fur Tierheilkund,121,341-355.Abst.2669.

NOCARD,E. & ROUX,E.R. (1898). Le microbe de la peripneumonie.Ann. Institute Pasteur,Paris,12,240-262.

NOEL,J.K., DEVOLT,H.M. & FABER,J.E. (1964). Identification of Mycoplasma gallisepticum in lesion tissue by immunofluorescence. Poultry Science,43,145-149.

ORGANICK,A.B. & LUTSKY,I.I. (1968). Pneumonia due to mycoplasma in gnotobiotic mice. IV.Localisation and identification of Mycoplasma pulmonis in the bronchi of infected gnotobiotic mice by immunofluorescence and by light microscope. Journal of Bacteriology,96,250-258.

ORGANICK,A.B., SIEGESMUND,K.A. & LUTSKY,I.I. (1966). Pneumonia due to mycoplasma in gnotobiotic mice. II.Localisation of Mycoplasma pulmonis in the lungs of infected gnotobiotic mice by electron microscopy. Journal of Bacteriology,92,1164-1176.

PIRIE,H.M. & ALLAN,E.M. (1975). Mycoplasmas and cuffing pneumonia in a group of calves. Veterinary Record,97, 345-349.

POLAK-VOGELZAND,A.A. & HAGENAARS,R. (1976). Identification of mycoplasmas and acholeplasmas by means of an indirect immunoperoxidase test. Proceedings for the Society for General Microbiology,3,(part4), 151. Society for General Microbiology,Reading, England.

POLAK-VOGELZAND,A.A.,HAGENAARS,R. & NAGEL,J. (1978). Evaluation of an indirect immunoperoxidase test for the identification of acholeplasmas and mycoplasmas. Journal of General Microbiology,106,241-249.

POSPISIL,Z.,GOIS,M., CERNY,M. & MENSIK,J. (1970). Demonstration of Mycoplasma hyorhinis in tissue cultures and pig lungs by means of immunofluorescence. Acta Veterinaria Brno,40,99-104.

POTGIETER,L.N.D. & ROSS,R.F. (1972). Demonstration of Mycoplasma hyorhinis and Mycoplasma hyosynoviae in lesions of experimentally infected swine by immunofluorescence. American Journal of Veterinary Research,33,99-105.

QUALMAN,S.J. & KEREN,D.F. (1979). Immunofluorescence of deparaffinised trypsin-treated renal tissues. Laboratory Investigation,41,483-489.

RADASZKIEWICZ,T., DRAGOSICS,B., ABDELFATTAHGAD,M. & DENK,H. (1979). Effect of protease pretreatment for immunomorphologic demonstration of hepatitis-B surface antigen in conventional paraffin-embedded liver biopsy material: quantitative evaluation. Journal of Immunological Methods,29,27-33.

RAZIN,S. (1978). The mycoplasmas. Microbiological Review,42,414-470. Abst.1202.

ST.GEORGE,T.D. & CARMICHAEL,L.T. (1975). Isolation of Mycoplasma ovipneumoniae from sheep with chronic pneumonia. Veterinary Record,97,205-206.

ST.GEORGE,T.D., HORSFALL,N. & SULLIVAN,N.D. (1973). A subclinical pneumonia of calves associated with Mycoplasma dispar. Australian Veterinary Journal,49,580-586.

ST.GEORGE,T.D., SULLIVAN,N.D., LOVE,J.A. & HORSFALL,N. (1971). Experimental transmission of pneumonia in sheep with a mycoplasma isolated from pneumonic sheep lung. Australian Veterinary Journal,47,282-283.

SAINTE-MARIE,G. (1962). A paraffin embedding technique for studies employing immunofluorescence. Journal of Histochamistry & Cytochemistry,10,250-256.

SCHULLER,W., LEHMKUHL,H.D. & SWITZER,W.P. (1976). A fluorescent antibody test for identification of Mycoplasma hyopneumoniae colonies. American Journal of Veterinary Research,37,477-478.

SMITH,W.D., DAWSON,A.MCL., WELLS,P.W. & BURRELLS,C. (1975). Immunoglobulin concentrations in ovine body fluids. Research in Veterinary Science,19,189-194.

STAMP,J.T. & NISBET,D.I. (1963). Pneumonia of sheep. Journal of Comparative Pathology,73,319-328.

STEVENSON,R.G. (1969). Respiratory diseases of sheep. Veterinary Bulletin,39,747-759.

STIPKOVITS,L., BELAK,S., PALFI,V. & TURY,E. (1975). Isolation of Mycoplasma ovipneumoniae from sheep with pneumonia. Acta Veterinaria Academiae Scientiarum Hungaricae, 25,267-273.

STREEFKERK,J.G. (1972). Inhibition of erythrocyte pseudoperoxidase activity by treatment hydrogen peroxide following methanol. Journal of Histochemistry and Cytochemistry,20,829-831.

SULLIVAN,N.D., ST.GEORGE,T.D. & HORSFALL,N. (1973). A proliferative interstitial pneumonia of sheep associated with mycoplasma infection.
a.Natural history of the disease in a flock.
b.The experimental exposure of young lambs to infection.
Australian Veterinary Journal,49,57-62,63-68.

SWOVELAND,P.T. & JOHNSON,K.P. (1979). Enhancement of fluorescent antibody staining of viral antigens in formalin-fixed tissues by trypsin digestion. Journal of Infectious Diseases,140,758-764.

THOMAS,L.H. & HOWARD,C.J. (1974). Effect of Mycoplasma dispar, Mycoplasma bovirhinis,Acholeplasma laidlawii and T-mycoplasmas on explant cultures of bovine trachea. Journal of Comparative Pathology,84, 193-201.

THOMAS,L.H. & SMITH,G.S. (1972). Distribution of mycoplasmas in the non-pneumonic bovine respiratory tract. Journal of Comparative Pathology,82,1-4.

TULLY,J.G., BARILE,M.F., EDWARD,D.G.ff,THEODORE,T.S. & ERNØ,H. (1974). Characterisation of some caprine mycoplasmas, with proposals for new species,Mycoplasma capricolum and Mycoplasma putrefaciens. Journal of General Microbiology,85,102-120.

UPPAL,P.K. & CHU,H.P. (1977). Attachment of Mycoplasma gallisepticum to the tracheal epithelium of fowls. Research in Veterinary Science,22,259-260.

WHITTLESTONE,P. (1972). Pathogenicity of mycoplasmas in animals. In:Microbial Pathogenicity in Man and Animals. pp217-250. Eds.H.Smith & J.H.Pearce,Cambridge University Press.

WHITTLESTONE,P. (1973). Enzootic pneumonia of pigs (EPP). Advances in Veterinary Science and Comparative Medicine, 17,1-55.

ZUCKER-FRANKLIN,D., DAVIDSON,M. & THOMAS,L. (1966). The interaction of mycoplasmas with mammalian cells.
a.HeLa cells,neutrophils and eosinophils.
b.Monocytes and lymphocytes.
Journal of Experimental Medicine,124,521-532, 533-542.

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MANILOFF,J. & MOROWITZ,H.J. (1972). Cell biology of mycoplasmas. Bacteriological reviews,36,263-290

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