AN *IN VITRO* STUDY OF THE REPLICATION, MORPHOLOGY AND DNA BASE COMPOSITION OF *MYCOPLASMA OVIPNEUMONIAE*

A thesis presented in partial fulfilment of the
requirements for the degree of
Master of Science in Microbiology at
Massey University, New Zealand.

Robert Malcolm Major

1977

ABSTRACT

Mycoplasma ovipneumoniae can almost invariably be isolated from the lungs of sheep with chronic pneumonia, which is a prevalent disease in New Zealand hoggets. At Massey University, a study is in progress to establish the part, if any, played by *M. ovipneumoniae* in the pathogenesis of the disease. This thesis represents an *in vitro* investigation of some properties of *M. ovipneumoniae*. It was undertaken as part of the larger study, and is presented in that context.

To establish a method for the production of high titre exponential phase inocula for use in disease transmission experiments, the growth of *M. ovipneumoniae* in FM4 broth was studied. It was found that a maximum titre of 1.0 to 3.0 x 10^9 CFU/ml was produced regardless of the inoculum size or degree of aeration. The organism had a minimum division time of 1.7 hr; had no stationary phase and in the late death phase was inactivated with a half-life of about 0.5 hr.

The organism was stored at -70° with little loss in titre (less than two-fold) over an 18 month period.

Shaking cultures became sufficiently turbid during growth to allow meaningful measurements to be made using an SP20 spectrophotometer. In defined conditions, viz. when a shaking culture is in the exponential phase and contains 2.0 to 10.0 x 10^8 CFU/ml, the viable cell count can be estimated from turbidity measurements.

Electron microscopy of *M. ovipneumoniae* showed that the cells are roughly spherical, 400 to 700nm in diameter, probably replicate by binary fission, contain ribosomes and fibrils of deoxyribonucleic acid, and are bounded by a trilaminar membrane bearing projections 12nm long. No specialized structural feature such as the attachment sites found in *M. pneumoniae* was detected.

The New Zealand isolate of *M. ovipneumoniae* was morphologically indistinguishable from the standard *M. ovipneumoniae* strain isolated in Australia.

Although the above description could be applied to many mycoplasma species, it should be noted that the

average cell diameter of M. ovipneumoniae (about 550nm) is larger than that found for most but not all species of mycoplasma.

The base composition of the DNA of M. ovipneumoniae determined by the thermal denaturation and buoyant density studies was 28.1% GC and 28.0% GC respectively. This relatively low GC content falls within the accepted range for mycoplasma species (23 - 40% GC) and within the much narrower range (26.8 - 28.5% GC) of glycolytic mycoplasmas causing respiratory disease in domestic animals.

ACKNOWLEDGMENTS

Firstly I would like to thank the Department of Microbiology and Genetics, Massey University for providing the opportunity and facilities for these studies, and the following members of the Department : Professor D.F. Bacon, Dr E. Terzaghi, Dr B.D.W. Jarvis, Mr P.L. Carter and Mr R. Cursons.

Other people who have been of particular assistance to me are:

Dr J. Lewis who provided technical advice in the ultracentrifugation studies.

Mr A.F. Green, without whom the electron microscopy would have taken a lot longer.

The Electron Microscopy Unit of the D.S.I.R.

Mrs P. van Doorn for first draft typing.

Mrs R. Storey for her generous assistance in typing the manuscript.

And last, but certainly not least, my Supervisor, Dr J.K. Clarke for his guidance and assistance.

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INTRODUCTION

Sheep are obviously an important domestic animal species, especially in New Zealand, and it is generally recognized that chronic pneumonia is one of the most prevalent diseases of sheep in this country. It is surprising, therefore, that the disease has received little attention until recently, and because it has not yet been studied intensively, the cause or causes of the disease have not so far been unequivocally established.

Although it could be argued that any investigation of the disease should avoid preconceived ideas, in practice it was found necessary, in this laboratory, to limit initial investigations to an area which appeared to be the one most likely to give positive results. For this reason, clues to the etiology of the disease were looked for by examining what was established for similar diseases in both domestic and laboratory animals.

Analogous diseases (reviewed in a later section) in animals, such as goats, cattle, swine and rodents have been shown to have, at least in part a mycoplasmal etiology. For this reason an investigation was undertaken (Clarke *et al.*, 1974) to isolate and identify mycoplasmas from the respiratory tract of normal and pneumonic sheep in New Zealand. Subsequently, two species of mycoplasma were isolated, one of which required arginine for growth and was identified as *M. arginini*. The other was a glycolytic mycoplasma which was found, by gel precipitin tests, to be related to an organism isolated three years earlier in Australia (St. George *et al.*, 1971) and later designated *Mycoplasma ovipneumoniae*. (Carmichael *et al.*, 1972).

The Australian workers (St. George *et al.*, 1971) claimed to have transmitted chronic pneumonia experimentally to sheep using broth cultures of *M. ovipneumoniae*, but attempts to repeat this in New Zealand (Alley & Clarke, personal communication) have either failed, or caused a disease not necessarily identical to the natural disease, in a low proportion of inoculated animals. However, the disease could be transmitted (Alley,

personal communication) experimentally by inoculating sheep intranasally with an aerosol of diseased lung homogenate.

There are several possible reasons for the failure to transmit chronic pneumonia by inoculating an *in vitro* culture of *M. ovipneumoniae* intranasally into lambs, e.g. the immune status of the lamb, or the size of droplet. However, since whole lung homogenate was successfully used to transmit the disease under similar conditions and using similar animals, it seems likely that other reasons must be sought, e.g. *M. ovipneumoniae* may not be the primary cause of the disease and is only a secondary invader, or it may be a primary invader, but only when in association with another organism.

The question of whether or not *M. ovipneumoniae* is a primary cause of chronic pneumonia in sheep can in principle be decided by intranasal inoculation of sheep or lambs which are known to be susceptible to the disease, but a technical difficulty arises when preparing the inoculum, which ideally should contain a high titre of actively growing organisms. This difficulty is due to the fact that mycoplasmas have little or no stationary phase, and die rapidly after the maximum titre has been reached. In practice, growth of mycoplasmas is indicated by a pH change of the medium, which, depending on the circumstances, may only be visible at, or even after the maximum titre has been reached. Consequently the preparation of a high titre inoculum of actively growing mycoplasmas requires a detailed study of the growth of the organisms. One section of the present study examines the growth and subsequent death of *M. ovipneumoniae*.

Further clues to the significance of *M. ovipneumoniae* with respect to chronic pneumonia in sheep could be sought by electron microscopic examination of infected sheep. However, a useful preliminary for *in vivo* electron microscopy is a detailed examination of the morphology of the organism which is best achieved using *M. ovipneumoniae* cultured *in vitro*.

Bearing in mind that mycoplasmas die rapidly following growth, it is surprising that many morphological studies of these organisms conducted using *in vitro* cultures have used mycoplasmas grown for an arbitrary period not determined from growth curve data (Anderson *et al.*,1965; Nelson *et al.*, 1965; Domermuth *et al.*, 1964 (i); Domermuth *et al.*, 1964 (ii)). However, this approach is likely to give a mixture of some living organisms among an excess of dead ones. For this reason, the present electron microscopic study was preceded by growth studies so that the phase of growth or death of the culture was known. The morphology of both New Zealand and Australian strains of *M. ovipneumoniae* are investigated in this thesis.

During the course of the present work, it became apparent that several basic properties of *M. ovipneumoniae* have not been previously determined. Among these properties was the base composition of the organism, a character of taxonomic importance. Thus, melting temperature and buoyant density studies of DNA isolated from *M. ovipneumoniae* were undertaken so as to determine its GC/AT ratio.

The following historical review examines previous investigations of mycoplasmas and mycoplasmal infections with particular reference to organisms found in the respiratory tract.

Introductions to each section of the experimental work are included at the beginning of the appropriate section.

b) HISTORICAL OVERVIEW:

a) Mycoplasmas : General Aspects

Contagious bovine pleuropneumonia was first recognized as a specific disease in the early eighteenth century. Although not highly lethal, it causes a significant mortality rate and serious economic losses. A century after its initial recognition, it was demonstrated that subcutaneous inoculation of a drop of the fluid present in the interlobular connective tissue of infected lungs produced an infection which spread through the subcutaneous tissue and contiguous areas to the lung and pleura. (see Sharp, 1970). Some animals died, but others recovered and were subsequently immune to further inoculation and natural infection.

Early attempts to isolate the causative agent failed until Nocard and Roux (1898) propagated the organism by inoculating bouillon broth with fluid from infected lung. The inoculated broth was placed in a collodion sac and inserted into the peritoneal cavity of a rabbit. When the sac was removed several days later, the inoculated broth was slightly cloudy, and the opalescence could be transmitted serially by subculturing to fresh broth in collodion sacs. After serial passage the opalescence retained its ability to produce the typical lesions of pleuropneumonia in cattle. Control broths remained clear. The same workers, in the course of their investigations, propagated the organism *in vitro* in serum enriched peptone broth, and subsequently (Dujardin-Beaumetz, 1900) it was cultured on solid media, leading to the first description of the classical fried-egg appearance of colonies which is now known to represent the colonial morphology characteristic of many mycoplasmas.

The organism of pleuropneumonia, (ultimately designated *Mycoplasma mycoides var. mycoides*,) although isolated on cell-free medium was still referred to as a "virus" and it occupied a unique position in taxonomy for 25 years, until *M. agalactiae* was cultured in serum broth inoculated with the joint fluid, milk and draining lymph nodes of sheep with contagious agalactia (Bridrē and

Donatien, 1923, 1925). Subsequently, several organisms with the cellular and colonial morphology of the bovine pleuropneumonia organism were isolated from a variety of sources and the lack of an acceptable classification scheme led to their being called "pleuropneumonia-like organisms" or PPLO.

Laidlaw and Elford (1936) recovered from sewage the first "mycoplasma" isolated from other than an animal source. This organism was also the first "mycoplasma" isolated which did not require serum which is a source of sterols for growth. Although it was named M. laidlawii at the time, it is now called Acholeplasma laidlawii, because, by the current definition, mycoplasmas, unlike acholeplasmas require sterols.

In 1956, a previously unrecognized group of mycoplasmas was isolated from the genitournary tract of men with non-gonococcal urethritis (Shepard, 1956). These organisms, designated T-strains differ from other mycoplasmas in that they require urea for growth, and have an optimum of pH 6.0 (Smith, 1971). They produce tiny (T) colonies on conventional mycoplasma media, are microaerophilic and do not ferment carbohydrates.

Mycoplasmas are now divided into three main groups, with respect to growth requirements:

(i) Those which ferment carbohydrates (Fermentative mycoplasmas). Most pathogenic species, are included in this group.

(ii) Those which derive their energy from the conversion of arginine to citrulline and ornithine via the arginine dihydrolase pathway. (Non-fermentative mycoplasmas) e.g. M. arginini.

(iii) Mycoplasmas requiring urea for growth. Although at present called T-strains, they may soon be classified as the genus Ureaplasma within the Mycoplasmatales.

b) Pathogenic Mycoplasmas in General

Mycoplasmas have been shown to cause, or are suspected of causing a wide variety of diseases in various organs of many animals, including man. These pathogenic mycoplasmas display a high degree of specificity of both the species parasitized and the organ or tissue colonised or invaded. They may affect the central nervous system, joints, pleura, peritoneum, upper and lower respiratory tract and genitourinary system. A list of pathogenic mycoplasmas and the diseases they cause is presented in Table I.

c) Mycoplasmas Involved in Diseases of the Respiratory Tract.

Although, as can be seen from Table I, mycoplasmas can, and do infect many sites in the body, the most prevalent type of infection caused by mycoplasmas are those of the upper and lower respiratory tract (Smith, 1971).

Before discussion various aspects of mycoplasmal infections of the respiratory tract, it is relevant to note that different designations have been used: thus mycoplasmas can be responsible for conditions variously referred to as "viral" pneumonia; atypical pneumonia; enzootic pneumonia; chronic respiratory disease and chronic pneumonia.

Clearly "viral" pneumonia should not be used to describe mycoplasmal infections. The terms "atypical" or "chronic" are used in practice to distinguish the pneumonia from the "typical acute" form of the disease caused by bacteria. Since in many animals, including sheep, "atypical" pneumonia is much more common than "typical" pneumonia, the term chronic pneumonia gives a more reasonable representation of the disease. Some pathological and epidemiological differences between the acute and chronic forms of lung disease in sheep are dealt with in a subsequent section. The term enzootic pneumonia can be applied to any pneumonia caused by organisms endemic in a population: thus it

TABLE I Diseases caused by mycoplasmas in a variety of hosts.

HOST	DISEASE	MYCOPLASMA SPECIES
Man	Primary atypical pneumonia	M. pneumoniae
	Urogenital tract infections, possibly causing infertility and non gonococcal urethritis)	T-strains
Cattle	Contagious bovine pleuropneumonia	M. mycoides var mycoides
	Mastitis	M.bovigenitalium
	Mastitis and arthritis	M.bovimastitidis
	Mastitis	T-strains
	Possibly chronic pneumonia of calves	M.dispar
Sheep & Goats	Contagious caprine pleuropneumonia	M. mycoides var capri
	Mastitis, contagious agalactia	M. agalactiae
Swine	Enzootic pneumonia	M. suipneumoniae (hyopneumoniae)
	Polyarthritis	M. hyosynoviae
	Arthritis and polyserositis	M. hyorhinis
Rodents	Rolling disease of rats and mice	M. neurolyticum
	Infectious catarrh and possibly pneumonia of mice and rats	M. pulmonis
	Arthritis of rats and mice	M. arthritidis
Fowl	Chronic respiratory disease	M. gallisepticum
	Infectious sinusitis	
	Cerebral polyarteritis of turkeys	
	Infectious synovitis & chronic respiratory disease	M. synoviae
	Air sac disease of turkeys	M. meleagridis
Cats	Conjunctivitis	M. felis

Adapted and extended from Hayflick, L. (ed) 'The Mycoplasmatales and L-phase of Bacteria' Appleton, New York, 1969, & Sharp J.T. (ed) 'The Role of Mycoplasmas and L-forms of Bacteria in Disease' Charles C. Thomas, 1970.

can be either acute or chronic. In practice however, the term is applied mainly to a particular chronic disease in swine caused by M. hyopneumoniae. Chronic respiratory disease can be applied to conditions of the upper and/or lower respiratory tract. Since this thesis is concerned only with infection of the lung, the term chronic pneumonia is used.

It is by no means simple to define the etiological role of a certain mycoplasma species in the production of chronic pneumonia in an animal. Although (as will be shown in a later section) it has been established that M. ovipneumoniae is involved in the chronic pneumonia syndrome in sheep, its exact role is uncertain. To illustrate that the presence of a mycoplasma in a pneumonic lung does not in itself establish a primary etiological role for the organism, four disease complexes are considered below. Any one of them might be an appropriate model system for M. ovipneumoniae infection in sheep, but the significance of the mycoplasma differs in each case. Respiratory diseases in man, swine, domestic fowl and rodents are discussed.

(i) Primary Atypical Pneumonia in Man.

Primary atypical pneumonia was the name given to a syndrome which was recognised as an infectious disease but which could not be associated with pathogenic microorganisms by the laboratory methods available in the late 1930's (Scadding, 1937). Patients with the disease frequently developed cold agglutinins (antibodies which agglutinate human O group erythrocytes at 0-4°, but not at 37°) during the course of the illness. It was believed at that time that this syndrome was caused by a specific virus (see Grayston, Foy and Kenney, 1969). However an agent was ultimately isolated (the "Eaton" agent) which could pass through 180-250nm pore size filters and could be serially passaged in embryonated eggs without discernable effect on the embryo. The passaged agent, when inoculated intranasally into hamsters and and cotton rats produced pneumonia. (Eaton,

Meiklejohn and van Herrick, 1944). This agent was, nevertheless, not accepted as the cause of primary atypical pneumonia in man until Liu, Eaton and Heyl (1959) demonstrated that epidemics of cold agglutinin-positive pneumonia were associated with the Eaton agent, by detecting significant antibody rises to the agent in paired sera. Marmion and Goodburn (1961) then showed that the Eaton agent was susceptible to chlorotetracycline, streptomycin and gold salts, and so could not be a virus but was likely to be a mycoplasma. This was confirmed by Chanock, Hauflick and Barile (1962) who cultivated the agent on cell-free agar medium, and subsequently demonstrated that the colonies were centreless and mulberry-like; rather than the fried-egg type typical of most mycoplasma colonies on solid media. The agent was named Mycoplasma pneumoniae by Chanock et al. (1963).

Koch's postulates relating M. pneumoniae to primary atypical pneumonia in man were fulfilled when, in a series of volunteer studies (Chanock, Steinberg and Purcell, 1970), 32 of 84 individuals free of growth-inhibiting antibody inoculated with cultured organisms developed a febrile respiratory illness, whereas only 3 of 71 volunteers with pre-existing growth-inhibiting antibody became ill. The finding that less than half the inoculated volunteers developed discernable respiratory tract illness should be interpreted in the light of the finding that natural infection with M. pneumoniae produces a spectrum of effects ranging from inapparent infection to bronchitis and clinical pneumonia (Grayston et al., 1969).

M. pneumoniae is one of the few mycoplasmas for which the mechanism of pathogenesis has been established. In cultured hamster trachea and fetal human trachea, M. pneumoniae interferes with normal ciliary activity. The toxicity involves a combination of cell-attachment and cell lysis mediated by the release of peroxide because the parasite has an attachment structure reminiscent of a phage tail, which can come into intimate contact with the host cell, and thus allow mediators of cell injury to pass directly to their target (Clyde, 1973).

From the above, it can be seen that M. pneumoniae has been established as the etiological agent of primary atypical pneumonia. This clear situation however is the exception rather than the rule when linking mycoplasmas with specific diseases.

(ii) Enzootic Pneumonia of Swine.

Enzootic pneumonia of swine is a chronic disease which has been estimated to affect 40 to 50 percent of all swine (Switzer, 1969). The severity and incidence of the disease has been shown to be increased by swine ascarid larvae infections and influenced by stress and environmental factors (Ross, 1973).

The etiological agent was propagated on cell-free media and shown to have the properties of a mycoplasma by Marē and Switzer (1965) who proposed the name M. hyopneumoniae for the organism, and by Goodwin, Pomeroy and Whittlestone (1965) who proposed the name M. suipneumoniae. Both groups of workers continue to use different names for the mycoplasma, and the nomenclature has not been resolved in the eighth edition of 'Bergey's Manual of Determinative Bacteriology', although the organisms have been shown to be strains of the same species (Goodwin, Pomeroy and Whittlestone, 1967).

Both Marē and Switzer (1965) and Goodwin et al. (1965) used pure cultures of the organism to produce pneumonia in SPF pigs on intranasal inoculation, although they had to inoculate the pigs on more than one occasion in the course of the experiment. (Betts, 1971). Since then, however, Hodges, Betts and Jennings (1969) have produced extensive pneumonia, indistinguishable from the natural disease, in gnotobiotic pigs by a single inoculation with a pure culture of M. hyopneumoniae. Clearly then M. hyopneumoniae is a cause of Enzootic Pneumonia in pigs but is it the sole cause?

Pneumonias caused by M. hyopneumoniae are commonly complicated with M. hyorhinis, a mycoplasma which has been isolated from both the upper and lower respiratory tract in swine with pneumonia and the upper respiratory tract of normal swine. (L'Ecuyer, Switzer and Roberts,

1961). Polyserositis and arthritis have been consistently produced experimentally with broth cultures of the organism (Ross, 1973), but there is some debate as to the role of M. hyorhinis in enzootic pneumonia. Many workers have been unable to produce respiratory disease by intranasal inoculation of M. hyorhinis in SPF pigs (Betts, 1969). However, Czechoslovakian workers Gois, Valicek and Sovadina (1971) using gnotobiotic pigs have produced rhinitis and a pneumonia with lesions similar to those of 'natural' enzootic pneumonia. In Britain, a mild transient pneumonia in gnotobiotic pigs has been reported (Poland, 1969) as the result of intranasal inoculation with broth cultures of M. hyorhinis, but the lesions were unlike the extensive pneumonia reported by Gois et al. (1971). When one of the Czechoslovakian strains isolated by Gois et al. was inoculated into gnotobiotic pigs in Britain, extensive pneumonia was produced in three of nine pigs (Poland, 1969). Baskerville (1972) and Baskerville and Wright (1973) produced enzootic pneumonia in pigs with a pneumonic lung homogenate from which only M. hyorhinis could be cultured. However M. hyopneumoniae has particularly fastidious nutritional requirements whilst M. hyorhinis is relatively easy to grow, so the apparent absence of organisms other than M. hyorhinis may have been due to technical difficulties.

It is concluded that M. hyopneumoniae is a cause of enzootic pneumonia in swine but there is some uncertainty as to whether it is the only cause of the disease with M. hyorhinis playing the role of a secondary invader, or alternatively if M. hyorhinis like M. hyopneumoniae can cause the disease on its own.

(iii) Air-Sac Disease in domestic Fowl.

Chronic respiratory disease in chickens is a relatively mild disease with a slow spread, long incubation period and long course. Exudate from the respiratory tract of infected chickens was inoculated into embryonated eggs and the infected yolk was seeded into heart-infusion broth enriched with horse serum by M arkham and Wong (1952). After passaging to remove possible viral

agents, the cultures were inoculated into embryonated eggs, and when the yolk from infected embryos was inoculated into the sinuses of turkeys sinusitis resulted. During propagation on cell-free media it was demonstrated that the organism was a mycoplasma, and was subsequently designated M. gallisepticum (Edward and Kanarek, 1960).

Thus, M. gallisepticum was shown to be the cause of this mild chronic respiratory disease. However, in the early 1950's, outbreaks of a much more severe respiratory disease were reported. This disease became known as "air-sac" disease or complicated chronic respiratory disease. E.coli was shown to be the most frequent complicating organism (Wasserman, 1954) and the disease was first produced experimentally by infecting chickens with combinations of E. coli plus M. gallisepticum, plus either infectious bronchitis virus or Newcastle disease virus (Gross, 1961). Fabricant and Levine (1962) inoculated fowl intranasally with combinations of M. gallisepticum, E. coli and infectious bronchitis virus and produced lesions indistinguishable from that of the natural field disease. In the course of the experiment, it was shown that E. coli does not readily invade the lower respiratory tract unless it has been previously infected with M. gallisepticum. A higher percentage of deaths and lesions produced in experimentally inoculated chickens occurred if, prior to E. coli infection, the birds were inoculated with both M. gallisepticum and infectious bronchitis virus.

Live virus vaccination with either infectious bronchitis or Newcastle disease virus, or natural infection with these viruses activates chronic respiratory disease in domestic hens and accelerates the spread of M. gallisepticum through a flock, especially when the birds are kept in close contact, as is the case in the modern poultry industry (Fabricant, 1969).

The resulting mild infection renders the lower respiratory tract susceptible to invasion by the ubiquitous E. coli and the birds subsequently develop the

severe 'air-sac' disease.

Although 'air-sac disease' in the fowl requires the presence of M. gallisepticum, without the complicating factors E. coli and a virus, only a relatively mild infection of the respiratory tract results. The situation with respect to M. ovipneumoniae and chronic respiratory disease of sheep is likely to be different, but the avian disease highlights the problems inherent in assigning an etiological role to an agent without taking many possible factors into account.

(iv) Chronic pneumonia of rodents

Initial studies of the etiology of chronic pneumonia of rodents were complicated by the isolation of the mycoplasma, later named M. pulmonis (Edward and Freundt, 1956), from rats and mice both with and without the lesions of respiratory diseases. The mycoplasma was isolated by:

1) Klieneberger and Steabben (1937) who designated the organism L3, from the lung lesions of rats with chronic bronchopneumonia.

2) Nelson (1937), from mice with infectious catarrh. He termed the agents "coccobacilliform bodies".

3) Edward (1940), from the lungs of normal mice.

4) Klieneberger-Nobel and Cheng (1955) from the nasopharynx of weanling rats.

They also showed that caesarean-derived rats of the same age were free of mycoplasmas.

It is now generally accepted that most strains of M. pulmonis are capable of causing upper respiratory tract infection, i.e. infectious catarrh. However the conclusion that it is the cause, or even a cause of chronic pneumonia in rodents is not universally accepted. Thus, Nelson (1967) regards the chronic respiratory disease syndrome in rats and mice as a complex of two independent diseases: infectious catarrh caused by M. pulmonis, and enzootic bronchietasis which he alleges is of viral origin, although the putative virus has not been characterized. Andrewes and Glover (1946) also ascribed the etiology of chronic pneumonia in laboratory mice to a virus which they called "gray-lung virus". The infection

is latent, but can appear after serial passage of lung material. The agent was later shown to be susceptible to tetracyclines and sodium aurothiomalate but not penicillin, so it cannot be a virus. Its fine structure, moreover, suggests that it is a mycoplasma (Tully, 1969).

In spite of such reports, some workers still maintain that M. pulmonis is the sole cause of chronic pneumonia in rodents: thus according to Cassell, Lendsey, Overcash and Baker (1973) three groups of workers, using pure cultures of M. pulmonis have produced lesions in SPF rats identical with those produced by placing the rats in contact with conventional animals with spontaneous chronic respiratory disease. All lesions were suppressed by oral administration of tetracycline. They admit however, that although all of the rat isolates tested produced upper respiratory tract infections, 28 days after inoculation, significant lower respiratory tract infection had been produced only occasionally and inconsistently. They also found that pulmonary clearance of M. pulmonis in the rat is much more efficient than that in the mouse, and believe that this relatively high clearance efficiency is important in explaining the difficulties of producing lower respiratory tract infection with M. pulmonis in the rat. They further suggest that impaired bronchial clearance due to a variety of natural stimuli might precipitate active lung disease.

In contrast to the above workers, Gay(1967) and Gay et al., (1972) produced chronicpneumonia in ten out of ten neonatal SPF rats by inoculating them intranasally with infected lung homogenate. From this lung homogenate he could isolate M. pulmonis and S. moniliformis, but when he attempted to produce experimental disease with an aerosol of these organisms, either individually or in combination, he had no success. However, he consistently produced a chronic pneumonia, histologically indistinguishable from the natural disease by inoculating neonatal SPF rats with lung material from a 4-week old conventional rat. No bacteria or mycoplasmas could be cultivated from the inoculum but mice inoculated with control lung

material from SPF rats remained healthy.

Attempts to culture the causative agent from the lung homogenate that caused this chronic pneumonia were unsuccessful, even though a wide variety of cell-free media and tissue culture systems were used. Electron microscopic inspection of the alveoli of the infected lungs, however, showed a large number of mycoplasma-like organisms. These organisms were predominantly narrow, elongated organisms which commonly occurred in parallel groups. When Gay et al. (1972) compared the lesions produced by this rat pneumonia agent, the "gray-lung virus" and the "enzootic bronchiectasis virus" of Nelson, he could find no difference macroscopically, and identical mycoplasma-like organisms could be seen in the lung lesions produced by each of the three agents. No virus-like particles were seen. Consequently he believed that chronic pneumonia of rodents is caused by a mycoplasma that has not yet been propagated.

Although rodents, being laboratory animals lend themselves to the study of the etiology of diseases affecting them it is obvious from the preceding section that the cause or causes of chronic pneumonia of rodents is still in dispute - and from the point of view of using chronic pneumonia in rodents as a model for chronic pneumonia in sheep, it is important to note that although M. pulmonis can be recovered from all natural cases of chronic pneumonia in rodents, it may not be the primary cause of the disease.

(d) Chronic Pneumonia of Sheep

Chronic pneumonia of sheep is a low mortality, high morbidity disease characterized by inadequate weight gain and exercise intolerance, and is thus of considerable economic importance. In New Zealand it has been estimated to affect 70% to 80% of lambs in some groups of animals, and although mortalities are usually low, the mortality rate can reach 15% in bad seasons (Smith, 1970 ; Davis 1970).

The chronic disease, unlike the acute form which can affect sheep of any age is prevalent only in lambs of three to ten months (hence the name "hogget pneumonia"). Acute pneumonias of sheep are typically caused by bacteria, e.g. P. haemolytica, while in the chronic form of the disease, bacteria is either absent or present in such low concentrations as to be inadequate to account for the disease (Alley, 1975). Furthermore, if the isolated bacteria are inoculated intranasally into susceptible animals, usually no lesions are produced, but occasionally a typical acute pneumonia appears. Acute pneumonia is characterized by rapid onset and short duration, and the chronic form by a long incubation period and slow progression. In practice, however, the two forms of the disease are distinguished by macroscopic and microscopic examination of the lesions of the lung. The acute form of pneumonia in sheep is characterized by intense congestion and varying degrees of red or grey consolidation of the ventral portion of one or both lungs. A cellular exudate composed of neutrophils, macrophages and detached alveolar epithelial cells with which many bacteria are closely associated, is present in the lungs. The alveoli are filled with large macrophages and small focal areas of neutrophil infiltration appear. In the "atypica'" or chronic lung disease, the macroscopic lesions vary from dark-red to grey areas of consoliation of the lung, to narrow branching bands of collapse in the anterior lobes of both lungs. The alveoli are infiltrated with macrophages and both lymphocytes and macrophages are present in the alveolar septa. The major factor underlying the pathological differences between acute and

chronic pneumonia is the degree and rapidity of destruction and damage to the alveolar epithelium, which is universal in the acute form of the disease, but less severe and more localized in the chronic form (Alley, 1975).

Since the earliest report of the isolation of a mycoplasma from the lungs of sheep (Grieg, 1955), refinements in techniques, and the realization that many mycoplasmas have complex nutritional requirements have led to reports of mycoplasma isolations from sheep in a large number of countries : Turkey (Dunusan and Dogyer, 1955; Cottew, Watson, Arisoy, Erdag and Buckley, 1968); Israel (Nobel, 1958); U.S.A. (Boidin, Cordy and Adler, 1958; Hamdy, Pounden and Ferguson, 1959; Barber and Fabricant, 1961); U.S.S.R. (Farzaliev, Khalimbekov, Dandamaeu and Aliev, 1962); Britain (Mackay, Nisbet and Foggie, 1963; Mackay, 1966; Mackay and Nisbet, 1966); Italy (Dieana and Cereto, 1967); Kenya (Krauss and Wandera, 1970); Australia (Cottew, 1971; St George et al., 1971; Carmichael et al., 1972; Sullivan, St George and Horsfall, 1973(i)) and New Zealand (Clarke et al., 1974: Alley et al 1975). Early attempts to produce chronic pneumonia with these isolated mycoplasmas were unsuccessful (Boidin et al 1958: Hamdy et al., 1959: Farzaliev et al., 1962; Hamdy and Pounden, 1959). The significance of these results is unclear, as it has been recognized that there are at least three species of "mycoplasmas" associated with sheep:

1) An arginine-requiring mycoplasma which does not ferment glucose, gives fried-egg shaped colonies and has been shown to be M. arginini (Clarke et al., 1974 Cottew, 1971; Carmichael et al., 1972; Krauss and Wandera, 1970).
2) A acholeplasma which does not require sterols for growth and has been identified as A. laidlawii (Krauss and Wandera, 1970).
3) A glycolytic mycoplasma that forms centreless colonies on solid media and was first reportedly isolated by Barber and Fabricant (1961) on a medium containing yeast hydrolyzate and horse serum.

This last organism now designated M. ovipneumoniae is generally believed (Carmichael et al., 1972; Clarke et al., 1974: Jones et al., 1976) to be the most significant from the pathological point of view, so an increasing amount of attention has been centred on it. This work is reviewed below:

Studies of M. ovipneumoniae in countries other than New Zealand:

A glycolytic mycoplasma was isolated by St George, Sullivan, Love and Horsfall (1971) from the lung of a sheep with chronic pneumonia. It did not grow on standard mycoplasma media, but was cytopathic in bovine testis cell cultures and could be propagated on Hanks medium plus lactalbumin hydrolysate, yeast extract and fetal calf serum. Colonies of the organism on solid media did not show the fried-egg shape reported for most mycoplasmas, but the centreless, 'lacy' or 'vacuolated' appearance typical of M. pneumoniae. The agent passed through a filter of pore size 220nm, sufficient to retain bacteria, and grew in the presence of thallium acetate, streptomycin and penicillin. It was chloroform-sensitive, and when examined by impression smears stained by the Dienes method, showed the morphology of mycoplasmas.

Carmichael, St George, Sullivan and Horsfall(1972) isolated a strain of mycoplasma, which they termed biotype Y-98, from sheep in a Queensland flock with a high incidence of chronic interstitial pneumonia. The Y-98 biotype was found to occur with the highest frequency in the nasal cavities, trachea and bronchi of pneumonic lambs, although on two occasions it was isolated from the nasal swabs of healthy adult sheep. The organism had centreless colonies on solid media, fermented glucose with production of acid, and had a marked hemolytic activity for ovine erythrocytes. It was serologically different from all other mycoplasmas isolated from the flocks studied, and was found to be antigenically unrelated to twelve additional ovine and caprine serotypes by metabolic inhibition, growth inhibition and immunodiffusion tests.

Since these properties indicated that it was a distinct species, Carmichael *et al* (1972) proposed the name *M. ovipneumoniae*.

Glycolytic mycoplasmas isolated from the respiratory tract of apparently healthy sheep, pneumonic sheep and sheep with pulmonary adenomatosis in Scotland (Jones *et al*., 1976) were shown by polyacrylamide gel electrophoresis, gel precipitin tests, metabolic and growth inhibition tests to be related to the Queensland Y-98 strain and could thus be classified as one species, viz. *M. ovipneumoniae*. Growth and metabolic inhibition tests showed, however, that intraspecific differences occurred with apparent polarization of strains from sheep with or without pulmonary adenomatosis. Jones *et al*. (1976) also pointed out that the serological relationship of *M. ovipneumoniae* to other members of the Mycoplasmatales had been insufficiently investigated and tested their strains, and the Y-98 strain against 40 hyperimmune sera to 33 named mycoplasma species and subspecies and six serogroups of bovine or caprine origin. They confirmed that the strains of fermenting mycoplasmas from the ovine respiratory tract were a distinct species and consequently approved the name, *M. ovipneumoniae*.

Strains of *M. ovipneumoniae* isolated from pneumonic lung have also been reported in Victoria (Furlong and Cottew, 1973; Cottew, 1971); New Zealand (Clarke *et al*., 1974); U.S.A. (St George and Carmichael, 1975); England (Leach *et al*., 1976), and Canada and Hungary (St George, 1976).

The Y-98 strain isolated in Queensland by Carmichael *et al*., (1971) was the only strain of *M. ovipneumoniae* reported by name in their paper, and has since been widely distributed and studied. Subsequently, Leach Cottew, Andrews and Powell (1976) proposed that it be the type strain of *M. ovipneumoniae* and deposited the strain in the National Collection of Type Cultures as NCTC 10151.

M. ovipneumoniae has been widely isolated from the lung lesions of lambs with chronic pneumonia and only inconsistently from apparently healthy sheep, and then from the nasal cavities. In order to establish any causal

relationship between chronic pneumonia and the mycoplasma, several workers have attempted to transmit the disease experimentally using a broth culture of M. ovipneumoniae, thus:

St George et al. (1971) isolated a mycoplasma (subsequently shown to be M. ovipneumoniae (Carmichael et al., 1972)) from the lung of a pneumonic sheep, propagated it in vitro for six subcultures, then investigated its ability to produce chronic pneumonia in caesarean derived and conventional lambs following intratracheal or intranasal inoculation. These were preliminary experiments, and poorly controlled because at least one lamb inoculated with sterile medium died. However, they produced evidence, which while not conclusive, indicated that the mycoplasma could play an important role in sheep pneumonia.

Sullivan, St George and Horsfall (1973(ii)) inoculated day-old lambs intranasally, or intravenously with M. ovipneumoniae and produced a proliferative interstitial pneumonia. The authors claimed that the disease produced closely resembled the chronic pneumonia seen in field cases. Similar lambs put in contact with the inoculated lambs also developed a chronic pneumonia. However, in no case was M. ovipneumoniae recovered from infected lungs, and this failure to recover the mycoplasma is difficult to account for if M. ovipneumoniae is indeed the cause of chronic pneumonia in sheep.

Foggie, Jones and Buxton (1976) inoculated SPF lambs intrabronchially with M. ovipneumoniae and produced small, discrete lesions in three out of six lambs. However, the lesions were milder than those of the natural disease. They recovered M. ovipneumoniae from the lungs of all infected animals, but not from the controls. SPF lambs in contact with infected animals, became infected with M. ovipneumoniae but the organism was recovered only from the upper respiratory tract, and none of the animals developed pneumonia.

We conclude that the role of M. ovipneumoniae in natural cases of chronic pneumonia in sheep remains to be clarified, as not all infected sheep get pneumonia,

and more significantly, the disease produced experimentally may not be identical to that seen in field cases.

Studies of M. ovipneumoniae in New Zealand.

Sheep play an important role in the economy of New Zealand, and chronic pneumonia has been estimated to affect 70% to 80% of lambs in many flocks (Smith, 1970). Nevertheless, until 1974, little or no effort had been made in this country to establish theetiology of the disease.

In an initial study undertaken to elucidate the etiology of chronic pneumonia in sheep, Clarke, Brown and Alley (1974) recovered a number of mycoplasmas from the respiratory tract of sheep with or without chronic pneumonia. The isolates could be divided into strains which fermented arginine and produced typical "fried-egg" shaped colonies on 1% agar, and glycolytic strains which produced "vacuolated" or "lacy" centreless colonies. The arginine-fermenting mycoplasma was identified as M. arginini, whereas the glycolytic mycoplasma was found to be indistinguishable from the Australian Y-98 strain isolated by St George et al (1971).

Alley, Quinlan and Clarke (1975) in a survey of the prevalence of M. ovipneumoniae in New Zealand sheep, recovered the mycoplasma from all of sixty pneumonic lungs sampled. The organism was found to be present in pneumonic lungs at a titre of 10^6 - 10^7 organisms per gram. However, the mycoplasma was also isolated from ten of forty normal lungs, although in only two cases did the titre exceed 10^3 organisms per gram. This low titre of mycoplasmas apparently present in normal lung could have been due to contamination of the lung by meat inspectors who handled the tissue before it could be sampled in the survey.

Although this high titre in pneumonic lungs, and 100% recovery rate from these lungs is suggestive that M. ovipneumoniae causes chronic pneumonia in sheep in New Zealand, it is possible that the mycoplasma is only an efficient secondary invader, so experiments to attempt

to transmit the disease experimentally to colostrum-deprived sheep using broth culture of M. ovipneumoniae were performed (Alley, personal communication). An aerosol of pneumonic lung homogenate consistently caused lesions in 60% to 70% of sheep inoculated, and the lesions produced were typical of the field disease. However, when an aerosol of M. ovipneumoniae was used as inoculum, lesions were produced only once in three attempts and with the exception of one lamb, they were not identical with those seen in natural disease. Several reasons could be advanced for these inconsistent results, but a fundamental necessity for consistent disease production is obviously the use of a consistent inoculum of micro-organisms. However, in all cases the mycoplasmas used for inoculation, were propagated until the day after a pH change was visible (Clarke, personal communication), and this can lead to large variations in the inoculum, as mycoplasmas die rapidly after reaching maximum titre. The in vitro studies of the growth of M. ovipneumoniae, reported in a later section, were undertaken so that high titre inocula could consistently be used to attempt to transmit the disease in sheep.

A parallel approach to transmission experiments investigating the role of M. ovipneumoniae in chronic pneumonia of sheep is to establish the presence of the mycoplasma in the lung by techniques such as fluorescent antibody studies or electron microscopy. Unfortunately, electron microscope studies do not usually distinguish between mycoplasmas, with the exception of the few mycoplasmas that have a characteristic feature, such as the blebs of M. gallisepticum. An in vitro electron microscope study of M. ovipneumoniae was nevertheless undertaken in order to elucidate its morphology, as this has not yet been reported. The electron microscopy was undertaken in conjunction with the growth experiments, so that the stage of growth of the mycoplasmas seen in electron micrographs was known.

Mycoplasmas have a low percentage of guanine plus cytosine in their DNA, thus, the base compositions reported for the DNA of all mycoplasmas except M.

pneumoniae, fall within the relatively narrow range of 23% to 35% GC. As the base composition of *M. ovipneumoniae* had not been unequivocally established, investigations were undertaken to find the melting temperature and buoyant density of the DNA, so that the base composition of *M. ovipneumoniae* could be determined by these two methods.

In summary therefore, the experiments reported in this thesis were undertaken as an *in vitro* study of *M. ovipneumoniae*. This study is thus part of the investigation being made at Massey University into the link between *M. ovipneumoniae* and chronic pneumonia of sheep.