ENZOOTIC PNEUMONIA OF PIGS (EPP) - DIAGNOSIS, PREVALENCE, EPIDEMIOLOGY AND ECONOMIC CONSEQUENCES

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DECLARATION

The work embodied in this thesis has been initiated, interpreted and reported by myself. Technical assistance was used for the conduct of routine laboratory testing, the field trial and in the feeding experiments.

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TABLE OF CONTENTS

		Page No.
ACKNOWLEDGMENTS		iv
SUMMARY		vi
ABBREVIATIONS		x
CHAPTER 1	INTRODUCTION: BIOLOGY OF MYCOPLASMAS	1
1.1	Taxonomy of mycoplasmas	2
1.2	Biological properties of mycoplasmas	17
1.3	Strain identification of mycoplasmas	55
1.4	Antibody detection	61
1.5	Immune response to mycoplasma infection	66
1.6	Diseases of man and animals caused by mycoplasmas	77
1.7	Mycoplasma infection causing pneumonia and respiratory tract infection in man and animals	87
1.8	Detailed consideration of enzootic pneumonia of pigs	96
1.9	Definition of unresolved problems and reasons for present investigation	138
CHAPTER 2	MATERIALS AND METHODS	142
2.1	Assessment of lung pathology	143
2.2	Giemsa staining of mycoplasmas	147
2.3	Complement Fixation Test	148
2.4	Immunofluorescence	149
2.5	Culture methods	155
2.6	Inoculation of pigs	157
CHAPTER 3	PRELIMINARY STUDIES ON LABORATORY PROCEDURES	160
3.1	Introduction	161
3.2	Materials and Methods	163
3.3	Results	168
3.4	Discussion	184

TABLE OF CONTENTS

		Page No.
CHAPTER 4	PREVALENCE OF LESIONS TYPICAL OF ENZOOTIC PNEUMONIA IN SOUTH AUSTRALIA - AN ABATTOIR SURVEY	192
4.1	Introduction	193
4.2	Materials and Methods	194
4.3	Results	195
4.4	Discussion	202
CHAPTER 5	EPIDEMIOLOGY OF ENZOOTIC PNEUMONIA IN SOUTH AUSTRALIAN PIGGERIES	207
5.1	Introduction	208
5.2	Materials and Methods	210
5.3	Results	217
5.4	Discussion	227
CHAPTER 6	INVESTIGATIONS INTO THE EFFECT OF ENZOOTIC PNEUMONIA ON GROWTH RATE AND FEED CONVERSION EFFICIENCY	235
6.1	Introduction	236
	Experiment I	
6.2	Materials and Methods	238
6.3	Results	241
6.4	Discussion	245
	Experiment II	
6.5	Materials and Methods	249
6.6	Results	252
6.7	Discussion	261
APPENDICES		268
I	Method of lung lesion calibration	269
II	Complement Fixation Test - Consumption Method	270
III	Complement Fixation Test - Direct Method	276

TABLE OF CONTENTS

		Page No.
IV	Paraffin sectioning for IMF staining	278
V	Culture of porcine mycoplasmas	279
REFERENCES		283
PUBLICATIONS		318

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SUMMARY

This study established rapid routine diagnostic tests which made investigation into the economic consequences of enzootic pneumonia of pigs (EPP) within the South Australian pig industry possible. Previously, serological procedures had not been in operation in South Australia and immunofluorescent testing had not been developed in Australia. Similarly, there had not been a comprehensive examination of the prevalence of lesions and the degree of losses caused by EPP, because of considerable speculation for some time that these would be much less than those demonstrated in colder northern hemisphere countries.

Diagnostic tests including Giemsa and immunoflourescent (IMF) staining of touch preparations of pneumonic lung, IMF staining of lung sections, and a complement fixation test for detection of Mycoplasma hyopneumoniae antibodies were established, and their sensitivity and specificity verified. It was found that a rapid diagnosis of enzootic pneumonia could be made by the demonstration of M. hyopneumoniae organisms via IMF staining of touch preparations or sections from lungs with active or recent infections. Good correlation was obtained between results of Giemsa and IMF staining of touch preparations and IMF staining of tissue sections. The presence of complement fixing antibodies to M. hyopneumoniae were associated ($p<0.001$) with typical gross lesions in corresponding lungs collected from 412 commercial pigs at slaughter.

Infection with EPP was found to be widespread, as typical gross lesions were found in 45.1% of 1 430 lungs from pork and bacon weight pigs sampled at 3 major abattoirs, EPP-like lesions were associated ($p < 0.001$) with *M. hyopneumoniae* antibody in corresponding serum samples. The prevalence of pleurisy was 14.5% among 712 lungs and was associated ($p<0.001$) with lesions of EPP, indicating that severe secondary bacterial contamination of lesions is common. The prevalence of EPP varied markedly between the abattoirs ($p<0.001$), being 22%, 42% and 71% respectively; emphasizing the need to sample lungs at several abattoirs to obtain a reliable estimate of prevalence within the industry, on a state or country basis. The seasonal prevalence of lesions varied ($p<0.1$) from its highest in summer of 51.1%, to the lowest in winter of 41.7%. This trend supports the view that infection is higher among young pigs during the cold winter months; these pigs being marketed 6 months later in summer.

A field study of the epidemiology of EPP in South Australia found similar predisposing factors operating to those demonstrated in several studies conducted in colder northern hemisphere countries. Environmental and management conditions of 15 piggeries with a high-prevalence (>70%) of EPP lesions were compared with those in 16 herds having a low-prevalence (<30%). A similar number of small herds, with 20-70 sows (sideline units) and large herds, with >100 sows (fully intensive units) were studied. Half the herds were visited in summer and half in winter to detect factors related to seasonal conditions. In small herds, factors commonly associated with a high-prevalence of EPP were: a higher gilt replacement rate

($p<0.1$); purchase of pigs for finishing ($p<0.07$); larger number of pigs per shed section ($p<0.001$); larger group sizes ($p<0.01$); and draughty farrowing and weaner accommodation ($p<0.01$). In large herds, factors commonly associated with a high-prevalence were: higher pen stocking rate ($p<0.05$); higher air-space stocking rate ($p<0.05$); and higher atmospheric ammonia levels in summer ($p<0.1$). Ammonia levels were also found to be dependant on the temperature at the recording site ($r = 0.43$, $p<0.05$). EPP was also associated with reduced production, as substantially more high-prevalence herds marketed pigs at a lighter bodyweight ($p<0.06$) and older age ($p<0.06$).

In the absence of any previous reports of the effect of EPP on production under Australian conditions, and because of the losses indicated by the field study, the economic consequences of EPP were investigated experimentally. EPP was reproduced using a local field strain of M. hyopneumoniae (Beaufort) under environmental and management conditions commonly found on commercial piggeries in South Australia. Both initial infection ("breakdown") and endemic stages of infection were evaluated. In the former case, growing pigs naturally challenged by inoculated transmitter pigs, recorded a substantial reduction ($p<0.01$) in growth rate of 12.7% between 50-85 kg liveweight. In the latter trial, inoculated gilts were used to naturally challenge piglets during suckling. Growth rate of infected piglets was also substantially reduced ($p<0.001$) by 15.9% between

8-85 kg liveweight, while the feed conversion rate was reduced ($p<0.06$) by 13.8% between 10-25 kg liveweight, when compared with piglets from uninfected sows. Severe reduction in performance during the post-weaning period, however, was not compensated for later in the growth period, as the performance of infected pigs between 50-85 kg only equalled that of control pigs. At slaughter, gross EPP lesions were considered mild; being present in only 44% of 25 lungs. However, this subclinical form of the disease, devoid of significant secondary bacterial invaders did reduce performance. Affected pigs had to be held for an additional 12 days to reach market weight of 85 kg. At current feed and production costs, the extra cost of production amounted to $7.63 per pig produced from the infected group, irrespective of presence or absence of gross EPP lesions.

These experiments demonstrate that substantial economic losses are caused by uncomplicated EPP under environmental and management conditions commonly found on commercial piggeries in South Australia. Furthermore, these current losses are similar in character and magnitude to those previously recorded in colder northern hemisphere countries. Consequently, South Australian producers would benefit from a greater emphasis on the control of EPP in their piggeries.

ABBREVIATIONS

$\overset{\circ}{A}$	-	angstroms 10^{-10}m
BSA	-	bovine serum albumin
C'	-	complement
CFT	-	complement fixation test
$C'H_{50}$	-	50% haemolytic end point
CPE	-	cytopathic effect
CMI	-	cell mediated immunity
ELISA	-	enzyme - linked - immunosorbent assay
EPP	-	enzootic pneumonia of pigs
IFAT	-	immunofluorescent antibody technique
FITC	-	fluorescein isothiocyanate
G + C	-	guanine plus cytosine
GPC'	-	guinea-pig complement
HEPES	-	N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
IHA	-	indirect haemagglutination
IMFT	-	immuno fluorescence test
IPP	-	indirect immunoperoxidase
LDH	-	lactate dehydrogenase
MH	-	M. hyopneumoniae
MI	-	metabolic inhibition
µm	-	micrometre 10^{-6}m
nm	-	nanometre 10^{-9}m
PBS	-	phosphate buffered saline
PHA	-	phytohaemagglutinin
RIA	-	radioimmunoassay
RIP	-	radioimmunoprecipitation
TP	-	touch preparation
VBS	-	veronal buffered saline

CHAPTER I

INTRODUCTION: BIOLOGY OF MYCOFLASMAS

1.1 Taxonomy of mycoplasmas

1.1 (i) Taxonomic position

The decision (Subcommittee 1967) to assign the mycoplasmas to a new and separate class, the Mollicutes, has remained essentially uncontested. The arguments presented at that time, were largely based on the characteristic colonial morphology, absence of a cell wall and an inability to synthesise cell wall muco-peptides. Significance was also attributed to the requirement of sterols for growth by a large majority of species and a wider guanine + cytosine (G + C) range 23 to 40 mol-% and lower G + C content than recorded for any known "eubacteria".

While data on G + C composition was sparse at the time, further studies have demonstrated a significant evolutionary gap between mycoplasmas and other procaryotes. These have demonstrated a genome size of 5×10^8 daltons for members of the Mycoplasmataceae (Bak et al 1969) which is half the size of the smallest bacteria, and studies on the electrophoretic mobility of ribosomal ribonucleic acids (r RNAS) of Mycoplasma and Acholeplasma species have shown differences in, base composition between mycoplasmas and bacteria (Kirk and Morowitz 1969), sedimentation properties in sucrose gradient (Reich 1967) and electrophoretic mobility in polyacrylamide gels (Harley et al 1973).

For the purpose of this review the term mycoplasma will be used when referring generally to organisms in the class Mollicutes; when specifically referring to species and other taxonomic groups the correct nomenclature will be given.

Contrary evidence to such a classification has been advanced by Niemark (1974 - cited by Freundt and Edward 1979) and relates to the presence of lactate dehydrogenases (LDHs) similar to those known to occur in Lactobacillaceae. While these were found to occur only in the Acholeplasmataceae, other similarities between these organism and streptococci including cellular proteins and immunologic analysis were interpreted as proof of their phylogenic relationship by the author. Considering the major differences between mycoplasmas and bacteria in base sequence homology and G + C studies these biochemical similarities are of minor taxonomic importance. They may however indicate that the Acholeplasmataceae occupy an intermediate phylogenetic position between the Mollicutes and bacteria of Class I of the Scotobacteria (Table 1.1)

The class Mollicutes contains only the order Mycoplasmatales, consequently the description for the order is the same as that for the class. The species contained are very similar except for differences in nutritional requirements for some sterols. Due to a lack of requirement for cholesterol by the Acholeplasma-

taceae it has been proposed that serious consideration be given to the elevation of this family to the next highest rank (Table 1.2).

The establishment of three families was endorsed by the Subcommittee (1977) after the proposal of Skripal (1977) to establish the family Spiroplasmataceae. Classification between families is based on nutritional and morphologic criteria, which is supported by differences in genome size (Bak et al 1969) and essential differences in the RNAs of the two families (Reff et al 1977). Of equal importance is the presence of LDHs in five Acholeplasma species examined, which are not found in members of the family Mycoplasmataceae (Neimark 1974 - cited by Freundt and Edward 1979).

The genera established within the families are shown in Table 1.2, indicating that only the family Mycoplasmatales is subdivided.

Two genera are recognised in the family Mycoplasmataceae, the Mycoplasma (Krass and Gardner 1973) and the Ureaplasma, which differ from all other mycoplasmas in possessing urea.

Two other genera are recognised in the class Mollicutes, the Thermoplasma and Anaeroplasma; however their correct taxonomic position is uncertain.

TABLE 1.1

Taxonomic position of the mycoplasma
(after Freundt and Edward 1979)

Kingdom: Procaryocyte

Division II: "Scotobacteria"

Class II: Obligate intracellular Scotobacteria in eukaryotic cells - Rickettsia.

Class III: Scotobacteria without cell walls - Mollicutes.

Criteria to define a species is again arbitrary, and is defined by the characteristics of the designated type strain. Further definition of a species was provided by the Subcommittee (1979) when referring to mycoplasma species as "clusters of morphologically identical isolates whose genomes exhibit a high degree of relatedness". Other methods used to determine the patterns of relationship are biochemical tests, gel electrophoresis of cellular proteins, and serology. Perhaps the best method to determine the genetic relationship between strains is to determine the extent to which the genomes are able to hybridize. A less direct method of genome description is the use of G + C ratios of DNA. Other characteristics used to define a species include morphologic and biochemical properties, patterns of cellular protein migration in gel electrophoresis and antigenicity. However, as few mycoplasma species possess a distinct cell morphology and biochemical pathways are often indistinct at species level, these methods are of limited value. Cell electrophoretic patterns of nett cell proteins provide a useful method of comparing genetic relationship provided it is performed under well defined conditions, as factors as medium composition and age of the culture influence the test result (Freundt and Edward 1979).

The most widely used marker is the antigenic pattern of the species, which are defined using

serologic tests. Previously, direct agglutination and complement fixation were used, these now being supplemented with a combination of growth inhibition, metabolic inhibition, and immunofluorescent tests.

Only one mycoplasma species is subdivided into a subspecies, namely Mycoplasma mycoides subsp. mycoides and capri. Classification is based on consistent differences in a number of properties while the species remain closely related by serological and nucleic acid hybridization tests.

1.1 (ii) Properties used in classification

Although mention of taxonomic criteria has been made, an assessment of the relative importance of each will be provided.

(a) Ultrastructure and morphology

The absence of a cell wall is the most important feature of the Mollicutes. Organisms are bound by a single membrane which allows cells to vary in shape, from coccoid forms to branched filaments (pleomorphic). The smallest viable units are about 300 nm in diameter.

Motility has been observed in three species,

M. pneumoniae, M. pulmonis and M. gallisepticum, all of which possess a specialised terminal structure which may assist movement by attachment to organelles. However, while gliding motility is associated with specialised structures these features are not used in classification of mycoplasmas.

Helical morphology, and rotatory and undulating movement are however, criteria by which the family Spiroplasmataceae are defined. Furthermore the posession of monotrichous flagella allowing swimming motility of Thermoplasma acidophilum is important in classification of this mycoplasma (Black et al 1979).

(b) Colonial appearance

The ability to produce a colony on solid medium with a "fried egg" appearance is typical of organisms belonging to the class Mollicutes. This ability is only shared with L-phase bacteria. Colonial morphology does not play an important role in the classification of species, although species often exhibit prominent differences. Colonial morphology is to some extent dependant on composition of the growth medium and the ability of the organsims to penetrate into the agar medium. For example, Ureaplasma urealyticum was originally observed as small atypical colonies, however on optimal medium they develop larger "fried egg" colonies.

(c) Growth requirements

The nutritional dependence on cholesterol which is characteristic of a large number of mycoplasmas, is a property unknown in any species of bacteria. As classification of Spiroplasmataceae depends on demonstration of helical morphology, the dependence on sterols for growth becomes a major criterion for the differentiation only between the Mycoplasmataceae and Acholeplasmataceae. The requirement for sterols may be determined indirectly by the digitonin test (Freundt 1973), in which digitonin inhibits growth by interacting with cholesterol in the cell membrane thereby increasing susceptibility to lysis.

Ability to metabolise urea is of paramount importance in the classification of the genus Ureaplasma which is the only mycoplasmas to contain urease.

Growth of mycoplasmas depend on the pH and temperature of the growth medium. The optimum pH for Thermoplasma acidophilum is 2-3 and temperature optimum of 56-60^{o}C, while the pH optimum for U. urealyticum is 5.5-6.5, about 0.5 lower than for most other mycoplasmas. Acholeplasmataceae generally grow over a wider temperature range with Acholeplasma laidlawii growing well at 22^{o}C.

Gaseous conditions for growth also vary with Ureaplasmas which require a gaseous mixture of 5-15% carbon dioxide in air or nitrogen, while Anaeroplasmas are distinguished by their dependence on strict anaerobic conditions for growth.

(d) Biochemical properties

Biochemical classification of mycoplasmas requires evaluation of the following properties: fermentation of glucose, hydrolysis of arginine, hydrolysis of urea and production of carotenoids (Razin and Claverdon 1965).

Species within these two biochemical groups show some serological relatedness, eg. M. pneumoniae with M. mycoides subsp. mycoides (Lemcke 1965) and M. orale with M. salivarum (Fox et al 1969) which does exist between groups. Furthermore, patterns of isolectric focussing of mycoplasma proteins were also found to differ in a characteristic way between groups. The correlation between biochemical properties and antigenic composition, together with the isolectric patterns of cell proteins attributes taxonomic significance to the capacity of Mycoplasma species to catabolize glucose and arginine. This may possibly serve as a basis for dividing the genus Mycoplasma into two distinct genera (reviewed by

Freundt and Edward 1979). Further tests may provide useful data, including fermentation of a number of carbohydrates, phosphatase activity, production of film and spots on agar plate medium containing egg yolk emulsion, proteolytic activity, tetrazolium reduction under aerobic and anaerobic conditions and haemolysis of sheep and guinea-pig red blood cells as well as testing for peroxidase as the haemolysin.

(e) Antigenic properties

A comparison of different serologic methods for species differentiation was conducted by Edward and Freundt (1969) who found direct agglutination, indirect haemagglutination, latex agglutination, growth inhibition, metabolic inhibition and immunofluorescence best to distinguish between species. However complement fixation and double immunodiffusion tests have been found to detect minor antigenic overlapping due to inclusion of antigens at sites other than on the cell membrane. Current recommendations in the Minimal Standards Document (Subcommittee 1979) designate the growth inhibition and epi-immunofluorescent tests as methods of choice to differentiate between species of Mycoplasma and Acholeplasma and between serotypes of Ureaplasma. As growth inhibition may be insensitive, even with high titre serum, the use

TABLE 1.2

Taxonomy of the Class Mollicutes*

Class: Mollicutes

Order: Mycoplasmatales

Family I: Mycoplasmataceae

1. Sterol required for growth
2. Genome size about 5×10^8 daltons
3. NADH oxidase localised in cytoplasm

Genus I: Mycoplasma (about 50 species current)

Do not hydrolyse urea.

Genus II: Ureaplasma (single species with serotypes)

Family II: Acholeplasmataceae

1. Sterols not required for growth
2. Genome size about 1×10^9 daltons
3. NADH oxide localised in membrane.

Genus I: Acholeplasma (7 species current)

Family III: Spiroplasmataceae

1. Helical organisms during some phase of growth
2. Sterol required for growth
3. Genome size about 1×10^9 daltons
4. NADH oxidase localised in cytoplasm .

Genus I: *Spiroplasma* (*S. citri* and others undefined)

Genera of uncertain taxonomic position.

Thermoplasma (single species)

Anaeroplasma (two species)

* from Tully (1978) - cited by Freundt and Edward (1979).

of metabolic inhibition is recommended when growth inhibition is not feasible. Complement fixation and double immunodiffusion were included to differentiate between group antigens common to different generic or sub-generic groups of mycoplasma species.

(f) Electrophoretic patterns of cell proteins

Currently minimal emphasis is put in the Minimal Standards Document on determination of the electrophoretic patterns of cell proteins. When the test is performed under well-specified standard conditions (Freundt and Edward 1979) the test is a valuable indirect measure of the genome of mycoplasma species. Unfortunately the electrophoretic pattern is influenced by the composition of the medium or the age of the culture. With further advances in culture techniques with media free of horse serum, the better techniques for protein extraction, the test will probably be used more extensively.

Generally the electrophoretic pattern of proteins is distinct enough to allow species differentiation. Thus Rosendal (1973) was able to differentiate 50 wild type isolates from dogs correctly as either M. canis, M. edwardii or M. spumans by the polyacrylamide gel electrophoresis test, even though at first patterns of M. canis and M. edwardii were difficult to distinguish from each other.

(g) Nucleic acid composition

As with other organisms a direct comparison of base sequence of nucleic acids of mycoplasma strains is the best way to determine genetic relatedness. The ways in which this may be assessed are by estimating genome size, determining the nucleic acid base ratios, determining sequence homology between nucleic acid by DNA to DNA or DNA to RNA hybridisation; also by determining the electrophoretic mobility of RNAs. These methods are used to establish the class itself and to classify the species and subspecies (see 1.1). Determination of nucleic acid homology by hybridisation experiments is limited to a few laboratories for technical reasons. It is therefore important to note the close agreement between serological and hybridisation studies; this allows the more readily performed serologic tests to reliably differentiate between species.

An example of the use of hybridisation studies to determine species or subspecies status is in the recent elevation of M. agalactiae subsp. bovis to M. bovis. Initial serological studies were conflicting. Hybridisation studies showed only a 40% homology between DNA of the two strains and further serologic studies were performed. The latter showed the two type strains to differ sufficiently and supported the establishment of a new species.

In the case of M. mycoides subsp. mycoides and subsp. capri the argument to retain these as the same species despite distinct serological differences is supported by a high genome sequence homology of about 80% between the DNAs of the two organisms.

In both cases final status of the taxa was determined by hybridisation data. However, the level of hybridisation required for species and subspecies status is again arbitary and is a matter of agreement between taxonomists. Despite this, nucleic acid homologies are still the most accurate way of determining genetic relatedness.

(h) Habitat and pathogenicity

The observation that mycoplasmas are not as highly host species specific as previously thought has led to a marked change in the understanding of host relationships of mycoplasmas. The Spiroplasma species present a striking example as they are capable of not only producing symptoms in a wide variety of unrelated plants, but also infect a number of insects over a wide phylogenic range (Whitcomb and Williamson 1975).

Classification of a new mycoplasma species previously required demonstration that the organism was antigenically different from all other species from the same habitat. However due to the recognition of

wider host relationships, comparison of new species is required with all other species in the genera .

A consequence of this wider host relationship is in the naming of new species. Initial isolations of an organism may be from a host which in time proves not to be the main host. For this reason one must be cautious in proposing a new species name, based on the name of the principal host or resulting disease.

1.2 Biological properties of mycoplasmas

1.2 (i) Morphology

Organisms of the class Mollicutes characteristically have no cell wall and range in size from 2-300 µm and are therefore capable of passing bacteria tight filters. Lack of a rigid cell wall together with the resulting osmotic sensitivity of these cells causes them to appear highly pleomorphic. The coccus form is most common; however branched filaments occur during reproduction. Filaments up to 98 µm in length have been observed in cultures of M. hominis prior to fragmentation into coccoid cells.

Studies of the morphology of mycoplasmas are complicated by the composition of the growth medium, the effects of preparative methods and the effect of

fixatives. Of major importance is the concentration of cholesterol and other lipid precursors in the medium provided. Lack of these cause unbalanced growth leading to extreme pleomorphism, lysis and death of cells. In cultures of glucose fermenting organisms the concentration of glucose and age of culture determines the concentration of acids, which cause swelling of cells due to action on the cell membrane. Damage to organisms may occur during preparation through distortion by mechanical traction, contact with a solid surface, distortion caused by centrifugation, the concentration of the buffer-fixative employed and the osmolality of the final solution.

Many of the physical properties of mycoplasmas are determined by the absence of a cell wall. As previously mentioned the organisms are susceptible to lysis by osmotic shock. They show an absolute resistance to penicillin.

The occurrence of similar pleomorphic organisms, known as bacteria L-forms which lack a cell wall, have been the cause of considerable confusion regarding mycoplasmas in the past. The occurrence of these organisms raise the question of the phylogenetic origins of mycoplasmas i.e. whether they are stable L-forms of bacteria. Absence of the cell wall causes a similar morphological appearance and physical properties to mycoplasmas, Methods differentiating these organisms

have been mentioned in the previous section on taxonomic differences from bacteria, however the main methods employed are Dienes staining (Dienes 1968), immunofluorescent staining, determination of G + C ratios and DNA and RNA homology studies. L-forms may occur naturally and were first detected in the 1930's in cultures of Streptococcus moniliformis by Klienberger (reviewed by Hayflick 1969). Induction of L-forms was first achieved by the use of penicillin which allowed further anatomical, chemical and immunological studies. Cultures characteristically revert to the original parent form once the inducing agent is removed; however stable forms do occur.

The application of genetic homology study evidence, indicates that the mycoplasmas and the L-phase variants of bacteria are not directly related in these particular cases where a relationship has been postulated.

Other forms of mycoplasmas are commonly observed, including the spherical budding forms of U. urealyticum, the helical filaments of Spiroplasma citri, the short filaments of M. pneumoniae, to the ovoid forms of M. gallisepticum with one or more terminal structure.

The presence of specialised organelles of mycoplasmas has been related to the capacity for movement. M. pulmonis may exist in two forms, coccoid with a stalk and a filamentous form with tapered ends (Andrewes and Welch 1946). M. gallisepticum is pear-shaped with a

distinct "bleb" structure at the forward end (reviewed by Bredt 1979) while M. pneumoniae appears elongated with a frontal projection and tail-like rear end (Bredt 1973). Generally the mechanism of movement of these species is unknown, however movement is in the direction of the specialised structure indicating contact with surfaces is important. There is little evidence for the presence of contractile structures, however M. pneumoniae contains a specific substructure in its specialised tip which may be associated with movement (Wilson and Collier 1976). Neimark (1977 - cited by Bredt 1979) reported the presence of actinlike material in M. pneumoniae which supports the theory of contractility; however there is no information on the localisation of this substance.

The movement of Thermoplasma acidophilum is probably caused by a flagellum (Freundt 1972) and the Spiroplasmas show rapid rotatory motion and flexational movements (Cole et al 1973). Motility may provide an advantage over non-motile competitors to survive in the host and may act as a pathogenicity factor, however not all respiratory tract pathogens are motile (eg. M. hyopneumoniae). Motility may assist in active invasion between cilia and thereby avoid host defences and enables organisms to leave environments which have become unfavourable (Bredt 1979). These potential roles of motility are however, only based on observations

of in vitro movement, which in vivo may not overcome the mucociliary clearance mechanism.

1.2 (ii) Ultrastructure

While the morphology is highly variable, depending on the several factors already mentioned, there is more agreement concerning the ultrastructure of the mycoplasmas.

The mycoplasmas are bound by a "unit" or trilaminar membrane consisting of a leaflet of lipid, bordered by monolayers of protein, with a total width of between 7.5 to 10 nm (Domermuth et al 1964). The presence of an amorphous or floccular material on the outer limiting membrane has been observed by many workers on many species (Domermuth et al 1964; Robertson and Smook 1976) and can be stained with osmium-ruthenium red complex which has an affinity for mucopolysaccharides (Luft 1971).

Profiles of the internal structure of mycoplasmas indicate the ultrastructure of the cytoplasm to be fairly uniform. Some variability in texture has been noted with the forms regarded as defining optimal ultrastructures as being either a loose cytoplasm with well-defined ribosomes and fibrillar nuclear material or a compact moderately electron dense cytoplasm with partially obscured nuclear material (Maniloff 1970; Boatman and Kenny 1970). A third form often resulting from in-

appropriate tonicity of fixative fluid is characterised by a very compact cytoplasm and nuclear material condensed into a small net-like area. Forms indicative of non-viable cells contain a very dispersed cytoplasm with virtually no nuclear material apparent.

During growth of the organisms, ribosomes (RNA) are distributed fairly evenly thoughout the cytoplasm of the cell, and are similar in structure to bacterial ribosomes with a sedimentation coefficient of 70S (Maniloff and Morowitz 1972). Ribosomes are found to form tetrahedral or helical arrays if protein synthesis is stopped or cells are centrifuged prior to examination (Allen et al 1970; Barker and Swales 1972).

The structure of the DNA genome of M. arthritidis (H_{39}) (Bode and Morowitz 1967) was found to be unbranched, circular and double stranded with an average length of 262 µm (5.1×10^8 daltons). This is packed intricately and yet functionally into a cell with a diameter of about 0.4 µm diameter. It has been proposed that the genome is attached to the cell membrane (Jacob et al 1963 - cited by Boatman 1979) to allow the cell to segregate replicating DNA during cell division to ensure the daughter cell receives a complete genome. The nuclear material is not bounded by a nuclear membrane, and there is no nucleolus. Further studies have revealed that in M. gallisepticum the DNA is found replicating in the membrane bleb region, together with

most of the ATPase. It has been proposed that these bleb complexes might be the "prokaryotic analogue of eukaryotic centrioles" (Boatman 1979).

The surface bleb itself is hemispherical, about 80 x 125 nm. Usually only one is seen, but in predivision of cells two blebs are present. When found in association with eukaryotic cell membranes M. gallisepticum organisms are found attached at the bleb end (Zucker - Franklin 1966). As mentioned earlier the bleb complex contains cellular DNA, it is also the site of major enzymatic activity and is considered to contain the membrane site and growing point of the "chromosome".

A specialised terminal structure has been a consistent feature of studies of the interaction between M. pneumoniae and human and animal respiratory tissue in organ culture (Collier and Clyde 1971). A short dense filament which is an extension of the unit membrane has been observed to attach to the membrane of epithelial cells. Presence of the filament alone does not confer ability to attach as biochemical studies with neuraminidase indicate the requirement for the presence of a membrane protein to mediate attachment (Powell et al 1976). Ability to attach to the surface of ciliated epithelial cells confers a pathogenic advantage on these organisms, which can then avoid the mechanical cleaning of airways by the muco-ciliary clearance mechanism. Organisms then in close association with eukaryotic

host cells are able to take up cholesterol and fatty acids from the host cell membrane itself. The potential for end-products of mycoplasmas, peroxide and ammonia to be cytotoxic is facilitated by this close contact. It allows them to be injected into the cell or accumulate in sufficient concentration to cause cell damage. This latter mechanism is less likely with peroxidase, due to inactivation by high levels of catalase in mucus coating these cells. Actual fusion of host and mycoplasma membranes has been proposed by Gabridge et al (1977) and is based on results gained by electron microscopy. If fusion does occur potentially cytotoxic nucleases and lipids could then be introduced directly into the host cell causing cilial loss and cell necrosis. The only study supporting this hypothesis' examined the attachment of M. gallisepticum to erythrocytes. All other micrograph studies have shown a gap of about 10 nm between the mycoplasma membrane and that of the host (Boatman et al 1977), a finding which does not support fusion.

Many cells also have a membrane-bound space, or vacuole (Hayflick 1969) which may be a common feature of some cultures, especially those of M. pulmonis. Some workers maintain that this apparent vacuole is really only an invagination of the surface membrane with an opening to the exterior, but despite this it is still termed a vacuole. Although usually empty, it sometimes contains smaller organisms or dense spherules "elementary bodies".

"Elementary bodies" were recognised as being the smallest form of these organisms with the potential to replicate (Kleinberger-Nobel 1962). These small elementary particles (in the 125 to 250 nm size range) are now recognised as non-viable involution forms not associated with reproduction (Rodwell and Mitchell 1979).

1.2 (iii) Chemical Composition

Analysis of the chemical composition and physical fractions of whole cells (Morowitz et al 1962; Razin 1963) from several species revealed the content of RNA to range from 8-14%, DNA from 4-7%, protein from 54-62%, lipid from 11-20% and of carbohydrate from 6-8%, although in M. gallisepticum this was 2.9%. When A. laidlawii strains were grown in media with cholesterol the content of cholesterol was lower and content of acetone-insoluble lipid higher. Membranes were found to comprise 35% of the dry weight of the whole cell.

The capsule of surface "fuzziness" observed on many species of mycoplasmas varies in composition and quantity. Even within species the quantity varies significantly, depending on the strain and growth conditions (Razin 1978). In cultures of M. mycoides subsp. mycoides the capsule is composed of a galactan with toxic properties (Razin 1973; Lloyd et al 1971). A. laidlawii is the only Acholeplasma capable of producing

a capsule which is composed of a hexosamine polymer which appears to be tightly bound to the membrane (Smith 1977).

Nearly all mycoplasma lipids are found in the cell membrane and are primarily comprised of phospholipids, glycolipids and neutral lipids. The trilaminar membrane is about 80-150A thick and contains protein which roughly comprises about two-thirds of the mass of the membrane, the balance being mostly lipid. The content of neutral lipid can be modulated by altering the concentration of exogenously supplied cholesterol (Smith and Rothblat 1960). Mycoplasmas have a growth requirement for sterol which is incorporated into their membranes when supplied in culture medium in a form identical to that supplied exogenously (Rothblat and Smith 1961). Acholeplasmas are capable of de novo synthesis of carotenoids which substitute for cholesterol or other sterols which have been demonstrated to be functionally proper, eg. cholestanol, ergosterol and B-sito-sterol, but can also use cholesterol. These are absorbed through tiny micelles and form hydrophobic bonds with the apolar regions of phospholipids.

The role of cholesterol in the membrane is as a regulator of membrane fluidity, allowing the organism to grow during changes in growth temperature and after alterations in the fatty acid composition of membrane

lipids (Razin 1975). In cultures of M. mycoides subsp. capri that were adapted to grow in lipid-depleted growth medium, a phase transition of membrane lipids occurred at about 25°C, which subsequently arrested growth. At this temperature most of the membrane lipids crystallised however in the native cholesterol-rich strain growth continued, though at a much slower rate than at 37°C (Rottem et al 1973).

The dependence of the Mollicutes on cholesterol for growth is associated with the absence of a cell wall. The suggestion is that cholesterol increases the tensile strength of the cell membrane, thus facilitating their survival and growth without the protection of a rigid cell wall. This is based on observations that cholesterol poor M. mycoides subsp. capri is osmotically unstable, and undergoes lysis even in growth medium (Rottem et al 1973).

Analysis of nucleic acid composition in several Mycoplasma species have shown that all studied contain both RNA and DNA in approximate ratios of 2:1 (Razin 1973). The mycoplasma genome is typically prokaryotic in consisting of a circular double stranded DNA molecule (Maniloff et al 1972 and Morowitz 1972), but differs from the genomes of all other prokaryocytes in its small size and low G + C content. Genome size varies with Mycoplasma and Ureaplasma species having a size of about 5×10^8 daltons (Table 1.3) and Acholeplasma, Spiroplasma and Thermoplasma species with a genome size of about 9×10^8

TABLE 1.3

Genome size and G + C content of members of the class Mollicutes*

Genus	Genome size (daltons)	Range of guanine + cytosine content (% G + C)
Mycoplasma	5×10^8	23 - 41
Ureaplasma	5×10^8	27 - 30
Acholeplasma	1×10^9	30 - 33
Anaeroplasma	Not known	30 - 33 and 40
Spiroplasma	1×10^9	25
Thermoplasma	$8.4\text{-}9.5 \times 10^8$	46

* from Stanbridge and Reff (1979)

a Although the G + C content of the sterol-requiring Anaeroplasma abactoclasticum has been reported as 30% (Robinson et al 1975), other candidates for the genus Anaeroplasma which are sterol-nonrequiring have a G + C content of approximately 40% (Robinson et al 1975). However, it is unlikely that these latter strains will be included in the genus Anaeroplasma (International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of Mycoplasmatales, 1977).

daltons (reviewed by Razin 1978). Assignment of the mycoplasmas to a separate class is supported by demonstration of a genome size in a considerable number of species which is half of that found in the smallest bacteria; Haemophilus influenzae with 1×10^9 daltons (Bak et al 1969). This distinction is less apparent when taking into account the genome size of the smallest rickettsia; R. quintana which has a genome size of 1.33×10^8 daltons (Myers et al 1979).

The low G + C contents, ranging from 23 to 41 mol-% are found to be at the theoretical minimum limit of G + C contents for coding DNA. Organisms with low G + C content are more susceptible to damage by ultraviolet irradiation. This would have the effect of limiting the range of potential environmental habitats for mycoplasmas and suggest a reason why mycoplasmas have entered into protected host-parasite relationships with animals and plants (Stanbridge 1976). The G + C contents of mycoplasmas can be subgrouped; Mycoplasma 25 to 29 mol-% (Neimark 1970), Ureaplasma 27.7 to 28.5 mol-% (Bak and Black 1968) and Acholeplasma 30 to 33 mol-% (Neimark 1970), with M. pneumoniae being set totally apart from all other mycoplasmas with a G + C content of 40 mol-% (Neimark 1979).

Mycoplasma ribosomes resemble those of other prokaryotes in having a sedimentation coefficient of about 70S, and 3 ribosomal RNA species 22S, 16S and 5S

(Maniloff and Morowitz 1972). Ribosomes are normally distributed randomly throughout the cytoplasm: however different fixative methods may lead to artificial arrays. RNAs are highly conserved molecules evolutionarily, as expressed both in molecular size and in base composition. Thus the G + C content of r RNA in wall covered bacteria only varies from 50 to 54%, whereas the G + C content of the total genome varies from 38 to 72% (Reff et al 1977). Mycoplasma r RNAs differ slightly from r RNAs of other prokaryotes , as indicated by their lower G + C content, 43 to 48%, which is well below that of bacterial r RNAs (Maniloff and Morowitz 1972). Data indicates that the low G + C content of the mycoplasma genome is not reflected in the G + C content of the mycoplasma r RNAs. M. capricolum with a 25% G + C DNA, has a t RNA of 53.8% G + C, only 4.4% less G + C than in E. coli and with A. laidlawii t RNA the difference is only 2% (Razin 1973).

Carbohydrates with a composition similar to those of the bacterial cell wall have not been found in mycoplasmas. However a galactose polymer (galactan) has been found to be synthesised by M. mycoides and comprises up to 10% of the dry weight of cells (Gourlay and Thrower 1968). This glucose polymer, also found in some other species of animal mycoplasmas has been found to make up the amorphous material, "fuzz", found at the

surface of the cell membrane (Domermuth et al 1964). There appears to be some difference however in carbohydrate content between species, as Morowitz et al (1962) and Razin (1963) found carbohydrate content of M. gallisepticum to be less than 3% of dry weight of cells. "Fuzziness" observed on the surface of sectioned A. laidlawii is due to a hexosamine polymer which is tightly bound to the membrane (Gilliam and Morowitz 1972). The polymer is only produced by A. laidlawii, but even in this species the quantity varies significantly between strains and growth conditions (Razin 1979).

Essentially all lipids found in mycoplasmas are associated with the cytoplasmic membrane. Total lipids of rapidly dividing cells comprise 3 to 20% of the dry weight of whole cells and 25 to 35% of the dry weight of membranes (Smith 1968). The lipid component of cell membranes is essentially composed of polar lipids, with smaller proportions of neutral and glycolipids. The proportions of these vary with the growth phase of the mycoplasmas and with the concentration of exogenously supplied cholesterol (Smith and Rotnblat 1960; Rottem and Razin 1973).

Sterol is found in all species of Mycoplasma, Spiroplasma and Ureaplasma, all of which have a growth requirement for sterol. Species of Acholeplasma (Smith and Henrikson 1966) and Thermoplasma (Langworthy et al 1972) are capable of incorporation of sterol into their

membranes when supplied in culture. When it is not present, these organisms can substitute sterols with other planar polyterpenes, eg. carotenol in the case of A. laidlawii. Bonding of the sterol is thought to occur by hydrophilic interaction with apolar regions of the phospholipids, since only lipid depletion of the membrane interferes with sterol uptake (Razin et al 1974).

The presence of acidic glycerophospholipids (polar lipids) is the only common feature of the lipids among all membranes of the Mollicutes (Smith et al 1973). Phosphatidyl glycerol and diphosphatidyl glycerol are ubiquitous. Unique polar lipids are found in Acholeplasma, Thermoplasma and Ureaplasma, including ceramides, phosphoglycolipid and aminophospholipids. Glycolipids on the cell surface were first indicated in studies of M. pneumoniae where an antiserum to the glycolipids was found to agglutinate the cells.

Glycolipids have been found distributed among the mycoplasmas with the glycosyl diglycerides occurring most frequently amongst the Acholeplasma and Ureaplasma and in many Mycoplasma species. Others frequently found are polyterpene glycosides and acylated sugars with a special type in Thermoplasma, diglycerol tetraether.

Mycoplasma proteins are concentrated into specific sites, eg. ribosomes and cell wall, as well as comprising

enzymes and proteins dispersed throughout the cytoplasm. Cell walls are primarily made up of proteins. They are comprised of peripheral proteins, eg. proteins held onto the membrane by ionic bonds or salt bridges (Singer and Nicholson 1972), integral proteins which are tightly bound to membrane lipids, and membrane-bound enzymes, eg. ATP and electron transport enzymes. The synthesis of proteins is consistent with mechanisms found in other free-living organisms, being synthesis from free amino acids. The molecular weights of mycoplasma membrane proteins range from 15 000 to over 200 000 (Morowitz and Terry 1969), well within the range of other biological membrane proteins (Guidotti 1972).

1.2 (iv) Cultural characteristics

Cultural characteristics deemed to be typical of mycoplasmas are:-

(1) The ability to grow in cell-free fluid media producing polymorphic organisms and filaments, with the smallest of these capable of passing bacteria-tight filters.

(2) The development of characteristic colonies on solid media, ranging from 15 to 600 µm.

Growth on soft agar medium is characterised by the formation of a ball-shaped colony embedded in the

agar surface, which is surrounded by a thin ring of surface growth, giving a typical "fried-egg" appearance (Fig. 3.1). This colonial morphology varies between species, eg. the porcine mycoplasma M. hyorhinis forms a typical "fried egg" colony, while M. hyopneumoniae grows on the surface producing a granular appearance. Colonial morphology is not of great value in discriminating between species as growth depends on the supply of essential media components and turgidity of the agar. As mentioned, growth of the colony is generally into the medium via penetration of interstices. Subculture therefore is performed by smearing inverted agar blocks with colonies on fresh plates and not by attempting passage of surface growth with a wire loop. Colony size, while not being a differential feature is an important feature of the Ureaplasma. Initially, embedded colony diameters of 10 $\pm$ 5 µm are often recorded, however these are a result of nutritionally inadequate culture medium. Typically they achieve a mature size of 15 to 25 µm, and were initially referred to as T-strains of mycoplasmas (tiny - form colonies).

On occasion mycoplasma colonies may have to be differentiated from morphologically similar artefacts (pseudocolonies) or from colonies of L-phase bacteria. Pseudocolonies may occur as a result of air bubbles in the medium, microdrops of condensed water, cells, and cell aggregates; all of which are not transferable. They may also occur as result of precipitation of medium

components. These pseudocolonies consist of circular agglomerates of granules which may or may not be radially arranged. They are composed of calcium and magnesium salts of fatty acids, and their size is dependant on the species from which the serum is derived, the presence of cholesterol and the distance between pseudocolonies (Brown et al 1940 - cited by Hayflick 1969). Differentiation from mycoplasma colonies may be made on morphological differences when stained with Dienes' (1968), failure to take DNA stains (Organick 1966), failure of inhibition by addition of merthiolate or formalin to medium and "growth" after autoclaving of the medium.

The distinction of mycoplasmas from L-phase bacteria (1.2(i)) may be required as they exhibit some morphological and cultural characteristics of mycoplasmas. L-phase bacterial colonies are generally larger, with heavy surface markings and greater opacity. They are more difficult to subcultivate, and the elementary bodies are larger than those of mycoplasmas (Edward 1954 - cited by Maxmion 1965). Other differentiating features are lack of mycelial forms and sharing of some cell antigens with the parent bacteria. Many forms are unstable and will revert to the parent bacteria upon removal of the inhibiting factor, eg. penicillin. Metabolically they resemble the parent bacterium, eg. pattern of sugar metabolism, and sensitivity to some respiratory pathway poisons, but

due to differences in respiratory pathway mechanisms between fermentative and non-fermentative mycoplasmas, these different sensitivities are of limited value. An important distinction is that L-phase bacteria do not have a growth requirement for cholesterol and do not incorporate it into the cell membrane (Marmion 1965). Further differentiation rests on morphological differences between colonies stained by Dienes' stain (Dienes' 1968), however empirical differences may now be shown by determination of G + C content and DNA hybridisation studies (1.1(i), (ii)). This is applicable if a relationship is postulated between a particular L-phase organism and a particular mycoplasma.

Culture in liquid phase is particularly useful in isolating organisms poorly adapted to the medium. Adaptation to the medium occurs on serial passage which facilitates later growth on solid medium of similar composition. Combinations of fluid and solid medium, ie., diphasic medium, are also of value in obtaining adaptation.

Embryonated hens eggs and tissue cultures may be used as culture substrates, but due consideration has to be given to contaminating mycoplasmas in these systems. Early studies of T-strains were misleading for this reason (Shepard 1958); fresh isolates of T-strains becoming contaminated with M. gallisepticum of egg origin. Contamination of cell cultures with mycoplasmas has become a major problem with up to 60% of cultures

being found to be contaminated (Hayflick 1969), primarily with M. orale type 1, M. hominis, M. salivarium, M. arginini and M. hyorhinis. The introduction of the latter, a porcine mycoplasma, has been proposed to be through use of trypsin of porcine origin in cell culture. Further discussion of mycoplasmas in cell cultures will follow.

1.2 (v) Medium composition

(a) Components

Despite the need for definition of the nutritional requirement of mycoplasmas for culture and investigation of their metabolic capabilities, completely defined media have been described for only 2 species: M. mycoides subsp. mycoides and A. laidlawii (Rodwell and Mitchell 1979). However despite this degree of definition some components are still added as crude mixtures providing several requirements at once, eg. serum, and unknown growth factors eg. yeast extract.

The major function of the serum component in mycoplasma medium is to provide fatty acids and cholesterol in a readily assimilable, non-toxic form for membrane synthesis (Razin 1973). As the Mycoplasma cell is

bounded only by a lipoprotein membrane it is extremely susceptible to the action of surfactant lipids.

M. mycoides is unable to synthesise or alter the chain length of either saturated or unsaturated fatty acids (Rodwell and Peterson 1970). These then must be supplied in serum which may comprise between 10-30% v/v of culture medium. The main source is either human ascitic fluid or mammalian serum, of which human, horse and pig are the most nutritious (Marmion 1965).

Of major importance is the supply of cholesterol which is essential for growth of all mycoplasmas except Acholeplasma species which can synthesise carotenol (Rothblat and Smith 1961) replacing cholesterol in the plasma membrane (1.2 (iii)).

Fraction C, an unidentified growth factor is present in fatty acid poor BSA. It has subsequently been shown (Rodwell 1969) that as well as binding fatty acids it also disperses cholesterol.

The serum protein is not utilised directly, however the albumin component functions as a carrier and detoxifier of fatty acids required for growth (Razin 1973). Its role as a regulator of uptake of sterol, fatty acids and lipids, protects the organism from surfactant lipids.

Most mycoplasmas utilise an unidentified component

contained in the watery extract of yeast cells. Extraction is performed at 80°C (Herderschee 1963) and is used as a supplement in culture medium usually at 10%. Yeast extract is required by M. hominis for vigorous growth zones, however at this level it is partially inhibiting to many strains of Ureaplasma in primary culture (revieved by Shepard and Masover 1979).

Further study by Smith et al (1975) found a fraction of the extract which produced a 21-fold increase in growth of Thermoplasma. Analysis of this fraction suggests the growth factors to be a polypeptide with an approximate molecular weight of 1 000, comprised of 8 to 10 amino acids. It is suggested that these polypeptides may function in providing essential amino acids in a permeable form, however due to the large amount required it may function as a collector of trace elements or protect the cell surface from H^+ ions. A further study by Rylance et al (1979) found that many tissues as well as yeast extract contain a water soluble, heat-stable material similar to glycerol-phosphoryl-R_A which might be involved. When this and like compounds were used all proved negative.

Some mycoplasmas can obtain their requirements for nucleic acid synthesis by degrading native RNA and DNA (Edward and Fitzgerald 1952; Razin and Knight 1960). DNA derived from calf thymus is usually added. The minimal requirement of M. mycoides

for nucleic acid precursors are met by the bases guanine, thymine and uracil. Nucleotide synthesis does not occur, however many nucleotide interconversion pathways have been found (Mitchell and Finch 1977). The requirement for A. laidlawii are less well understood, but four nucleosides, adenosine, guanosine, cytidine and thymidine are recognised (Razin 1962).

The vitamins, coenzyme A, riboflavin, nicotinamide and thiamine are essential for growth of M. mycoides, however, no response was found with the supplementation of biotin, folonic acid or vitamin B_{12}. Pantotheine, but not pantothenate can be substituted for coenzyme A for growth of avian strain J (Lund and Shorb 1966).

A. laidlawii strain A requires nicotinic acid, pyridoxin or pyridoxal, thiamine, and, in the absence of thymidine, folinic acid (Razin and Cohen 1963). Tourtellotte et al (1963) found that coenzyme A was essential for strain B and that it could not be substituted with pantothenate, pantetheine not being tested.

Spermine and spermidine, while not essential were observed to stimulate growth of M. mycoides (Rodwell 1967). Polyamines frequently stimulate growth in a range of bacteria (Guirard and Snell 1964) and consequently a requirement by mycoplasmas would not be surprising.

Carbohydrate fermenting strains require metabolisable saccharide as a carbon and energy source. Large amounts are required as energy is low. M. mycoides can also utilise glucose, maltose, mannose and fructose, while A. laidlawii can use glucose or maltose but not fructose, galactose, sucrose or lactose (Razin and Cohen 1963).

Glycerol is essential for some strains of bovine and caprine M. mycoides (Plackett 1967) while addition of lactate in rotated-tube cultures stimulates growth and spares the requirement of glycerol in aerated cultures.

Acetate is an essential component in medium for A. laidlawii strain B, being incorporated into saturated fatty acids (Pollack and Tourtellotte 1967) and into carotenoids (Smith and Rothblat 1962).

The only nutritional requirement defined for growth of ureaplasmas is urea (Ford and MacDonald 1967). The main source of urea is in the mammalian serum enrichment of the culture medium. Normal horse serum has proved to be the best source of enrichment for growth of T-strains of mycoplasmas. The function of urea in the growth of ureaplasmas remains a perplexing problem as studies by Masover et al (1974) and Masover et al (1977) do not support the presence of urea as an essential factor, while Kenny and Cartwright

(1977) found that growth was proportional to urea concentration. Despite this latter observation there is no known pathway in ureaplasmas by which this energy can be trapped or utilized (Razin 1978).

Considering that ureaplasmas are found commonly in the urogenital tract, the case for dependence on urea for growth is well supported.

(b) Culture conditions

Most strains grow well within an osmotic pressure range of between 7 to 14 atmospheres (Leach 1962) with optimum growth occurring between 10 to 14 atm. However it is interesting to note that pathogenic mycoplasmas grow well in the host at an osmotic pressure of 7.6 atm. The sensitivity to osmotic lysis is influenced by the presence of stabilizing cations and by the lipid composition of the membrane.

Most strains grow well within the pH range of 7 to 8, while T-strains require pH 5 for optimum growth (Shepard and Lunceford 1965). Carbohydrate fermenting strains produce large amounts of lactic acid, therefore strongly growing strains need to be well buffered, however, the amount of added buffer salt is limited by the hypertonicity tolerated.

The effect of aeration of cultures depends upon the respiratory system of the species, whether pre-

dominantly fermentative, flavin-terminated or cytochrome-linked. Many species are aerobes or facultative anaerobes, but some prefer microaerophilic conditions with the addition of carbon dioxide, while others are strict anaerobes.

Dependence on temperature is expressed by the range of temperature permitting growth, and by the temperature that is optimal for growth. Temperature requirements throughout the class are uniform with one major exception, A. laidlawii. This species has a lower optimal temperature than most (30°C for freshly isolated strains) and is able to grow at 22°C. In addition M. gallisepticum has been shown to have an optimal temperature of 38°C (Gill 1962), which is slightly higher than that recorded for other "parasitic" mycoplasmas of 36° to 37°C. For human Mycoplasma species the lower temperature at which growth will occur is between 20 to 27°C, while several T-strains will grow at 22°C (Hayflick 1969). On the contrary Thermoplasma has an optimum temperature for growth of 59°C, however the growth range spans the limit of 40 to 62°C (Belly et al 1973).

(c) Suppressors

Culture medium for mycoplasmas contains a number of bacterial suppressors which allow selective culture and isolation of mycoplasmas.

Absence of a cell wall allows growth in the presence of antibiotics which inhibit cell well synthesis, eg. penicillin, bacitracin and polymixin B. Penicillin (Ampicillin) is frequently included to prevent bacterial overgrowth which enters by way of contamination or in the primary inoculum.

Since slow-growing Mycoplasma colonies are often overgrown by bacteria and fungi found in natural materials, thallium acetate is added at a low concentration to prevent the growth of other than mycoplasmas (Hayflick 1969).

Avoidance of substances which bind to sterol in the cell membrane, eg. saponin, digitonin and polyene antibiotics filipin and amphotericin B is important, as sterol requiring species are lysed by substances which complex with cholesterol. Another polyene antibiotic, nystatin, has little activity against cholesterol dependent species of mycoplasmas (Newnham and Chu 1965) indicating different functional classes within the polyenes (Cirillo et al 1964).

1.2 (vi) Growth on cell cultures

Robinson et al (1956) reported the first isolation of a mycoplasma from a contaminated HeLa cell culture. Since then, mycoplasmas have been established as common

and often troublesome contaminants. Primary cell cultures are rarely contaminated, however continuous cell culture lines, and those subject to high passage are more frequently contaminated. All types of cell cultures are susceptible, including primary or continuous cultures, fibroblastic, epithelial and lymphocytic cultures. Species vary in their ability to cytabsorb (eg. M. hyorhinis and M. fermentans avidly cytasorb), consequently the methods of demonstrating contamination vary. For species which cytabsorb, use of specific immunofluorescence or non-specific DNA-fluorochrome staining are best, while for non-cytabsorbing species dilution - colony count procedures are recommended (Barile 1979).

The main contaminants involved are bovine, human or porcine species of mycoplasmas. In studies reviewed by Barile (1979) 45% of cultures were contaminated with bovine species, ie. M. arginini, M. bovis, M. bovoculi and Acholeplasma species, 33% consisted of human oral species, ie. M. orale, M. hominis and M. salivarium, while 21% of isolates were M. hyorhinis. The source of these contaminants has been associated with, the source of medium components, ie. bovine strains introduced via contaminated bovine serum and M. hyorhinis via contaminated porcine trypsin and bovine serum, and inadequate quality control in the case of human mycoplasma contaminants. The origin of other less frequent contamination by murine, avian or canine species of

mycoplasmas is probably via contaminated organs used to establish primary cell lines.

To prevent contamination, methods to control the sources and reduce the spread of mycoplasmas are used successfully (reviewed by Barile 1979). The major spread is by aerosol, contaminated equipment or reagents. Methods routinely employed to reduce the risk of contamination are: the use of primary cell cultures, avoidance of mouth pipetting, filtration of bovine serum and trypsin prior to use, use of laminar flow hoods which are cleaned regularly and discarding of contaminated cells immediately.

Elimination of mycoplasmas from cell culture is very difficult, prevention being the most effective method of quality control. Methods used include the use of antibiotics (eg. tetracyclines, kanamycin) tricine, triton-X, prolonged heat and inclusion of specific antiserum. Although some methods have generated optimism there is no universally effective way of eliminating mycoplasmas from cell cultures.

Cell cultures were used for the initial isolation of M. pneumoniae (Chanock et al 1960) and M. hyopneumoniae (Goodwin and Whittlestone 1963) as these agents were first considered to be viral diseases. Growth in cell cultures can be detected by the type of cytopathic effect (CPE), by subculture to agar medium and specific

immunofluorescent staining of infected cells. Contamination may go unnoticed and cell changes may be minimal, resulting from depletion of amino acids, sugars and nucleic acid precursors. Changes can normally be reversed by replenishment with fresh medium. Many species of mycoplasmas however are cytopathic, producing characteristic CPE eg. M. gallisepticum produces a stunted, abnormal growth with round degenerated cells with "moth-eaten" edges, while M. hyorhinis causes destruction of the entire monolayer (reviewed by Barile 1979). Detachment of cells from the glass is a characteristic of fermenting mycoplasmas which produce large amounts of acid, eg. M. hyorhinis, M. pulmonis, M. capricolum and A. laidlawii. The effects of arginine depletion by some species of Mycoplasma and Acholeplasma (1.2(vii)) produces alterations in protein synthesis, cell division and growth, lymphocyte blast formation and virus propagation.

The intimate association between adhering mycoplasma and their host cells is an important factor in their pathogenesis. The close proximity may allow concentrations of excreted H_2O_2 to become toxic to host cell membranes, prior to degradation by catalase and peroxidase present in the extracellular body fluids. Hydrolytic enzymes produced by mycoplasmas may cause damage to the host cell membrane and enzymes and lipids may even be introduced directly into the host cell. The possibility of membrane fusion has been discussed

in section 1.(2)(ii), where only minimal evidence was available to support this pathogenic mechanism.

Adherence to the epithelial linings of the respiratory tract and the urogenital tract allow the classification of mycoplasmas as surface parasites. While preventing their elimination by the action of the ciliated epithelium and urine, this pericellular association also allows utilisation of the fatty acids and cholesterol of the host cell membrane.

The nature of the eukaryotic cells to which the mycoplasmas attach has been established for M. pneumoniae, M. gallisepticum and M. synoviae. Since attachment of these is affected or even abolished by pretreatment of host cells with neuraminidase (Gesner and Thomas 1965) it is accepted that these mycoplasmas attach to sialic acid moieties on the host cell surface. The residual "background" attachment, however, may involve receptors other than sialic acid.

Treatment of mycoplasmas with neuraminidase does not affect their ability to adhere, so sialic acid can

be ruled out as a binding site (Manchee and Taylor-Robinson 1969a). The binding sites of M. pneumoniae and M. gallisepticum are most likely of protein nature, since heat, merthiolate or trypsin pretreatment abolishes their ability to bind to tracheal epithelial cells, erythrocytes or to plastic or glass beads (Gesner and Thomas 1966). Nevertheless, M. hominis and M. salivarium sheets adhering to plastic could not be dislodged by trypsin (Manchee and Taylor-Robinson 1969a, b) making generalisations regarding the chemical nature of binding sites impossible.

1.2 (vii) Metabolism

Important metabolic characteristics used to distinguish between genera are the ability to ferment carbohydrates and catabolise urea and arginine.

Fermentative Mollicutes are described as having a flavin terminated respiratory chain (VanDemark 1969). This characterisation is based in part on the presence of flavins and the absence of cytochromes and quinones. Only small amounts of quinones are present in Acholeplasma, Mycoplasma and Spiroplasma species (Hollander et al 1977) raising the doubt that they comprise any energetically useful respiratory chain systems. The same study reported the absence of cytochromes in all fermentative and non-fermentative strains tested. The hallmark of oxygen-

linked electron disposal mediated by flavoprotein catalysis is peroxide formation. Peroxide is potentially toxic and may be inactivated by peroxidases and catalases. While it has been implicated as a major pathogenic factor produced by mycoplasmas, ever since it was shown to be responsible for lysis of erythrocytes by mycoplasmas in vitro (Razin 1969), it is clear that hydrogen peroxide (H_2O_2) by itself does not determine pathogenicity. In humans pathogenic M. pneumoniae produced no more H_2O_2 than less pathogenic strains. A. laidlawii is non-pathogenic but produces H_2O_2 in similar amounts as the pig pathogens M. hyopneumoniae and M. hyosyoviae (Razin 1978). The bulk of H_2O_2 produced by mycoplasmas is quickly destroyed by the host catalase and peroxidase activities. For the H_2O_2 to exert its toxic affect, the mycoplasma must adhere close to the surface to maintain a toxic level which may cause damage by lipid peroxidation of the host cell membrane (Cohen and Somersen 1969). Peroxide has been demonstrated to be the major haemolysin associated with mycoplasmas (Cole et al 1968). Typically species M. pneumoniae, M. fermentans and M. orale type 2 produce rapid B-haemolysis on guinea pig cells. With the development of improved standardisation techniques for its demonstration, increased use has been made of haemolysis as a criteria for classification.

The nature of the energy sources of the non-fermentative species is less clear. Schimke et al (1966) showed that M. arthritidis derives sufficient energy from

metabolism of arginine via the arginine dihydrolase pathway Subsequently most non-fermentative species tested have been found to possess arginine deiminase (Tully and Razin 1977), the enzyme catalysing the first reaction in this pathway, and of those tested all contain ornithine transcarbamylase, the second enzyme (Barile et al 1966). It can be assumed that the complete dihydrolase pathway exists in species shown to contain arginine deiminase (Rodwell and Mitchell 1979). The end-product of arginine metabolism, as well as metabolism of urea by Ureaplasma species (1.2(vi)) is ammonia. Stalheim and Gallagher (1977) related the presence of ureaplasmal epithelial lesions to ammonia levels by adding ammonia or urease to uninfected tissue cultures which produced a duplicate effect. In the case of other cell cultures contaminated with arginine-splitting mycoplasmas it has been demonstrated that the toxic effects are often due to the depletion of the essential amino acid, arginine, rather than the toxic end-product (Barile 1973).

Attention has been drawn to the major metabolic difference between Acholeplasma and Mycoplasma; the ability to synthesise carotenoids by the Acholeplasma substituting for exogenously supplied cholesterol. All species of Acholeplasma are capable of synthesising carotenoids and contain specific enzymes of the biosynthetic pathway for polyterpenes starting from acetate.

Lipolytic activity of mycoplasmas resulting in the production of small spots consisting of calcium and magnesium soaps, or a "pearly" film containing cholesterol and phospholipid was described by Edward (1954). The production of these characteristic deposits however varies widely between culture mediums. Best results were obtained by using a combination of porcine serum and egg yolk in the medium which improved sensitivity and standardised the culture medium (Fabricant and Freundt 1967), allowing differentiation between species in the ordinary microbiology laboratory.

The reduction of 2, 3, 5-triphenyltetrazolium from its colourless oxidised form to reduced red formazan (Jensen 1964) by M. pneumoniae, has been utilised in the growth inhibition test. Inclusion of this in the medium as a colour indicator, allows detection of growth suppression caused by the addition of specific antibody.

1.2 (viii) Reproduction

(a) Replication of the Genome

Studies of genome replication, primarily with M. gallisepticum have revealed essentially the same mechanisms as in other prokaryotes. Quinlin and Maniloff (1972) presented evidence which showed the DNA growing-point to be membrane bound. In later studies attachment to the

membrane-bleb-infrableb site was found to be the site of DNA-replication (Maniloff and Quinlin 1974). It is unknown whether the bleb plays any role in DNA replication but it is likely that both the cell attachment site and the DNA-replication complex are localised in the same region of the cell.

The mechanism of segregation of daughter chromosomes of mycoplasma is unknown. One possibility is that the membrane plays an active role in chromosome segregation by a primitive form of mitosis. Evidence for this has been presented by Bredt (1970 - cited by Bredt 1979) and Bredt et al (1973) who observed active constrictions of the cell membrane during cellular division of M. hominis.

(b) Cell division

The cell multiplication of mycoplasmas does not differ in any fundamental way from that of other prokaryotes (Razin 1973). Binary fission has been described as the characteristic mode of reproduction of M. gallisepticum (Morowitz and Maniloff 1966), M. pneumoniae (Bredt 1968), M. hominis (Robertson et al 1975) and other species. While this may be true for the first two species, in which a specialised terminal structure appears to play a central role in cell division, it is doubtful if it is the sole mode in any species. For binary division to occur it must be fully synchronized with genomic replication, and this is by no means always

the case. If cell division lags behind genome replication, multinucleate filaments result. This occurrence is in keeping with the findings that synthesis of lipids, DNA and proteins are not synchronized. Formation of branching filaments during growth may be influenced by the lipid composition of the medium. Once formed these filaments fragment into mononucleate daughter cells (Turner 1935). A third means of replication is the process of budding, in which a budlike process is "pinched off" to form daughter cells.

The process of reproduction in the two motile species M. gallisepticum and M. pneumoniae appears to be similar, being associated with the terminal structure. Cell division is always preceded by the appearance of a second bleb, usually at the opposite pole, with the cell then dividing by constriction at the middle (Morowitz and Maniloff 1966). While replication of the genome and separation of the daughter cells are not closely coordinated events for most species, it appears that the specialised terminal structure is associated with the machinery for synchronisation in these two species.

1.2 (ix) Mycoplasmaviruses

The isolation of a virus that could infect mycoplasmas was first reported by Gourlay (1970). Since then more than 50 isolates have been reported (reviewed by

Cole and Ward 1979 and Maniloff *et al* 1979), all being DNA viruses. Most mycoplasmaviruses have been isolated from *Acholeplasma laidlawii* strains, with only a few of these strains having been shown to produce plaques. A consequence of this is that mycoplasma strains used to propagate viruses may also carry viruses, however, the carrier state does not appear to interfere with virology studies.

Further virus particles, morphologically distinct from those isolated from *Mycoplasma* and *Acholeplasma* species have been isolated from *Spiroplasma* cultures. Since it has not been possible to propagate these, little biochemical and virological data is available.

Since the occurrence of these bacteriophages is ubiquitous, further studies to clarify the viral role in *Mycoplasma* and *Spiroplasma* disease states are required. Their occurrence will also allow new approaches to the study of the molecular biology of the mycoplasmas.

1.3 Strain identification of mycoplasmas

Serology is an indispensable means for the identification of organisms, both for diagnosis and comparison of antigens for taxonomy. From the point of view of routine diagnosis strains are commonly identified by growth inhibition, gel diffusion and immunofluorescent

techniques. However, to obtain all the information possible for taxonomic purposes, a wide range of serological methods have been used, including agglutination, agglutination of erythrocytes coated with fragments of organisms and metabolic inhibition.

1.3 (i) Growth inhibition

Growth inhibition techniques have been very useful in the study of mycoplasmas. The technique measures a decrease in the number of colonies on an agar medium or reduced tubidity in broth, as a result of inclusion of specific antiserum. The method using antibody impregnated paper disks was first described by Huijsmans-Evers and Ruys (1956 - cited by Hayflick 1969). Antibodies active in the test are induced by immunisation with membrane preparations (Kahane and Razin 1969), and the results of the method are considered to be little influenced by antigenic variations between strains of a species (Kenny 1979). The use of antiserum produced in rabbits is recommended (Marmion 1965) because of simplicity, economy of antigen and specificity. The specificity may result from the action on cell wall antigens or because antibody detectable by other methods, eg. CFT, may not inhibit growth due to the bulk of activity against intra cellular antigens.

Modifications of this method may be used with M. pneumoniae and include the estimation of reduced colony

numbers after plating a standard dose of organisms with antiserum (Jensen 1963) and inclusion of colour indicators, eg. triphenyl tetrazolium chloride or phenol red with glucose, in the growth medium.

1.3 (ii) Gel diffusion

The agar gel diffusion test allows detailed analysis of the antigenic pattern of mycoplasmas. The technique allows identification of minor antigenic relationships which may only be detected by other methods with difficulty and permits demonstration of minor antigenic differences between strains that are otherwise indistinguishable.

The test is fairly insensitive, requiring both concentrated antigens and strong antiserum for detection of antigen and antibodies to mycoplasmas (Kenny 1971). Antigenic comparison has been made for a wide range of species: human species (Taylor-Robinson et al 1963) Thermoplasma (Bohlool and Brock 1974) and a variety of other species (Lemcke 1965; Kenny 1969).

The main problem with the technique is that with the Mycoplasmatales many of the antigens are located in the membrane and hence poorly soluble, unlike the cytoplasmic fraction, in aqueous solvents. The use of non-ionic detergents to extract membrane antigens, together with washing and staining of the precipitin lines greatly

enhances the sensitivity of the method (Hollingdale and Lemcke 1969). However, problems arise with M. pneumoniae glycolipid precipitin lines as these are not stable to washing, and therefore must be photographed directly (Kenny 1979).

Use of this method has led to the demonstration of a substantial degree of cross-reaction between human Mycoplasma species, other than M. pneumoniae which is antigenically distinct (Lemcke 1965). The complexity of antigenic components demonstrated were well beyond those revealed by Taylor-Robinson et al (1965a). Work by Lemcke contributed significantly to the discussion regarding whether M. mycoides var. mycoides and var. capri be kept as subspecies, by showing that according to their precipitin lines these organisms were more closely related than any other serological types examined.

1.3 (iii) Immunofluorescence

The highly specific immunofluorescent technique was used extensively for the identification of M. pneumoniae (Chanock et al 1962; Marmion and Hers 1963). The test is species-specific giving a similar result to that of growth inhibition. Marmion (1965) records that absorption of antiserum and conjugate with tissue or other powders representing antigenic components in the medium as being highly desirable. While the method is laborious and

technically difficult to perform it has the advantage of detecting accidental mixtures of serotypes in agar cultures (Lemcke 1964) which are otherwise difficult to detect.

1:3 (iv) Production of specific immune serum

The major problems in preparing antigens of organisms in the Mycoplasmatales are the poor yield of organisms and the contamination of the antigenic preparation by medium components. Cultures harvested for the preparation of antigen should be in log phase or late log phase since aging cultures show substantial biochemical changes in lipids and proteins (Rottem and Greenberg 1975) and substantial degradation of structure (Boatman and Kenny 1970). Control of pH for organisms which ferment glucose or utilise arginine or urea is important since immunogenicity was impaired by low pH for M. pneumoniae (Pollack et al 1969), a problem which may be overcome by the use of HEPES buffer. For these reasons cultures for antigen should be harvested in log phase or late log phase.

Washing procedures are sufficient to remove highly soluble components, eg. albumin but other components may be concentrated into the pellet. Yaguzhinskaya (1976) produced evidence that specific serum components coprecipitate with the pellet during centrifugation. While these components cannot be removed by washing they may be removed by sucrose gradient techniques.

Extensive precipitation of medium components in medium prepared with whole peptones and filtered yeast extract may be partially overcome by substituting with soy peptone and yeast extract dialysate. The serum component which undergoes ready denaturation *at* 37°C presents a larger problem. This problem may be circumvented to a degree by using "agamma sera" (serum chemically fractionated to a reduced gammaglobulin content). The RIA, ELISA, CFT and IFAT are highly sensitive to medium component antibody. Accordingly it is important to prepare antiserum to the serum component of the culture medium for immunization, and to use this serum to demonstrate that the antigen tested does not contain medium components.

To overcome the immunologic problem associated with the medium component use has been made of homologous serum to supplement culture broths. Most commonly "agamma" rabbit serum has been used for antigen production, prepared for antiserum production in rabbits. Serologic test antigens were then grown in the same medium.

The method of choice of antiserum production to a particular immunogen largely depends upon the purpose for which the immunogen is to be used. If the antiserum is to be used for detection of as many antigens as possible of an organism, the intensive immunisation with large amounts of organisms and Freund 's incomplete adjuvant is warranted. If the antigen is highly immunogenic or the serological testing system is highly sensitive

then a less intensive programme may be followed. Intensive immunisation is required for production of antibody suitable for growth inhibition studies on agar (Clyde 1964). For production of highly specific antiserum lower doses of immungen and shorter immunisation schemes are used. Such antiserum may be used for typing or organisms by the immunofluorescent method.

1.4 Antibody detection

Several techniques discussed in this section have also been used for antigenic definition and where relevant, attention will be given to the role. Reviews of the serology of mycoplasmas have been presented by Marmion (1965), Purcell et al (1969) and Kenny (1979).

1.4 (i) Agglutination

Use has been made of the agglutination reaction in serological work with human mycoplasmas. When the tube agglutination test was used for study of the antigenic structure of organisms, large amounts of antigen were required. Since, then the test has been adapted to slide agglutination which requires a smaller amount of antigen. The test is useful in detecting small differences in surface antigenic structure between strains.

Agglutinating antibody titres with hyperimmune serum are as great in magnitude as those obtained with the complement fixation test; therefore being of similar sensitivity. The sensitivity of the test was increased for avian mycoplasma work by the use of antiglobulin (Adler and Damassa 1964). The tanned erythrocyte method appears to be more sensitive than the CF method, as antibody in convalescent serum was detected by this method, when CF tests were negative (Taylor-Robinson et al 1965b).

Further use of the reaction has been made in the development of a growth agglutination test which incorporates organisms, complement and specific antiserum in microtitre wells. Clumping of organisms in the bottom of the well confirms the presence of specific antiserum.

The ability of some species of Mycoplasma, eg. M. gallisepticum and M. pneumoniae to haemagglutinate erythrocytes has been used for the measurement of antibody. Presence of specific antibody inhibits haemagglutination, and it seems the sensitivity of this reaction is at least as sensitive as complement fixation, but less sensitive than metabolic inhibition for the quantitative measurement of antibody.

Colonies of some mycoplasma species have the ability to haemadsorb sheep erythrocytes, use being made of this in the identification of mycoplasmas by pretreating with specific immune serum, prior to application of the

erythrocytes. Inhibition of haemadsorption indicates specificity of the antiserum for the isolate.

1.4 (ii) Indirect haemagglutination, latex agglutination

Indirect haemagglutination was first used by Cottew (1960) in the study of M. mycoides, when crude polysaccharides were used to sensitise sheep erthrocytes. Tanned erythrocytes coated with M. pneumoniae sonicates have been used to demonstrate antibody rises in humans infected with M. pneumoniae and M. hominis, however lack of uniformity of erythrocytes have led to problems in maintaining reproducibility. While indirect haemagglutination tests for mycoplasmas were found to be sensitive and specific, their value is diminished by lack of uniformity and instability of the antigen-RBC complex. To overcome these problems mycoplasma antigen may be complexed to latex particles. This method of antibody detection was found to be as sensitive as complement fixation, but not as sensitive as the indirect haemagglutination technique.

1.4 (iii) Complement fixation

Since it was first used with M. mycoides (Campbell and Turner 1936 - cited by Hayflick 1969) the complement fixation test is found in most laboratories involved in the study of mycoplasmas. Many isolates have been studied

by this method, although those from birds which agglutinate erythrocytes, have been more commonly studied by haemagglutination inhibition. Antigen for the complement fixation test first prepared in liquid medium by (Chanock et al 1962) and the method was used for epidemiological studies for serodiagnosis of M. pneumoniae infections.

The complement fixation test has a major advantage in that the antigens tested need not be soluble as required in most other serologic tests, therefore it is highly useful in measuring antibody to glycolipids. The test is usually carried out with whole organisms or sonicates, and yields a titre which is the sum of surface and intracellular antigen and complement fixing antibodies that are present. The major antigens present have been demonstrated to be lipids for M. fermentans and M. pneumoniae, heat-stable proteins for M. pulmonis and carbohydrate containing antigens for M. mycoides subspecies mycoides.

1.4 (iv) Metabolic inhibition

The metabolic inhibition test measures inhibition of growth in liquid medium, which is assessed by the formation of a metabolic product which can be detected by a colour reaction, eg. acid from glucose, ammonia from urea and arginine, or the reduction of tetrazolium. The addition of specific antibody inhibits growth and the

formation of the end product; the end point can be measured by the titration of the antibody against a fixed number of "colour changing" units of the organism. Antibodies detected are those active against membrane antigens, eg. glycolipids in the case of M. pneumoniae and membrane protein for A. laidlawii (Williams and Taylor-Robinson 1967; Dorner et al 1976).

In general metabolic inhibition reactions are more likely than growth inhibition tests to reveal heterogeneity within a species, since the metabolic inhibition test requires far less antibody. This is understandable as the growth inhibition on agar is less likely to demonstrate intraspecies heterogeneity as the end point can be measured only by zone diameters and not by serum dilution.

1.4 (v) Fluorescent antibody

The indirect method of fluorescent antibody testing for serodiagnosis is very useful because antibodies to the specific immunoglobulin classes can be determined (Biberfeld and Sterner 1971). These may also be determined by use of other antibody capture methods using anti-α, anti-μ and anti-γ globulins.

1.4 (vi) Radioimmunossay and ELISA

Brunner and Chanock (1973) developed a radio-

immunoassay for the detection of antibody to M. pneumoniae by using organisms grown in ^{14}C oleic and palmitic acids as antigen. The organism - antibody complexes were precipitated with antiimmunoglobulin. Further modifications (Brunner et al 1973) allowed detection of specific immunoglobulins.

The enzyme-linked immunoabsorbent assay (ELISA) described by Engvall and Perlmann (1971) has been adapted for the detection of antibody and antigen of M. pneumoniae (Voller et al 1976 - cited by Kenny 1979) and M. hyopneumoniae (Bruggmann et al 1977). The advantages of the test are that performance of the test is simple, radioactive reagents, are avoided and conjugated reagents are available which permit detection of antibody to specific immunoglobulin classes.

1.5 Immune response to mycoplasma infection

The immune response of humans and animals to mycoplasma infections has been reviewed by Whittlestone (1973) and Fernald (1979).

1.5 (i) Humoral immunity

The study of the progressive appearance of immunoglobulin in the serum of naturally challenged cattle

infected with M. mycoides (Pearson and Lloyd 1972) found that IgM always appeared in the serum, but that IgG tended to be associated with the more severe forms of the disease and did not always appear in mild cases. IgA was not detected in the serum. The progressive appearance of immunoglobulins in the serum of mice infected with M. pulmonis (Cassell et al 1974) demonstrated IgM levels peaking by 4 weeks, IgG_1 and IgG_2 by 5 weeks and IgA by 6 weeks; by single radial diffusion in gel (qualitative) and IMF (quantitative) methods. In this study the nature of cells infiltrating the peribronchial and perivascular spaces was investigated by IMF and histologic methods. Cells producing IgM appeared early and were followed by cells producing IgG_1 and IgG_2. Cells producing IgA were present as early as 2 weeks and became the principal type by 3 weeks. Results of these studies suggest that these serum antibodies were produced locally and diffused into the general circulation and that antibody may be a major component of acquired immunity to M. pulmonis.

It appears that serum from some convalescent or hyperimmunized animals will protect the recipient from the development of mycoplasmal pneumonia, but does not prevent colonization of the upper respiratory tract. The passive transfer of serum from convalescent and subcutaneously immunized cattle to susceptibles was found to provide protection from subsequent natural challenge (Lloyd and Trethewie 1970; Masiga and Windsor 1975).

Pearson and Lloyd (1972) further attempted to define the protective component of serum by comparing serum known to be protective and others that were nonprotective. No quantitative differences were revealed between the IgM and IgG specific immunoglobulins contained. Similar protection has been conferred by immune serum in pigs exposed to infection with M. hyopneumoniae (Lam and Switzer 1971b) and M. hyorhinis (Gois et al 1974). In the latter study it was found that M. hyorhinis colonized the upper and lower respiratory tracts, but not extrapulmonary sites. In this experiment both IgM and IgG given intraperitoneally to piglets conferred considerable resistance to challenge.

The protective role of humoral antibody was further investigated in piglets from sows vaccinated prior to farrowing with a Tween 80 antigen extract of M. hyopneumoniae in paraffin. Colostral antibody to M. hyopneumoniae was detected by IHA in both sows milk and serum of piglets (Durisic et al 1975a). Levels were high in piglets during the first week but soon fell to low levels which persisted to between the second to eighth week of life (Durisic 1975b). This passive antibody did not greatly interfere with humoral response to vaccination at 7 weeks of age. At slaughter at the end of the fattening period only 20% of vaccinated piglets from vaccinated sows had gross lesions of EPP, while 50% of unvaccinated piglets from vaccinated sows and 80% of control piglets free of M. hyopneumoniae antibody at 7 weeks of age had gross

lesions. While the presence of circulating IHA antibody was associated with a reduced prevalence of gross lesions, the presence, class and protective role of antibody in the respiratory secretions was not established.

The presence of circulating antibodies, IgM and IgG have been detected in sputa of patients with M. pneumoniae infection (Biberfeld and Sterner 1969, 1971) by IMF and radioimmunoprecipitation (RIP). In secretions IgG_1 and IgG_2 antibodies were detected first at 2 weeks, IgM at 4 weeks and IgA by 5 weeks. It was felt that these IgM and IgG antibodies were probably derived from serum, as a result of transudation following bronchial inflammation. Despite this a closer association between the circulating immune system and respiratory infection is suggested by the local synthesis of IgM and IgG by large numbers of plasma cells near bronchi in mice infected with M. pulmonis (Cassell et al 1974) and in humans infected with M. pneumoniae (Biberfeld and Sterner 1971). While it is evident that IgM and IgG may afford some degree of protection when present as local antibody on the respiratory epithelium its mechanism of action is not yet defined. Potential opsonic activity of antibody promotes phagocytosis of mycoplasmas by macrophages, and IgM and IgG antibodies cause fixation of complement leading to lysis.

1.5 (ii) Secretory immune system

Detection of respiratory mucosal antibody (reviewed by Fernald 1979) to mycoplasmas has become feasible through the recent introduction of very sensitive tests: immunofluorescence, radioimmunoassay and radial diffusion. Earlier studies of bronchial washings for antibody of M. pneumoniae infection were negative due to insensitive tests. Indirect immunofluorescence was used by Biberfeld & Sterner (1971) to detect IgA antibody to M. pneumoniae in sputum samples of patients with pneumoniae. IgA antibody was present in all 31 cases, and was the only antibody detected in 5 cases, with IgG present in 24 and IgM in 13 cases.

The secretory IgA molecule is a dimer with attached secretory piece with sedimentation coefficient of 11S, while the serum form (monomer) is primarily 7S, although 9S, 11S and 13S often occur (Tomasi 1968). Immunoglobulin A and albumin are the most abundant proteins in nasal sections (Remington et al 1964); IgA was found to comprise between 21 and 50% of total nasal washing protein in 15 volunteers (Rossen et al 1966). Part of the IgA in nasal secretions is derived from serum 7S form which is not modified in any way, but by far the majority is in the 11S dimer form, which is produced locally and actively secreted.

IHA antibodies to M. hyopneumoniae were detected in tracheobronchial secretions of pigs within 2 weeks of infection (Holmgren 1974c). Some of the pigs which were positive 2-4 weeks post-inoculation had no detectable IHA antibody in their serum. In one pig the major IHA activity in tracheobronchial secretions was localised in the fractions containing IgA. Brunner et al (1973) measured IgA specific M. pneumoniae antibody in nasal washings and in sputa in a group of experimentally infected volunteers. Men with pre-existing titres of nasal IgA antibody showed most resistance to the challenge infection. Levels of serum antibody were statistically unrelated to resistance. This report confirmed that resistance to M. pneumoniae infection was more dependant upon secretory than serum antibodies. It is not known whether there is a similar relationship between IgA level in porcine respiratory secretions and protection against M. hyopneumoniae infection.

Since IgA has been found to specifically inhibit attachment of Streptococci to epithelial cells (Williams and Gibbons 1972) it is likely that this is the role of IgA in preventing mycoplasma infection of the respiratory epithelium. Prevention of adsorption of mycoplasmas would prevent the secretion of toxic substances, eg. H_2O_2 in apposition to epithelial cells, thereby allowing inactivation by catalase and peroxidase before cell damage can occur. Unattached mycoplasmas would also be more readily cleared by the muco-ciliary clearance

mechanism. IgA has little opsonic activity (Beinenstock and Perey 1972) but may activate complement by the alternative pathway.

1.5 (iii) Cell-mediated immunity

Clinical evidence to support the role of cell-mediated immune phenomena in the development of immunity to mycoplasmal respiratory disease was provided in cases of M. pneumoniae infection in patients suffering from primary antibody deficiency syndromes (Foy et al 1973). Whilst disease was more severe these patients recovered and stopped excreting the organisms, suggesting that part of the host defense to M. pneumoniae infection may be mediated by cellular immune mechanisms. Further association of this part of the immune system is indicated by the histological similarity between the peribronchial and perivascular lesions to that of delayed hypersensitivity (Fernald 1969). While direct evidence of local cell-mediated response has not been demonstrated, CMI responses exhibited in vitro by lymphocytes and macrophages from extra respiratory sites support the system's involvement (Fernald 1979). Lymphocyte stimulation in vitro to M. pneumoniae organisms appears soon after natural infection of humans and persists for many years (Biberfeld et al 1974). Similar lymphocyte stimulation has been demonstrated in guinea pigs (Brunner 1974 - cited by Fernald 1979). Occurrence of this phenomena in humans

infected with M. pneumoniae, however, appears to be less common than lymphocyte transformation (Biberfeld 1974).

The effect of T-cell depletion on M. pulmonis infection of mice was studied by Denny et al (1972). Infected, T-cell depleted mice had less marked perivascular and peribronchial lymphoid cuffs and had greater numbers of mycoplasmas in the respiratory tract, suggesting that recovery from infection was also T-cell dependant.

Mycoplasma pneumoniae has also been demonstrated to generate delayed cutaneous hypersensitivity in humans (Mizutani et al 1971; Suzuki 1976). Further studies on skin sensitivity reactions to M. hyopneumoniae (Roberts 1973) show development of nodules 24 to 72 hours after intradermal inoculation, which are typical of delayed type hypersensitivity reactions on histological examination. As with the in vitro parameters these reactions indicate that T-cells are sensitised during infection but shed no light on their role in the local immune response in the lung.

T-cells could operate by stimulating macrophages to kill or stimulate polymorphs to migrate toward mycoplasmas, or by stimulating the development of antibody producing cells in the lungs.

1.5 (iv) Immunosuppression

There is an increasing body of evidence that indicates mycoplasma infection can cause a suppression of the host's immune response, be it initially in the early course of infection. In respiratory diseases caused by M. hyopneumoniae (Adegboye 1975), M. pneumoniae (Liu et al 1972) and M. mycoides (Gourlay 1964), it is cell-mediated immunity which is selectively depressed as there are no reports of decreased antibody response. However, with M. arthritidis infection, both humoral and cell-mediated components are suppressed. Adegboye (1978) postulates that the ability of mycoplasmas to linger on in lesions may be due to this phenomenon.

Clinical evidence of possible mycoplasma mediated immunosuppression mediated by mycoplasmas has been observed as persistence of organisms at sites of infection, anergy to intradermal testing and potentiation of concurrent bacterial infections.

For instance, M. mycoides subsp. mycoides has been recovered from contagious bovine pleuropneumonia lesions at least 10 months old (Turner 1954), M. hyopneumoniae has been recovered as late as 262 days after infection and M. pulmonis has been reisolated from rat lungs up to 712 days after inoculation (reviewed by Whittlestone et al 1972) but no evidence has been presented to support the view that persistence is due to immuno-suppression.

The ability of M. pneumoniae infection to produce anergy to tuberculin in humans suggests an association with immunosuppression. Fiala et al (1974) reported that an originally Mantoux positive woman became anergic to tuberculin during the acute phase of her illness, but regained her responsiveness after treatment. Similarly, Biberfeld and Sterner (1976) found in Sweden that 22 out of 36 patients infected with M. pneumoniae were tuberculin negative, with 16 of these patients becoming responsive when tested several weeks or months later. It has also been observed that cattle with CBPP lesions were anergic to intradermal injection of purified membrane antigen of M. mycoides, whereas cattle recovering or that had apparently recovered from the disease gave typical delayed-type skin reaction to the antigen (Windsor et al 1978).

The potentiation of bacterial infections by M. pneumoniae indicating an immunosuppressive effect was reported by Liu et al (1972) who showed M. pneumoniae augmented pneumococcal septicaemia of hamster . It was found that active multiplication of M. pneumoniae was required for this effect to occur.

Results of in vitro tests during infection of pigs with M. hyopneumoniae support the role of mycoplasmas in suppression of cell-mediated immune response. Adegboye (1975) detected no CMI response to both lymphocyte transformation and the delayed-type hypersensitivity skin tests during the first few weeks post-infection, when pigs were expected to have

pneumonia and a high titre of organisms. However, these responses were recovered in later stages when pigs were recover ing from the active phase of the infection. It was also noted that peripheral blood lymphocytes collected from infected pigs responded poorly to phytohaemagglutinin (PHA), when compared to control pigs (Adegboye 1978). Kaklamanis and Pavlatos (1972) also showed that lymphocytes obtained from rats during active infection with M. arthritidis failed to undergo blast formation in the presence of PHA, indicating some form of immunosuppression.

Suppression of humoral immunity has only been observed in association with M. arthritidis. When injected concurrently with Pseudomonas phage 5 humoral antibody response was reduced to the phage (Kaklamanis and Pavlatos 1972). Similarly antibody response was reduced to Escherichia coli or Straphylcoccus aureus infection when membrane antigens were injected concurrently (Bergquist et al 1974).

Mechanisms for mycoplasma-induced immunosuppression is unclear, however several have been proposed. Anergy to intradermal testing may be due to engagement of the majority of responding cells at the infection site (Adegboye 1975), leaving insufficient for delayed-type skin reaction. It was also proposed that because of a paucity of sensitised lymphocytes in peripheral blood during the pneumonic stage, the peripheral blood lymphocytes

population might not give sufficient blastogenic response. A more attractive explanation could be in the observation that the organism is not readily ingested by phagocytic cells.

1.6 Diseases of man and animals caused by mycoplasmas

Members of the Mycoplasmatales are the causative agents of a wide variety of animal diseases which most commonly result in respiratory and joint infections, together with infections of the urogenital system. Most of these infections are chronic in nature, suggesting an intimate host-parasite relationship.

1.6 (i) Pneumonia

Pneumonia caused by mycoplasma infection is a major disease of livestock throughout the world. Of major importance are contagious bovine pleuropneumonia, contagious caprine pleuropneumonia, chronic respiratory disease of poultry, enzootic pneumonia of pigs, together with atypical pneumonia of humans. These conditions and others will be reviewed in the following section on mycoplasma infections of the respiratory tract.

1.6 (ii) Arthritis

While it has long been recognised that arthritis may be associated with the classical mycoplasmal diseases, contagious bovine pleuropneumonia, contagious caprine pleuropneumonia and contagious agalactia, many species are primary arthritogenic agents. Arthritis in farm animals primarily results from infection of the joint, with organisms most commonly entering from the blood. The majority of cases of arthritis are polyarticular, and bilaterally involve the larger joints. Young animals appear to be particularly susceptible to mycoplasma-induced arthritis (reviewed by Gourlay 1981).

Typically infection follows an acute suppurative phase which is usually followed by a chronic disease featuring synovial cell proliferation, mononuclear cell infiltration and cartilage destruction. The ability of some of the species which cause arthritis, to survive in the respiratory tract provides a potential reservoir for continued infection.

Infection of calves with M. mycoides subsp. mycoides (small colony type) often result in development of arthritis. Subcutaneous inoculation of cattle will produce arthritis, particularly if they have been pre-inoculated with M. mycoides subsp. mycoides polysaccharides (Hudson et al 1967). Arthritis can also be caused by M. bovis following intratracheal injection of gnotobiotic calves (Gourlay et al 1976).

Arthritis in sheep and goats can be induced by subcutaneous inoculation of M. agalactiae (Watson et al 1968) while strains of M. mycoides subsp. mycoides (large colony type), M. mycoides subsp. capri and M. sp. F38 have not been observed to produce arthritis after experimental inoculation.

M. hyorhinis and M. hyosynoviae are able to produce spontaneous arthritis in pigs (Ross and Duncan 1970; Duncan and Ross 1973) with potential to cause serious economic loss. M. hyorhinis causes arthritis in piglets less than 8 weeks of age. Although organisms can be readily recovered up to 57 days post-infection, they are only rarely recovered from joints after this time (Cole and Ward 1979). Further studies using immunofluorescence have demonstrated M. hyorhinis antigen in synovial membranes several months after viable organisms can no longer be isolated suggesting non-cultivable myco-plasmas may be responsible for the continued inflammation.

Arthritis in poultry is most commonly caused by M. synoviae, while M. gallisepticum has only been rarely recovered from field cases (reviewed by Jordan 1981).

While M. pulmonis has been isolated from spontaneous arthritis in rats only intravenous inoculation will produce arthritis in both rats and mice (reviewed by Cassell et al 1981). Strains of mice and rats have been observed to vary markedly in their susceptibility and

response to infection. M. arthritidis also produces a naturally occurring arthritis in rats which is acute and self-limiting.

The occurrence of mycoplasma arthritis in various animal species with a very similar pathological character of lesions as seen in rheumatoid arthritis (RA) of humans, and the ability to experimentally induce arthritis, have been the main reasons for searching for evidence of mycoplasmal infection in the joints of patients with RA. Initial attempts to recover mycoplasmas from cases of RA disease yielded spurious results which have not been repeated, probably because mycoplasma -contaminated cell cultures were used (reviewed by Cole and Ward 1979). Despite encouraging results of Williams (1968) who isolated M. fermentans from a significant number of cases of RA disease, these results have not been repeated. However a recent study by Hernandez et al (1977) reported arthritis as a sequel to M. pneumoniae infection. In a further study on immunocompetant patients, M. pneumoniae isolated directly from the joint of a hypogammaglobulinaemic patient (Taylor-Robinson 1981), further raising the question of mycoplasma involvement in human arthritis. Ureaplasmas have also been isolated from joints of 2 patients with hypogammaglobulinaemia who had septic arthritis (Webster et al 1978 and Stuckey et al 1978 - cited by Taylor-Robinson 1981).

1.6 (iii) Urogenital tract infection

Mycoplasma species commonly populate the urogenital tract of animals and humans, existing as pathogens and commensals. Examples of known pathogens are M. bovingenitalium (Erno 1967) a cause of mastitis and possibly granular volvovaginitis in cattle (Afshar et al 1966), M. bovis a cause of mastitis (Hale et al 1962) and possibly infertility and abortion (Gourlay and Howard 1979), M. agalactiae a cause of mastitis in sheep and goats (reviewed by Cottew 1979), M. gallisepticum a cause of infertility and foetal death in chickens and turkeys (reviewed by Jordan 1979) and Ureaplasma urealyticum a cause of non-gonococcal urethritis (NGU) in humans (reviewed by Taylor-Robinson et al 1981).

Ureaplasma urealyticum is now the second member of the Mycoplasmatales to be established as a disease producing pathogen in humans. Taylor-Robinson et al (1977) produced NGU by intra-urethral inoculation of human volunteers with the organism, which was recovered from urinary tract lesions and responded to tetracycline therapy. A problem encountered in establishing the role of mycoplasmas as a cause of NGU is that is is not the only possible cause, as Chlamydia trachomatis accounts for probably 50% of cases of NGU. Other studies using antibiotics which differentiate between ureaplasma and chlamydia or selectively suppress mycoplasmas eg. rifampicin and minocycline indicated an aetiological assoc-

iation of mycoplasmas with NGU. Recently Taylor-Robinson *et al* (1981) isolated a new mycoplasma on 2 occasions from cases of NGU. At this stage there is no data relating this mycoplasma to NGU although its presence in the urethra, ability to adhere, and specialised structures including a flask shaped appearance with a terminal rodlike structure have been associated with pathogenicity in other mycoplasmas.

M. hominis has been incriminated in a number of human uro-genital infections (reviewed by Taylor-Robinson and McCormack 1979). The most striking evidence to support the pathogenic role of *M. hominis* is its isolation from the blood from patients who had febrile abortions, but not from women who had afebrile abortions.

Uncertainty over the aetiological role of many mycoplasmas in reproductive failure is highlighted by the isolation of several species from aborted foals (revieved by Lemcke 1979). While isolates of *A. laidlawii*, *A. equifetale*, *A. hippikon*, *M. equigenitalium*, *M. subdolum*, *A. oculi* and *M. bovigenitalium* have been obtained from many internal sites, there is no answer to the question whether the invading mycoplasma killed the foetus or whether it invaded subsequent to foetal death.

Many reports indicate association between isolation of *M. bovigenitalium* and disease in cattle (reviewed by

Gourlay and Howard 1979). Problems associated with these isolates are herd infertility, chronic vesiculitis of bulls, granular vulvovaginitis and abortion. As the organism is common in the urogenital tract of healthy cows and bulls, the role of M. bovigenitalium as the primary cause of these infections is regarded as no more than a possibility. However, M. bovigenitalium has been reported from outbreaks of mastitis, a role which has been confirmed experimentally (Ernø 1967).

The ability of the respiratory pathogen M. gallisepticum to infect the urogenital tract of chickens and to cause salpingitis is a major problem to the poultry industry (reviewed by Jordan 1979). While reduced egg production is a problem attributed to the infection, the transmission of the organism between generations via contaminated fertilized eggs creates a major problem in the control of the disease.

1.6 (iv) Conjunctivitis

Several pathogenic mycoplasmas have been frequently isolated from infectious bovine keratoconjunctivitis (IBK); these include M. bovirhinis, M. bovis and Ureaplasma spp. but their role in the disease is not clear-cut (reviewed by Gourlay 1981). M. bovoculi is most frequently isolated, however inoculation into the eyes of gnotobiotic calves failed to produce any sign of the disease.

A. oculi and M. conjuctivae have been incriminated in infectious keratoconjunctivitis in sheep and goats (Al-Aubaidi et al 1973; Trotter et al 1977). Inoculation studies support their pathogenic role, however when goats are inoculated subconjunctivally a more severe syndrome is produced than occurs naturally, making interpretation of results difficult.

Isolation studies on cats indicate that 100% of cases of conjunctivitis are infected with mycoplasmas (reviewed by Rosendal 1979) while only 20% of healthy cats harbour mycoplasmas. Most commonly M. felis is recovered, however M. gateae has been found. Several investigations have established a close association between M. felis and conjunctivitis while the condition has been induced in kittens following conjunctival instillation of M. felis broth cultures (Cole et al 1967).

1.6 (v) Infections of the central nervous system

Nervous symptoms are infrequently caused by mycoplasma infection; with the best known disease being "Rolling" disease of mice caused by M. neurolyticum (Cassell and Hill 1979). The disease is characterised by the abrupt onset of continuous rolling which persists for several hours terminating in death. Rats are also affected in a like manner following iv. injection of a neurotoxin; however, the disease has not been observed naturally.

Ataxia in turkeys is a clinical sign of M. gallisepticum infection which is associated with brain lesions. Pale areas in the cerebrum are observed (reviewed by Jordan 1979) and result from arteritis of the meningeal and parenchymatous arteries and arterioles.

M. hyopneumoniae is classically thought to have a tropism for ciliated epithelial cells of the porcine respiratory system. Recently Williams (1980) has demonstrated that this organism is capable of propagation on central nervous system (CNS) ciliated ependymal cells in vitro and in vivo. Whether this is reflected in CNS pathology is unknown and nervous symptoms have not been associated with infection in the past.

CNS complications of a wide variety have been reported in association with M. pneumoniae infection and include syndromes of psychosis, meningitis, meningoencephalitis, cerebellar ataxia, transverse myelitis and Gillian-Barre radionucleopathy (reviewed by Clyde 1979). These syndromes may be mediated by potentially pathological immune mechanisms as demonstrated by Biberfeld (1971 - cited by Brunner 1981) who reported the presence of antibodies to brain tissue in 80% of pateints with M. pneumoniae infection.

1.6 (vi) Sequellae of mycoplasma infections

The possibility that immune mechanisms may be involved in the pathogenicity of M. pneumoniae as well as cytopathic alterations has been suggested by previous seroepidemiological evidence. Other syndromes (reviewed by Clyde 1979) which have been described in association with M. pneumoniae infection are otitis media, bulbous myringitis, erythema and multiforme exudativum, nervous symptoms, haemolytic anaemia and polyarthritis.

Evidence suggests that the disease caused by M. pneumoniae infection may, in part, be immunologically mediated in a previously sensitised host. Smith et al (1967) noted that respiratory disease in humans which failed to respond serologically to M. pneumoniae vaccination was more severe than that in unvaccinated controls. Observation of more severe disease in sensitised hosts and the detection of M. pneumoniae antibodies in sera of young children from which M. pneumoniae could not be isolated, led to the search for other sensitising agents with similar shared glycolipid antigens (Brunner 1981). Subsequently it was found that these antigenic determinants were shared with certain foods, including spinach, and bacteria, which may sensitise children to M. pneumoniae infection. The occurrence of vaccine enhanced disease has been associated with previous sensitisation with a low potency vaccine in several infections including Rocky Mountain

spotted fever and measles (reviewed by Smith et al 1967).

Immunologically mediated complications of M. pneumoniae infection occur in extrapulmonary sites. While nervous sequelae as previosly mentioned occur, antibodies known as cold agglutinins directed against the I-antigen of erythrocytes, a shared antigen with M. pneumoniae, develop in 50% of patients with mycoplasmal pneumonia (reviewed by Brunner 1981). Elevated cold agglutinins are more prevalent among severe cases complicated by haemolytic anaemia (Fiala et al 1974).

1.7 Mycoplasma infection causing pneumonia and respiratory tract infection in man and animals

Mycoplasmas have been demonstrated to be a primary cause of pneumonia in humans and most species of domestic animals. In animals they cause chronic respiratory disease with high morbidity and low mortality. Especially where animals are intensively housed, eg. pigs, poultry and laboratory rodents, where a large number of debilitating factors may predispose to the appearance and severity of pneumonia.

1.7 (i) M. pneumoniae infection of humans

Recent reviews of M. pneumoniae infections of man have been published by Clyde (1979) and Brunner (1981).

Mycoplasma pneumoniae represents one of the most common aetiologic agents of the lower respiratory tract of man. In a 12 year surveillance period in the U.S.A., infection rate varied from 2% in endemic years to 35% in epidemic years (Foy et al 1979). In the study 20% of all pneumonias were due to M. pneumoniae infection. Epidemics of atypical pneumonia followed a cyclic pattern, occurring at intervals of 4 to 6 years (Lind 1971; Foy et al 1979). Primary atypical pneumonia most commonly occurred in children 5 to 19 years, denoting children as an important vector of the disease.

Most infected people have relatively mild symptoms which are only infrequently accompanied by complications. A dry cough, malaise, fatigue and rhinitis are most frequently observed. It has been estimated that approximately half the infections occurring in adults are completely asymptomatic; probably reflecting some degree of protective immunity (Clyde 1979). However, natural reinfection with M. pneumoniae may occur within 1 to 3 years periods in children (Fernald et al 1975) and asymptomatic disease may reoccur in adults within 5 years (Foy et al 1979); indicating that protective immunity following natural infection may be limited in degree and duration.

1.7 (ii) Mycoplasma pneumonia in cattle, sheep and goats

Ruminants are infected by some highly pathogenic species of mycoplasma which are the cause of diseases of major importance, including contagious bovine pleuropneumoniae (CBPP) and contagious caprine pleuropneumonia (CCPP). The agent of CBPP, M. mycoides subsp. mycoides was first isolated as long ago as 1898 (Nocard and Roux - cited by Gourlay and Howard 1979) and while eliminated from many countries still causes serious economic loss in many parts of Africa, India and possibly Mongolia.

Reviews of CBPP have been made by Hudson (1971) and Gourlay and Howard (1979). The natural disease spreads slowly and is difficult to eradicate. CBPP may be quite acute, causing death within a week or it may be chronic. The condition may become arrested by the walling off of infected lung foci, in which case the animal may appear to have recovered, but the sequestra may breakdown at any time with reappearance of symptoms and shedding of organisms. Infection is characterised by a fibrinous interstitial pneumonia with pleurisy.

Initially CCPP of goats was attributed to M. mycoides subsp. capri, however, more recently CCPP has become a less specific term and now refers to any contagious mycoplasmal pneumonia of goats. M. mycoides subsp. mycoides has been incriminated aetiologically as

well as a new strain, F38 (McMartin *et al* 1980) which closely resembles CCPP in historical reports (reviewed by Cottew 1979; Gourlay 1981). Isolates of *M. mycoides* subsp *mycoides*, large colony type, isolated from cases of CCPP have failed to produce disease in challenged cattle (Cottew and Yeats 1978).

Other species of mycoplasmas, including *M. bovis*, *M. dispar* and *Ureaplasma* spp. are frequently isolated from pneumonic lungs of calves. When inoculated into gnotobiotic calves *M. bovis* and *Ureaplasma* spp. produce "cuffing" pneumonia and *M. dispar* an interstitial pneumonia. These subclinical pneumonias in gnotobiotic calves closely resemble subclinical pneumonia present in young claves. These mycoplasma species can also be isolated from severe chronic cases of calf pneumonia from which many different species of mycoplasmas can be isolated. The exact role of these mycoplasmas in the clinical syndrome is not clear.

Investigations into atypical pneumonia of sheep by Foggie *et al* (1976) and Jones *et al* (1978) concluded that *M. ovipneumoniae*, *M. arginini* and *Pasturella haemolytica* may commonly be the aetiological agents involved in ovine pneumonia. When *M. ovipneumoniae* was inoculated endobronchially into 2 lambs only 1 developed microscopic lesions. The organism was then naturally transmitted to 6 in-contact lambs, being isolated from nasal passages only, but produced no lung lesions in

these. When inoculated in conjunction with cloned isolates of M. arginini and P. haemolytica lesions indistinguishable from field cases of atypical pneumonia were produced.

1.7 (iii) Mycoplasmosis in poultry

Subclinical respiratory tract infections may occur with M. gallisepticum, M. synoviae and M. meleagridis. Clinical disease is often observed in young birds possibly following infection through the egg, however incidence and severity of disease are subject to predisposing factors, including trauma, environmental ammonia, cold and social stress (reviewed by Jordan 1981)

M. gallisepticum (Mg) frequently causes clinical disease in chickens and turkeys, and signs observed include rales, coughing, sneezing, nasal discharge and swelling of the infraorbital sinuses especially in turkeys. While M. synoviae infection most commonly causes arthritis, it may also produce respiratory signs similar to Mg in chickens and less frequently causes sinusitis in turkeys. In M. meleagridis infection there are rarely any respiratory signs.

Chronic respiratory disease caused by mycoplasma infection has a worldwide distribution and is of major economic importance when stock are reared under intensive

management systems. Mortality in poultry is relatively rare and economic loss is caused by reduced production of layers, broilers and turkeys, downgrading of carcasses and sub-optimal hatchability.

1.7 (iv) Mycoplasma spp. causing respiratory disease in pigs

Enzootic pneumonia of pigs (EPP), which is typically a chronic pneumonia affecting pigs housed under intensive conditions, is caused by M. hyopneumoniae (Mare and Switzer 1965; syn. M. suipneumoniae, Goodwin et al 1965) infection, and is the subject of this study (reviewed in next section).

Initially it was thought that M. hyorhinis infection only played a secondary role in the production of chronic pneumonia in finishing pigs (Switzer 1967; Whittlestone 1979). Further studies demonstrated the role of this organism in the production of primary pneumonia in piglets (Friis 1971b) and that strains vary in virulence. The natural disease is characterised by pneumonia and polyserositis producing pleural, pericardial, and peritoneal adhesions, with polyarthritis in piglets.

M. flocculare was first isolated by Friis (1972) in Denmark and further isolates have been obtained from

pneumonic lung and nasal cavities (Friis 1976). Isolations of this species in the U.K. (Whittlestone 1979) have been obtained from pneumonic lung generally free of M. hyopneumoniae. Piglets experimentally inoculated with aerosols of this organism developed small pneumonic lesions and the organism was recovered from the brain and pleural and pericardial cavities (Friis 1973a, 1974c, 1976). The role of this organism in the pneumonic complex is uncertain, as infections with this mycoplasma in SPF pig herds in Denmark are not associated with clinical pneumonia (Friis 1976).

1.7 (v) M. pulmonis infection in rodents

Murine respiratory mycoplasmosis (MRM) is a major disease problem encountered in laboratory rats and mice (reviewed by Cassell and Hill 1979; Cassell et al 1981). They not only present diagnostic problems, but also interact with host physiology including changes in biological responses which may adversely effect studies in many fields eg. inhalation toxicology.

Transmission of infection may occur in utero or by inhalation of infective aerosol in the first few weeks of life (Cassell and Hill 1979). Once infected, progressive respiratory disease develops which may eventually resolve or persist for life with these animals acting as reservoirs of infection. The incidence and severity is influenced by many environ-

mental, microbial and host factors in a manner similar to chronic respiratory disease of poultry. Of particular interest is the finding of Broderson et al (1976) who showed that levels of intracage ammonia from 25 to 250 ppm significantly increased the severity of rhinitis, otitis media, tracheitis and pneumonia. In fact, the prevalence of pneumonia positive correlation with increasing ammonia levels.

M. pulmonis respiratory disease is clinically silent, with only weight loss, roughened hair coat, nasal and ocular discharges developing in the terminal stages of the disease. Correlation between clinical signs and pathological alterations is almost non-existent and lesions vary considerably between animals in the same cage. Although clinical disease is rare, epizootics of disease sometimes occur.

1.7 (vi) Respiratory tract mycoplasmas of dogs and cats

Pneumonia in dogs is very often associated with mycoplasmal infection, however, organisms are also recovered from normal lungs at a low frequency. (Rosendal 1979). M. cynos was initially isolated from a case of pneumonia (Rosendal 1972) and when inoculated endobronchially into week old pups caused severe bronchitis and bronchiolitis. The organism was recovered for a period of 3 weeks post-

inoculation (Rosendal 1978). Other species isolated from cases of distemper include M. bovigenitalium, M. canis, M. spumans and M. gateae and when incoulated into pups only M. bovigenitalium caused morphological lesions, being a mild bronchitis.

While M. felis is most frequently associated with feline conjunctivitis (reviewed by Rosendal 1979), intranasal and conjunctival inoculation of 6 to 10 week old kittens resulted in 2 developing foci of interstitial pneumonia (Tan and Miles 1974). A further isolate of M. arginini (Tan et al 1977) was examined for experimental pathogenicity but none of the 6 to 8 week old kittens developed any respiratory signs, despite colonization of the upper respiratory tract.

1.8 Detailed consideration of Enzootic Pneumonia of Pigs

1.8 (i) History

Chronic non-fatal pneumonia is frequently a major problem when large numbers of growing pigs are housed together in confined air space. Many specific agents and non-specific factors contribute to the pneumonic conditions of pigs, but the most common chronic pneumonic syndrome occurring throughout the world is enzootic pneumonia of pigs (EPP). The disease has been referred to by a variety of names, including infectious pneumonia, "virus pneumonia" as well as the current enzootic pneumonia. Since definition of the aetiologic agent the disease has also been termed mycoplasmal pneumonia of swine.

Prior to 1957 it had been presumed that a virus was the causal agent of chronic porcine pneumonia. This view had arisen as filtrates of lung suspensions passed through bacteria-retaining filters, were infective to pigs and the infectivity was unaffected by penicillin or sulphonamides (Whittlestone 1958 - cited by Whittlestone 1973).

Evidence suggesting the agent to be non-viral was first provided by Betts and Campbell (1956) who demonstrated that the establishment of EPP could be prevented by prior administration of tetracyclines. Following this, Whittlestone (1957; 1958 cited by Whittlestone, 1979) demonstrated that the size of the causal agent

agent was between 200 and 450nm and that similar sized organisms could be seen in Giemsa stained touch preparations of pneumonic lung. At this time Whittlestone postulated that this organism seen in association with pneumonia was the causal agent of EPP, most likely being a fastidious mycoplasma that could not be cultivated at that time. The organism was first grown in tissue culture (Betts and Whittlestone 1963; Goodwin and Whittlestone 1963) and then in media free of living cells (Goodwin and Whittlestone 1964). This latter report demonstrated conclusively that the agent was non-viral by repeatably passaging organisms grown in broth culture beyond the theoretical limiting dilution through which a hypothetical viral agent could have been carried. At this time the agent was cultivated in the United States by L'Ecuyer and Switzer (1963) who propagated the agent in cell culture, however the isolate lost infectivity for pigs during passage. Ma're and Switzer (1965) then reported that the agent cultivated in broth was able to produce pneumonia, confirming it to be non-viral. Colonies were cultivated from their broth cultures which were distinguishable from M. hyorhinis and Acholeplasma granularum, but it was not known whether these colonial organisms, called M. hyopneumoniae could produce pneumonia, or were the same organisms as those in broth cultures which induced pneumonia. This proof was provided by Goodwin et al (1965) who obtained colonies from broth cultures, passaged them serially to preclude mechanical carryover of other agents, and then induced EPP in pigs with the final colonies, later recovering this organism

from the affected pigs. This isolate, named M. suipneumoniae was found to be unrelated to 42 other strains of mycoplasmas by the growth inhibition tests; and the pneumonia-inducing mycoplasma isolated in broth culture of Ma're and Switzer (1965) was indistinguishable from M. suipneumoniae (Goodwin et al 1967).

Both names for this mycoplasma have been used since, and several reports of the Subcommittee on the Taxonomy of Mycoplasmatales (Subcommittee 1967, 1972, 1975) have discussed the taxonomic difficulties. While the name M. hyopneumoniae had priority as to date of publication, the original culture lodged with the American Type Culture Centre (ATCC 25617) was lost because it could not be maintained serially in sub-culture (Rose et al 1979), being subsequently replaced by another culture obtained by Switzer. However, as the passage history of this culture did not include cloning by appropriate methods, it is doubtful whether it is identical with the strain on which the description of M. hyopneumoniae is based, and hence a neotype had to be designated. As strain J, the type by monotypy of M. suipneumoniae is easier to grow and more workable than strain 11, from which it is serologically indistinguishable (Rose et al 1979) the J strain (ATCC 25934, National Culture Type Centre 10110) has been adopted as the neotype of M. hyopneumoniae. The J strain was purified prior to deposition as the type strain by 5 serial single - colony passages on

agar medium. The parent strain induces EPP but the cloned culture does not (Whittlestone 1979), however no serological difference exists between the parent and cloned strains.

1.8 (ii) Clinical Aspects

Upon initial introduction of the disease into a herd, all pigs are fully susceptible to infection, including adult boars and sows which may be overwhelmed by fulminating lesions from which they sometimes die. Initial symptoms in these cases are usually anorexia, acute respiratory distress with or without fever (Blood and Henderson 1974).

In the endemic situation, the disease is a chronic non-fatal condition (subclinical), characterised by a dry non-productive cough, retarded growth rate and ineffective feed utilisation. Where management practices and environmental conditions predispose pigs to pneumonia, lesions may be extended and complicated by secondary bacterial invaders. It is important to note that coughing and pneumonia are different criteria of the disease. Coughing is more symptomatic of bronchitis than pneumonia and it partly depends upon the influence of secondary bacteria whereas pigs with extensive enzootic pneumonia may not cough.

In cases where the disease is severe, or where extensive secondary bacterial infection occurs, occasional deaths or runting of pigs may occur. On a herd basis these symptoms cause greater uneveness in bodyweight than otherwise expected, leading to a "tail in production".

1.8 (iii) Pathology

The gross and histologic appearance of EPP lesions are described in detail in Chapter 2, 1, (i) (ii) (iii).

The progressive development of EPP lesions was described by Whittlestone (1972) who recorded that after inoculation of pigs with cultures of M. hyopneumoniae or EPP lung suspensions, gross pneumonic lesions could be detected within 7 to 10 days. Gross lesions in most pigs persisted up to 6 weeks post-inoculation and there was progressive recovery after 10 weeks. Residual lung lesions were detected in a few pigs up to 37 weeks.

The healing process following experimental EPP described by Bertschinger et al (1972)-cited by Whittlestone (1979) - is similar to that described by Whittlestone (1973) in Chapter 2. 1 (ii). In that report gross lesions were found less frequently 2 months after infection but the peribronchial reaction persisted for the full observation period of 4 months.

Pneumonia caused by M. hyopneumoniae infection in pigs is of longer clinical and pathological duration than M. pneumoniae infection in humans. In experimentally infected pigs, healing of lesions occurred after 10 weeks, however gross lesions were detected in a few pigs up to 37 weeks (Whittlestone 1972). By comparison M. pneumoniae infection of humans lasts as an acute febrile disease with coughing and malaise for approximatel 1 week. When infection is accompanied by pneumonia, lassitude may persist for 3 weeks or more (reviewed by Clyde 1979). In pigs, the pneumonia typically involves the alveoli and is characterised by infiltration of polymorphonuclear neutrophils and mononuclear cells, together with proliferation of alveolar cells producing consolidation of alveolar tissue. In humans, alveoli are usually spared with apparent consolidation being due to atelectasis caused by exudate from the bronchial infection obstructing the airway.

The duration of gross pneumonic lesions in pigs is even greater under field conditions. At normal slaughter age of 20-28 weeks, between 16-100% of lungs have been observed affected (Table 1.4). This may be attributed to the microbial complexity of the field condition (L'Ecuyer et al 1961; Gois et al 1975), which is commonly secondarily contaminated with Pasteurella multocida (Carter and Bain 1960 - cited by Smith et al 1973; Bolske et al 1980; Gois et al 1980) which augment damage in lung already injured by EPP (Smith et al 1973).

Other factors which promote the chronicity of M. hyopneumoniae infection are levels of atmospheric ammonia in pig houses which interfere with the muco-ciliary defence mechanism (Drummond et al 1978) and the accumulation of high levels of aerosol bacteria in closed or poorly ventilated airspaces. The role of these secondary bacteria is further supported by the demonstration that the number of bacterial species in the lung increase with the extent of lung lesions (Gois et al 1975). As a consequence, treatment of the condition often centres on determining what type of secondary bacteria are present and their antibiotic sensitivity. The antibiotic of choice is therefore one with a broad spectrum of activity or a combination of 2 drugs to treat the primary mycoplasmas and secondary bacteria, respectively. Application of a vaccine against known secondary invaders may reduce the severity of the condition, however it is likely that under these unfavourable conditions the effect of pure mycoplasma infection would itself be economically significant and still warrant the development of its own vaccine.

1.8 (iv) Diagnosis

Despite major advances in recent years in techniques for the isolation of M. hyopneumoniae its serological identification and detection of specific antibody, few laboratories have developed sufficient expertise to use these techniques routinely (Whittlestone 1979). Extensive use is therefore still made of non-specific methods of diagnosis (McKean et al 1976, 1979; Giger et al 1977).

(a) Non-specific methods

The presence of EPP in herds previously free of disease is indicated by a rising prevalence of lungs at slaughter with catarrhal pneumonia. Lesions may be present even in the absence of obvious clinical signs, and when present in adults is extremely suspicious. Clinically, chronic coughing may be evident in all age groups of stock.

Many of the histopathological lung changes which occur in EPP are considered indicative of, but not pathognomonic for, the disease. Pneumonia characterised by migration of polymorphonuclear neutrophils, vacuolation, enlargement and proliferation of alveolar cells, and accumulation of lymphocytes, macrophages and plasma cells in the perivascular and peribronchiolar tissues may often prove to be cases of EPP (Whittlestone 1973). As the pulmonary changes are non-specific Jericho (1977) advises that diagnosis is best based on the presence of chronic pneumonia in a large percentage of growing pigs. Caution must also be used in interpretation of changes as other pneumonic conditions may appear similar, e.g. swine influenza, Jubb and Kennedy (1963). Despite this, McKean et al (1976) advised the use of histologic examination in the diagnosis of EPP in conjunction with other tests.

In a later study (McKean et al 1979) where both non-specific and specific diagnostic methods were used on the same specimens, histopathology was a useful tool in determining EPP status of a herd. However the occurrence of similar histologic lesions in EPP free herds confirms that this test should not be used as the sole diagnostic test on an individual herd.

The examination of Giemsa-stained touch preparations of pneumonic lesions for organisms with the morphology of M. hyopneumoniae has been used as a rapid diagnostic method in the U.K. and Swiss EPP control schemes (Goodwin and Whittlestone 1967; Keller and Bertschinger 1968 - cited by Whittlestone 1979; Keller 1976). Typically M. hyopneumoniae organisms appear as delicate bipolar or ring forms with thickenings at 1 or more points (Whittlestone 1973). The complete absence of organisms of this morphology from several early pneumonic cases from a suspected outbreak is significant negative evidence; whereas the presence of large numbers of organisms with clear morphology of M. hyopneumoniae indicates the disease is virtually certain to be EPP. Problems arise when other porcine lung mycoplasmas are present. False positives may occur in cases where lungs carry M. flocculare (Whittlestone 1979) and some experience is required to differentiate M. hyorhinis that consist mainly of cocci and comma-shaped organisms with fewer ring forms. There is a good correlation between this method and isolation (Whittlestone 1979)

or the IMF and immunoperoxidase tests (Giger et al 1977). The continuing use of these non-specific diagnostic aids in EPP control schemes may be justified by the demonstration that a high degree of accuracy in diagnosis of EPP may be achieved by using combinations of non-specific with the more specific serological methods (McKean et al 1979).

(b) Specific diagnosis

CULTURE: While isolation of M. hyopneumoniae in broth followed by serological identification, is the most common specific diagnostic method used, isolation remains difficult and laborious even under the best conditions (e.g. Wilson 1976; Slavik 1976; Pijoan and Roberts 1973). Initially isolation was only achieved in a small percentage of cases (Goodwin et al 1968b) from field material due to problems with the fastidious growth requirements and overgrowth by M. hyorhinis which is much less fastidious and also produces a colour shift due to glucose fermentation. The degree of purity of medium constituents and chemical cleanliness of vessels are also very critical.

To selectively isolate M. hyopneumoniae in the presence of M. hyorhinis 4 main methods have been used. Firstly, M. hyorhinis antiserum (Goodwin and Hurrell 1970; Yamamoto et al 1971) can be incorporated at 6% into the culture medium and when used in conjunction with dilutions of the primary inoculum from 10^{-1} may selectively grew M. hyopneumoniae. Secondly, inclusion of the antibiotics kanamycin sulfate (Yamamoto et al 1971) or cycloserine (Friis 1971d) has a selective action against M. hyorhinis. Thirdly, Goodwin (1976) found that optimum growth of M. hyopneumoniae was obtained in 27% pig serum, and Whittlestone (1976a) found that addition of 5% rabbit serum gave a stonger colour change. Finally, Gois et al (1975) found that M. hyorhinis was generally dead after 5-7 weekly passages, but M. hyopneumoniae continued to grow. Failing this hyperimmune antiserum against M. hyorhinis was included and cultures further passaged.

The culture method used by Whittlestone (1979) is similar to that used by Friis (1975) and Goodwin (1977) and uses aseptically collected pneumonic tissue where feasible. If contaminated, pieces of lung are surface sterilised by boiling in water for 8-10 seconds. A 10% suspension of lung tissue is made in non-selective broth and dilutions made to 10^{-8} in both selective and

non-selective broth. M. hyopneumoniae strains that grow readily show an acid shift after 3-10 days when they should be passed. Friis (1974a - cited by Whittlestone 1979) noted that the growth of M. hyopneumoniae is strongly promoted by the roller technique. Farrington and Switzer (1976 - cited by Whittlestone 1979) recommended that a minimum of 5-10 blind passes at 3-5 day intervals should be made before calling a culture negative; Whittlestone (1979) recommended they not be abandoned until they had been incubated for 2 months.

Once a colour shift is produced in broth by the organism, the method of choice for identification is the metabolic inhibition test, using hyperimmune rabbit antiserum prepared against the neotype J strain (Goodwin 1977; Whittlestone 1979). As M. hyopneumoniae generally fails to form well defined colonies (Gois and Kuska 1975 - cited by Whittlestone 1979) the DGI test cannot be used but the GP test may give results of equal specificity to MI. Limitation of the GP test has been described by Friis (1977) who found cross-reactions between M. hyopneumoniae and M. flocculare when agar plates were incubated for prolonged periods.

The diagnosis of the disease would be greatly simplified, if direct isolations could be made from EPP lungs. Two earlier publications (Mare and Switzer 1966 ; Goodwin and Pryor 1970) report the direct isolation of M. hyopneumoniae on solid medium, however

few reports support this work.

The first report of growth of mycoplasma type colonies on solid media from pneumonia-inducing fluid cultures was from Mare and Switzer (1965) who incorporated 1% agar in fluid medium of Goodwin and Whittlestone (1964). Goodwin et al (1965) first provided details of methods and results of colony culture; incubation being at 37°C in a moist atmosphere containing 5 to 10% CO_2 in air. Convex mycoplasma colonies developed in 3 days on agar prepared from A_{22} broth media (incorporating boiled-lung tissue), and after passage maintained their ability to induce EPP. Apart from further successes in growing colonies of M. hyopneumoniae (Whittlestone 1972c; Goodwin 1972a; Friis 1971c) there appears to have been a general problem, even amongst experienced workers, in growing colonies of M. hyopneumoniae. Whittlestone (1979) reports difficulty in obtaining colony growth even from cultures already adapted to liquid medium and known to contain large numbers of colony forming units.

Direct isolation of M. hyopneumoniae on solid medium from experimentally infected and field cases of EPP was reported by Goodwin and Pryor (1970). However, subsequent batches of medium using different porcine serum did not give rise to colonies so regularly (Goodwin 1972b). Earlier Mare and Switzer (1966) mentioned they could isolate M. hyopneumoniae directly

from field cases but gave no indication of methods, subcultivation and identification used. Friis (- cited by Whittlestone 1973) was able to isolate M. hyopneumoniae directly from experimental cases but not field cases.

Growth of M. hyopneumoniae in embryonated chicken eggs was possibly first achieved by Dinter et al (- cited by Whittlestone 1979), however due to lack of suitable serologic techniques for identifying the organisms or detecting an antibody response in pigs the results are difficult to interpret. Since isolation became feasible in cell free media there have been few reports of propagation of isolates in embryonated chicken eggs. Mare and Switzer (1966) could find no evidence of growth of their American strain VP11 in chicken embryos, nor were lesions of EPP induced in pigs inoculated with the material. Strain J of M. hyopneumoniae was successfully passed via yolk sac of 6-7 day embryonated chicken eggs (Goodwin et al 1968a), and on the 17th passage representing a dilution of 10^{-32} of the original ground pneumonic lung, induced EPP in inoculated pigs, which was naturally transmitted to other pigs with M. hyopneumoniae being recovered from these.

Despite the original successful isolations of M. hyopneumoniae in plasma clot tissue cultures (Betts and Whittlestone 1963; Goodwin and Whittlestone 1963),

pig lung monolayers (Mare and Switzer 1966) and pig testicle cell cultures (L'Ecuyer 1969), this technique has been neglected since the introduction of cell free mediums. Perhaps with better cell culture techniques now available attempts to isolate M. hyopneumoniae on tissue cultures should be resumed (Whittlestone 1979). Isolates of M. pneumoniae using HeLa cells inhibited by cycloheximide have been obtained after 3 days incubation (pers. comm. T.W. Weng 1983).

IMMUNOFLUORESCENCE: Since the initial demonstration of M. hyopneumoniae organisms lining the bronchial and bronchiolar epithelium by the IMF method (L'Ecuyer and Boulanger 1970; Meyling 1971; Livingston 1971), the method has been adopted as a rapid diagnostic technique (Holmgren 1974c; Gois et al 1975; Giger et al 1977; Whittlestone 1979; Weng 1980; Amanfu et al 1980).

A problem was initially encountered by L'Ecuyer and Boulanger (1970) who found that conjugates prepared from normal and hyperimmunized rabbit antiserum tended to give a faint yellow-white coloration to the tissue sections. The non-specific staining property probably directed against porcine serum components in the material used to immunise could be removed by repeated absorption with pig liver powder, however its disappearance was accompanied by a decrease in the specific staining properties of the conjugate. In view of the potential development of antibodies in

animals against medium components in mycoplasma antigens used in their immunization, it was concluded that serum from infected pigs rather than hyperimmunized rabbits be used. Non-specific staining of frozen tissue sections was not seen subsequently in preparations stained with the various porcine globulin conjugates. In further studies porcine serum has been replaced by horse or rabbit serum in media for antigen production, (Livingston 1971; Gois et al 1975; Whittlestone 1975) to be used in rabbits. With the advent of M. hyo pneumoniae strains adapted to grow in rabbit broth medium, the indirect method applied to frozen sections has become a reliable diagnostic method (Whittlestone 1979). Amanfu et al (1980) using hyperimmune M. hyopneumoniae serum prepared in pigs for conjugation found, by means of the direct plate epiimmunofluorescence test, some cross-reaction with colonies of M. hyorhinis on agar. This was reduced by cross-absorbing concentrated conjugate with a desoxycholate extract of M. hyorhinis.

Specific fluorescence is limited to the surface of the epithelium of the bronchi and bronchioles and to exudate filling the smaller airways. The antigen appears as a granular, intensely fluorescent, yellow-green layer closely following the epithelial folds of the larger passages (L'Ecuyer and Boulanger 1970).

Fluorescence was not detected until 25 days post-infection using experimentally infected pigs, and was regularly seen in pigs killed thereafter (L'Ecuyer

and Boulanger 1970). Livingston et al (1971), again using experimentally infected pigs, demonstrated specific fluorescence as early as the 14th day post-infection, and up to the 42nd day. Active lesions are reported to give best results, due to reduced numbers of organisms in chronic lesions (L'Ecuyer and Boulanger 1970; Giger et al 1977). Giger et al (1977) also applied IMF to both frozen sections and bronchial smears. Similar results by both methods indicated that the bronchial smear method was a significant improvement in the diagnosis of EPP, however in chronic cases where there is a low number of organisms several lungs should be examined.

The IMF method is used in conjunction with culture to obtain specific diagnosis in the greatest number of cases possible. Whittlestone (1979) reported that in the Danish SPF scheme negative IMF results from suspicious pneumonias are checked culturally. Gois et al (1975) assessed the presence of M. hyopneumoniae in 39 pneumonic lungs from a severely affected herd by both culture and IMF. M. hyopneumoniae was detected in 10 by culture and IMF, 11 were positive by IMF only, and 18 were positive only by culture. This work stresses the importance of applying more than one diagnostic method. In other studies IMF has identified M. hyopneumoniae in 70% of 50 lungs with lesions obtained from abattoirs and in 73% of 49 affected lungs from piglets between 2-12 weeks of age (Meyling 1971; Holmgren 1974c).

Detection of antibody response

COMPLEMENT FIXATION (CF): Complement fixing antibodies to M. hyopneumoniae were first detected by Roberts (1968) using the direct method which used unheated serum from experimentally infected pigs. Initial use of the test on serum collected at abattoirs (Hodges and Betts 1969b) demonstrated antibodies to M. hyopneumoniae in 61% of pigs with microscopic lesions of pneumonia and in 12% of animals free of gross lesions. The test was further utilised in experimental and field studies (Hodges and Betts 1969a; Goodwin et al 1969a,b; Wallis and Thompson 1969; Roberts and Little 1970; Eskildsen and Schjerning-Thiesen 1971).

The direct modified CF test was used by Boulanger and L'Ecuyer (1968), Takatori et al (1968, 1969). Pig serum was heated to 56°C and the inactivated supplementing factors were provided by the addition of serum from calves between 3 to 8 months of age (Boulanger and L'Ecuyer 1970). Slavik and Switzer (1972) used complement provided by desiccated guinea pig serum reconstituted in serum from young, pneumonia-free pigs, and standardized production of the antigen which was heated at 50°C to 54°C for 15 minutes. Although the authors do not discuss the reasons for doing this, it presumably overcame the problems of poor complement (C') binding and procomplementary activity of the antigen. Eskildsen (1975) showed that porcine antigen-antibody complexes would not fix guinea pig C' optimally unless the heat labile porcine complement component C' 1q was present.

Demonstration of low levels of antibody in pig serum are often difficult as these are usually very procomplementary (Whittlestone 1973; Eskildsen 1973 - cited by Etheridge and Lloyd 1980). To overcome this problem, Etheridge and Lloyd (1980) used a complement dilution method; by testing one low dilution of serum in the presence of a range of C' concentrations the procomplementary effect for that serum is kept constant and the amount of C' fixed by antibody - antigen complex can be calculated. The test also uses 1% serum from young pigs to enhance complement fixation which is reduced in heated serum (Boulanger and L'Ecuyer 1968; Slavik and Switzer 1972).

During the course of experimental EPP, CF antibodies can be first detected after 2 to 3 weeks and reach maximum titres after 1 to 9 months (Boulanger and L'Ecuyer 1968; Roberts 1968; Hodges and Betts 1969a; Roberts and Little 1970). Thereafter, titres progressively fall so that by 9 to 14 months they are low (Boulanger and L'Ecuyer 1968; Goodwin et al 1969a). During natural outbreaks of EPP, antibodies develop to M. hyopneumoniae (Takatori, 1968, 1969; Roberts 1968; Hodges and Betts 1969b; Wallis and Thompson 1969). Isolates of M. hyopneumoniae from America (strain 11), Canada (strains 29 and 33), and Britain (J strain isolates 6234 and 10 110) are all antigenically similar by this method (Roberts and Little 1970).

The CF test exhibits a high degree of specificity. Cross reactions could not be demonstrated between pig serum containing CF M. hyopneumoniae antibody and M. hyorhinis or A. granularum antigen (Takatori et al 1968; Boulanger and L'Ecuyer 1968). However, Hodges and Betts (1969a) did find that 2 pigs infected with A. granularum and 1 of 16 infected with M. hyorhinis produced low-titre cross reactions with M. hyopneumoniae antigen. CF antibody was detected in 5% of serum samples from 6 pneumonia-free herds and in a further 8 of 34 samples from 2 herds believed to be free of EPP, but affected with other types of pneumonia (Hodges and Betts 1969b). In a larger study Woods et al (1976) found CF antibody in 9.4% of 458 serum samples from 8 EPP-free herds, compared with 51.7% of samples from EPP infected herds. Titres recorded in EPP-free herds were also generally lower than titres recorded across infected herds. False negatives in infected pigs have also been reported by Roberts and Little (1970) and at low dilutions the variable procomplementary activity of porcine serum may be responsible (Eskildsen 1972). The occurrence of non-specific positive reactions are most likely due to cross-reactions with antibodies against similar glycolipid antigens shared by other organisms (McCormick et al 1974). Thus the CF test cannot yet be used as a reliable check for the continuing absence of EPP in herds, since the significance of positive reactions cannot be readily interpreted.

IMMUNOFLUORESCENCE: The indirect IMF method has been used for the detection of antibodies to M. hyopneumoniae (Meyling 1972 - cited by Whittlestone 1973). Antibodies were first detected 3 weeks post-infection with maximum fluorescence being recorded at 6-8 weeks. Of 265 serum samples collected at slaughterhouses 67% were positive when serum was diluted 1:10. Six of 375 serum samples from EPP-free herds gave trace reactions, which when re-evaluated were considered negative.

INDIRECT HAEMAGGLUTINATION: Use has been made of the IHA in detecting serum antibodies to M. hyopneumoniae, however the technique has not received widespread acceptance due to technical difficulties. Goodwin et al (1969a) when testing pigs 4 weeks post-inoculation failed to detect antibodies, however IHA titres were detected at 16 to 18 weeks when CF titres were low. Holmgren (1974c) found that 2 to 5 week old piglets in infected herds were negative, but many 10 to 12 week old pigs were positive as were a few gilts and sows. IHA titres were found to be high in inoculated pigs when slaughtered up to 60 weeks post-infection.

Difficulties were encountered by Lam and Switzer (1971a) as they failed to demonstrate IHA titres with untreated mycoplasmas. This might have been due to a lack of attachment. After treating antigen with sodium lauryl sulphate, dialyzing and finally adding thimerosal, and by using pig erythrocytes they found 92% of experimentally infected pigs with gross lesions had developed IHA titres (Lam and Switzer 1971b).

Furthermore the IHA test is difficult to standardize, as it has been found that it is often difficult to obtain reproducible results due to variation in the suitability of sheep (Hodges 1969) and porcine erythrocytes (Switzer 1972). Holmgren (1974c) made improvements by using formalin-treated tanned porcine erythrocytes and sonicated antigen, enabling the preparation of large batches of sensitized erythrocytes which could then be stored in small aliquots for use.

While early field results of Lam and Switzer (1971a) suggest that the IHA is not very specific, later studies by Gois (1972) gave hope that the test would be useful in the field diagnosis of EPP. The former study indicated that several supposed EPP-free herds were infected with M. hyopneumoniae despite contrary clinical and pathological evidence. In the latter study 2 of 120 pigs in EPP-free herds had low IHA titres, while 179 of 373 pigs in infected herds were positive to M. hyopneumoniae.

LATEX AGGLUTINATION: To overcome problems of erythrocyte variability Slavik (1976) developed the latex agglutination test to detect antibodies to M. hyopneumoniae. These were detectable by 3 weeks post-infection and remained detectable until slaughter at 24 weeks, by which time gross lesions had resolved. Latex agglutination titres have been reported to persist for 48 weeks after experimental infection (Slavik 1976).

METABOLISM INHIBITION: Results of MI with EPP have not been as successful as attempts to demonstrate MI serum antibody to mycoplasmas in man. Goodwin et al (1969a) detected non-specific inhibitory substances in serum of some pigs both before and after infection. Takatori (1970 - cited by Whittlestone 1973) was unable to detect MI antibody in post-inoculation serum.

INDIRECT IMMUNOPEROXIDASE (IIP): The ENZYME-LINKED IMMUNOSORBENT ASSAY (ELIZA) : test was used by Bruggman et al (1976) to examine serum from EPP infected and non-infected herds. Drops of M. hyopneumoniae cultured in broth were used as the antigen substrate. In infected herds there was a 96% positive correlation between the serological test and presence of the disease, whereas all serum from EPP-free herds were negative. The test was shown to be more sensitive than the CFT, especially in the case of chronically infected pigs. Other advantages of the test were its high specificity as no cross reactions with M. hyorhinis or M. flocculare were obtained, and its ability to detect separate immunoglobulin classes.

A quantitative ELIZA for the detection of M. hyopneumoniae antibody (Bruggman et al 1977) was developed using pigs experimentally infected with EPP, which became positive as early as 2 weeks post-infection. All serum tested was positive between 3 and 50 weeks post-infection. While the test is highly sensitive some cross-reactions with M. hyorhinis were detected. When compared with the

CF test (Armstrong *et al* 1980) using samples from 18 pigs with suspected pneumonia, all samples were CF positive while only 13 were ELISA positive. Further work by Armstrong *et al* (1980) using sonicated whole cells as antigen found minor cross reactions between *M. hyorhinis* antiserum and *M. hyopneumoniae* antigen as well as a more pronounced cross-reaction with one *M. flocculare* antiserum. When comparing the ELISA with the CFT using serums from naturally infected pigs the ELISA was less sensitive in determining early infection. This was not surprising in as much as purified IgG was only used to prepare the alkaline phosphatase "conjugate". While the CFT and IHA are currently the most useful techniques for immunodiagnosis the ELISA has advantages as a herd test since it can be used on an automated basis. However it was concluded that a more sensitive and specific *M. hyopneumoniae* antigen was required for the ELISA. Nicolet and Paroz (1980) reported the extraction of membrane proteins in an effort to purify *M. hyopneumoniae* antigen and found that a purified protein antigen extracted in Tween 20 gave comparable activity but higher specificity than whole-cell antigen. Furthermore Nicolet *et al* (1980) considered that medium components which contaminate antigen are responsible for some non-specific ELISA reactions. By extracting antigenic cell wall proteins (M.W. 40 000 - 60 000 daltons) with neutral detergents as Tween 20, growth medium contaminants can be avoided. Preliminary results with this antigen during a controlled experiment detected antibodies in pigs 3 weeks after contact with infected animals. The epidemiological significance of this increased sensitivity is being evaluated.

1.8 (v) Immunity to EPP

Resistance following infection has been reviewed by Whittlestone (1973; 1976a; 1979).

After natural infection with M. hyopneumoniae pigs are apparently protected against reinfection. In endemically infected herds it is typically the young stock which are clinically affected. This pattern is not due to an age related resistance, as older stock are as susceptible when infection is first introduced. Furthermore, pigs infected experimentally and allowed to recover, resisted reinfection up to 60 weeks after primary infection (Goodwin et al 1969a). While these early observations demonstrate the presence of a persistent active immunity following natural infection, the nature of the immunity was not indicated.

The role of serum antibody was investigated by Lam and Switzer (1971b) who transferred serum from vaccinated to susceptible pigs, which were then challenged. Gross EPP lesions were recorded in fewer of the pigs receiving serum than in controls, and lesions were less extensive. Other attempts to associate serum antibody detected by CFT (Goodwin et al. 1969 a,b) or IHA (Goodwin et al 1969b; Lam and Switzer 1971a) have found no correlation between presence of serum antibody and immunity.

The presence of antibodies in tracheobronchial secretions has been investigated by Holmgren (1974a) and in colostrum by Durisic et al (1975a). Antibodies to M. hyopneumoniae have been demonstrated in tracheobronchial secretions of experimentally infected pigs within 2 weeks of infection and persisted for at least 13 weeks. While some pigs had IHA activity localised totally in tracheobronchial secretions i.e. no detectable serum IHA antibody, and IHA activity localised in the fraction containing IgA, the relationship between mucosal IgA and protection is not known. Similarly Durisic et al (1975a) demonstrated antibodies by IHA in colostrum of vaccinated sows which was associated with a reduced prevalence of gross lesions in these piglets when compared to piglets from unvaccinated controls (Durisic et al 1975b).

The potential protective roles of the different antibodies produced in response to M. hyopneumoniae infection are opsonization and prevention of attachment of organisms to the respiratory mucosa. IgM and IgG antibodies present in the transudate which collects at the site of inflammation opsonize organisms which enhances phagocytosis. IgA secreted onto the mucosa may inhibit attachment allowing clearance of organisms by the mucociliary mechanisms.

In pigs the massive peribronchial lymphoid reaction following infection suggests that cell-

mediated immunity could play an important part in provision of active immunity. Specific activation of the CMI system was first demonstrated by lymphocyte transformation (Roberts 1973) which was shown to reach maximum levels between 15-44 weeks post-infection (Abegboye 1975). Other indicators of CMI activation detected are macrophage and leucocyte migration inhibition (Roberts 1973; Nicolet and Rivera 1976) and delayed type hypersensitivity skin reactions (Adegboye 1975), however their role in protection from infection is unknown.

1.8 (vi) Epidemiology

The most common means of assessing presence and severity of EPP has been to record the prevalence of EPP-like lesions at slaughter. There appears to be good correlation between the prevalence of pneumonic lesions at slaughter and their extent. Aalund et al (1976) found that as the prevalence of lesions increase within a herd, they also become more extensive, so that it would be unusual to find a herd with 85% of pigs affected at slaughter but with most of the lungs having slight lesions. Therefore the prevalence of lesions in a substantial and continuing sample of slaughtered pigs is probably one of the best indicators of economic loss.

Reports of prevalence, which vary widely (19-100%) are summarised in Table 1.3. In the northern hemisphere where the disease is considered to be of greater

significance the prevalence has been shown to be high, 35-100%, while EPP surveys in Australia have demonstrated prevalences ranging from 19-60% in slaughterweight pigs. Here the disease is observed more frequently as a clinical disease in the southern states of Victoria and Tasmania; however as indicated by the surveys EPP is widespread in Australia. Supporting this view is the recent result of an abattoir survey in Townsville, Qld., where a prevalence of 40% was recorded in porker pigs. This result was double that recorded by Edwards et al (1971) in pork and bacon weight pigs in N.S.W., which may be partly explained as porkers in a previous study (Pullar 1949a) had a higher prevalence than older bacon weight pigs. Up to 1973 it was considered by Whittlestone (1973) that the prevalence of pneumonia, based on abattoir surveys throughout Australia had declined, probably due to improved husbandry standards and disease awareness accompanying intensification of the industry.

M. hyopneumoniae is an inhabitant of the respiratory tract of the pig, which is the only known host of the organism. It has been shown experimentally that M. hyopneumoniae can be recovered from the nasal cavities and lungs of experimentally infected pigs that have not developed or are not showing pneumonia macroscopically (Goodwin 1972b; Etheridge et al 1979). Under field conditions where there is a variable degree of passive immunity, pneumonia may not immediately follow

TABLE 1.3.

Prevalence of Enzootic Pneumonia lesions at Slaughter

Author	Year	Country	Pig Age	Prevalence % (No. exam.)
Betts	1952	England	Baconer	42 (1000)
Macpherson & Shanks	1955	Scotland	Baconer	55 (1000)
Switzer	1967	USA (Iowa)	Market Weight	35 - 60*
Huhn 1970b quoting Audi	1962	Yugoslavia		'Approacing 100'*
Huhn 1970b quoting Kono	1967	Japan		'Similar to other countries'*
Pullar	1949a	Australia (Victoria)	Small Porker	55 (85)
			Porker	68 (152)
			Baconer	65 (110)
			Sow	32 (960)
Rees	1964	N.Z.	Porker	69 (326)
Edwards et al	1971	Australia (N.S.W.)	Porker & Baconer	19 (960)
Norton	1975	Australia (N. Qld.)	Porker	40 (324)
Mercy	1981	Australia (W.A.)	Porker & Baconer	22 (winter)* 16 (summer)*

*unknown number examined.

exposure to infection, and it may never occur in some pigs (Durisic et al 1975b). M. hyopneumoniae has also been recovered from CNS tissue (Williams 1980) where organisms were found in close association with ciliated ependymal cells udergoing pathological changes involving cilia. The finding suggests a greater role in the disease processes of pigs by causing cytopathogenicities in both pulmonary and CNS tissues, however, this CNS involvement does not appear to be associated with any clinical condition.

Transmission via direct pig-to-pig contact and airborne routes have been demonstrated. Transmission in endemically infected herds is primarily from the sow to the suckling piglets (Whittlestone 1973). Further transmission and exacerbation of existing lesions occurs as a result of grouping and stress in pigs at weaning. It is during this post-weaning and growing period that the highest clinical incidence of the disease is seen. The incidence of clinical pneumonia declines with increasing age to the point where adults show no clinical signs because they are strongly immune (Lannek and Bornfors 1957; Goodwin et al 1969a). It is therefore less likely that older sows in endemically infected herds will transmit infection to their litters. The transmission of M. hyopneumoniae, M. hyorhinis and A. laidlawii from dam to foetus was investigated by Heitmann and Kirchhoff (1982). Infection was not transmitted to foetuses in dams intravenously inoculated with these organisms, however A. laidlawii was recovered from a nasal swab

from 1 of 24 piglets at birth. The potential introduction of EPP into a healthy herd through boar semen used for artificial insemination was first raised by Schulman and Estola (1974) who isolated M. hyopneumoniae from 1 of 101 samples. Further studies by Mandrup et al (1975), Whittlestone (1979) and Kobisch and Goffaux (1980) failed to isolate the common respiratory mycoplasmas from a total of 341 samples. However the latter study reported the isolation of A. laidlawii and M. verecundum from 6 of 79 samples. The possibility that lungworms may act as a reservoir for M. hyopneumoniae was investigated by Preston and Switzer (1976). Lungworm larvae-infected earthworms were fed to EPP-free pigs, none of which developed lesions or CFT antibody against M. hyopneumoniae. Goodwin (1972a) encountered difficulty in achieving in-contact transmission from inoculated pigs to EPP-free pigs, however Etheridge et al (1979) and Etheridge and Lloyd (1980) using an Australian isolate, designated the Beaufort strain, had no difficulty in producing EPP lesions in contact pigs.

The main reason why EPP is important is that it reduces the efficiency of feed utilisation or, on a given feed scale, slows the rate of growth. Since this effect is variable within any group of pigs, smooth production becomes difficult because pigs starting together at similar weights may finish at very different times. This results in under-utilisation of increasingly expensive spaces. Some

previous experimental reports have suggested that EPP depresses liveweight gain between 7% to 22% (Betts and Beveridge 1953: Betts et al 1955; Huhn 1970b) while others found no effect (Euglert and Eisemack 1964 - cited by Mercy 1981; Eikemier and Mayer 1965 and Bjorklund and Henrieson 1965 - cited by Huhn 1971a). Two further studies in herds which became infected with EPP give an accurate assessment of the disease under field conditions. Goodwin (1963) demonstrated greater profitability in a herd prior to infection and again after eradication of the disease, but did not quantify reduced performance in actual growth characteristics. Braude and Plonka (1975) in a similar exercise found a significant deterioration in both daily weight gain (5.5%) and efficiency of feed utilisation (4.6%) when comparing performance of 3 years both pre and post-infection. Huhn (1970b) found that the rate of body weight gain was depressed in proportion to the severity of EPP lesions in naturally infected pigs, however Mercy (1981 - personal communication) did not find this association in a severely affected herd. Discussion of reduced performance corresponding with extent of lesions on an individual basis probably complicates the determination of the effect of the disease in the field, as pigs may be affected at any age. For instance pigs with extensive lesions at slaughter may have only been recently infected (when there would be little effect on growth rate) whereas, another pig might have no gross lesions at slaughter because severe infection early in life may reduce growth to an extent that lesions had time to

resolve (Goodwin 1982). Goodwin (1971) pointed out that the disease should be assessed for its effect on a herd, not on an individual basis and there appears to be good correlation between the prevalence of pneumonic lesions at slaughter and their extent (Willeberg 1979) ie. as prevalence increases so does the extent of lesions. Therefore the prevalence of lesions in a high proportion of lungs continuously at slaughter is probably one of the best indicators of the economic loss.

Field studies show marked variation in EPP disease levels between herds which is attributed mainly to differences in management and environmental conditions and also the presence of different types of secondary bacteria. There has been no direct evidence of varying pathogenicity between strains of M. hyopneumoniae which could account for this variation (Muirhead 1979). Where pigs are housed indoors at a high density there is clearly an effect of seasonal climatic fluctuation on the disease. In a review by Done (1971) all authors agreed that the condition was at its worst clinically during the winter months. External climatic conditions can indirectly affect EPP, as with low external temperatures ventilation rates are reduced in order to maintain shed temperature. Probable reasons for the development of more extensive lesions of EPP under conditions of poor ventilation are likely to be accumulation of noxious

gases (ammonia) which damage ciliary function, accumulation of infective droplets and overloading of clearance mechanisms of the lung by inhalation of large numbers of bacteria (Drummond et al 1978).

Severe drops in temperature are often associated with the development of acute cases of pneumonia in pigs in poorly insulated buildings. It is likely that body chilling reduces the effectiveness of the ciliary clerance mechanism allowing inhaled bacterial contaminants to assume a pathogenic role (Whittlestone 1976b).

Accumulation of atmospheric ammonia has been associated with pneumonia in pigs and other species. In a field study of 41 piggeries in Germany, Mickwitz et al (1975) found that presence of pneumonically sick pigs was associated with ammonia levels of 12.4ppm or greater. From those results it was recommended that levels be kept below 10 ppm to avoid problems. The mechanism by which ammonia exerts its effect is by damaging the cilial lining of the respiratory tract, thereby reducing clearance of inhaled bacteria by impaired function of the mucociliary clearance mechanism (Drummond et al 1978; Oyetunde et al 1978; Drummond et al 1981). Levels even as low as 3 ppm cause ciliostasis in rat trachea in vivo (Dalhamm 1956 - cited by Broderson et al 1976). The interaction between ammonia and M. gallisepticum infection of poultry (Sato et al 1973) and M. pulmonis infection of rats (Broderson et al 1976) have been studied experi-

mentally. Results show that even after short term exposure (3 days) to 25 ppm of ammonia there was an increased prevalence and enhancement of lesions which continued to increase as the ammonia exposure was raised. Studies with M. hyopneumoniae have not been conducted in pigs but is to be expected that this association occurs, as the studies of Drummond et al (1978) and Drummond et al (1981) demonstrated a similar effect in piglets challenged intranasally with Bordetella bronchiseptica and Escherichia coli. Even in the absence of M. hyopneumoniae infection, exposure of pigs to ammonia is of considerable significance. Kalich and Schuh (1979) demonstrated, in repeated experiments, that in pigs exposed to 30ppm of ammonia, growth rate was reduced by 17.5% and feed conversion efficiency by 13.1%.

Survival of the organism in aerosols is important in transmission of EPP: factors important in organism survival outside the host are temperature, relative humidity, drying and ultraviolet radiation. While there are no direct reports on the survival of M. hyopneumoniae in aerosols or exhaled sputum, studies on the survival of organisms in mucus on bronchial surfaces are probably comparable with survival in moist sputum. Organisms survived for 4 hours at 37°C, for 2 hours at 42.5°C and for 3 to 7 days at 17°C to 25°C (reviewed by Whittlestone 1976b). Interaction between temperature and relative humidity has been reported, whereby viability is enhanced under drier

conditions (RH 13%) even at normally damaging high temperatures (43°C) (reviewed by Whittlestone 1976b). It was noted by Gordon (1963) that the incidence of clinical pneumonia was lower in pigs from houses with a high relative humidity and temperature ("Sweat house" system). It was reported that sedimentation of particles of 1 to 3 um diameter which are normally retained in the lower airways was increased under this system, thereby reducing the mycoplasma challenge to the lungs.

The effect of drying on survival of M. hyopneumoniae and M. hyorhinis was studied by Friis (1973b) who found organisms would survive at room temperature (20°C to 25°C) for up to 8 days. Ultraviolet radiation is most effective in destroying mycoplasmas at low humidity, as it was found that RH 95% afforded some protection against UV damage (Wright and Bailey 1969 - cited by Whittlestone 1976b).

Pigs exposed to contaminated premises after removal of infected pigs do not readily contract EPP. Whittlestone (1958 - cited by Whittlestone 1978) found that pigs exposed to a dark, cold (5°C) straw hut which had housed EPP-infected pigs 24 hours earlier did not develop EPP, nor could M. hyopneumoniae be recovered from pigs placed in pens from which infected pigs had been removed 5 minutes previously (Goodwin 1972a). Betts (1953 - cited by Whittlestone 1976b) did find that one pig contracted EPP after being placed in a pen that had

been vacated 10 minutes previously. Survival and spread in aerosols occurs over short distances (Whittlestone 1976b however unexplained infections that occur over longer distances raise the possibility of spread by smaller particles which remain suspended for longer periods.

The extent and severity of lesions of EPP may be increased by concurrent viral or parasitic infections. Pneumonic lesions were more severe in pigs inoculated with porcine adenovirus and M. hyopneumoniae, than in pigs inoculated with either agent alone (Kasza et al 1969). Lesions were also more severe in pigs infested with lungworms, Metastrongylus elongatus (Mackenzie 1963), and migrating Ascaris suum larvae (Underdahl and Kelley 1957; Zimmerman 1971 - cited by Whittlestone 1979). Augmentation of M. hyopneumoniae infection also occurs when secondarily infected with P. multocida (Smith et al 1973).

1.8 (vii) Control

(a) Antibiotics and chemotherapeutic drugs.

The prospects of treatment and control of EPP on a herd basis is good. The efficacy of chlortetracycline in preventing infection with EPP given at concentrations practical for commercial pig production was demonstrated by Huhn (1971). Previously it was demonstrated that the drug could only be used prophylactically as the drug did not eliminate infectivity of established lesions (Betts and Campbell 1956). Subsequently Etheridge et al (1979) have

demonstrated resistance of M. hyopneumoniae to to chlortetracycline in Australia.

Nitrofurans given intramuscularly were shown to reduce the incidence and extent of pneumonia (Durickovic et al 1964 - cited by Whittlestone 1973) however wide use of these drugs has not occurred as the effect observed was most likely to be due to suppression of secondary infection only. The use of tylosine during suckling to a week post-weaning has been reported to prevent establishment of EPP lesions (Schuller and Glawischnig 1972). These results on the naturally transmitted disease are more encouraging than experimental studies with the drug (Huhn 1971).

A new diterpene antibiotic, tiamulin hydrogenfumarate (Dynamutilin, E.R. Squibb and Sons) has been demonstrated to have high activity against mycoplasmas both in vivo and in vitro (Goodwin 1979; Laber and Schutze 1976; Glawischnig and Steininger 1970). Levels of 200ppm produce considerable resolution of the lung reaction and a lower incidence of clinical pneumonia amongst weaner pigs. Alexander et al (1980) used tiamulin in a medicated early weaning programme, designed to produce piglets free of enzootic pneumonia and swine dysentery when weaned at about 5 days of age. After prefarrowing treatment of the sows and daily dosing of piglets orally with tiamulin powder, the progeny of 51 sows have remained free of mycoplasma infection.

(b) Vaccination

Formalinised M. hyopneumoniae antigen appears to have no protective effect against EPP (Goodwin et al 1969b; Lam and Switzer 1971b). Vaccines prepared from disrupted mycoplasmas and administered with Freund's incomplete adjuvant did markedly reduce the number of pigs developing gross lesions of pneumonia (Lam and Switzer 1971b). When mixed with Freund's complete adjuvant Goodwin et al (1969b) found some protection against low dose challenge of EPP lung. Protection comparable to that demonstrated by Lam and Switzer was achieved by Goodwin and Whittlestone (1973) using formalinised antigen with either Freund's complete adjuvant or Bayol or Arlacel. Reactions produced by these adjuvants are so severe that the vaccination sites would be unacceptable in carcasses used for human consumption. In a field trial using aluminium hydroxide as an adjuvant no protection was afforded (Goodwin 1973). In another field trial using a lower dose of antigen, there was no evidence that the vaccine reduced the incidence or extent of pneumonia, and there was no evident improvement in performance of vaccinated pigs (Goodwin 1973). Farrington (1976) subsequently found that an antigen-peanut oil vaccine may have given some protection. Stimulation of colostral immunity was investigated by Durisic et al (1975a) who suggested that intramammary inoculation of antigen - Tween 80 - paraffin oil vaccine afforded protection against natural challenge; however insufficient pigs were used in the trial to fully assess the vaccine.

The development of a temperature sensitive mutant of M. hyopneumoniae capable of growth only in the upper respiratory tract may lead to the developed of an effective vaccine. Such a vaccine would be more effective in stimulating local IgA and cell mediated immunity, both of which are probably very important in immunity to mycoplasmal respiratory diseases (Whittlestone 1979).

(c) Management and environment

As part of the variance in EPP level between herds may be attributable to the different types of secondary bacteria, the maintenance of a self-contained herd or purchase of pigs for finishing or breeding from as few herds as possible, will assist in control. Even introduction of EPP-free stock to infected herds may reduce risk to the recipient herd; these pigs show few problems when introduced to infected herds (Muirhead 1979). The age structure of the herd is an important factor in disease control, as litters from older sows are possibly protected by passive immunity or older sows transmit the disease less frequently. Litters from young sows are most frequently affected, therefore gilts should be reared in well ventilated housing where disease is present but sub-clinical.

Provision of "all-in-all-out" accommodation, especially in the farrowing and weaning areas is an effective method of preventing disease establishment

and perpetuation. Provision of small compartments with thermal and fan equipment to provide constant conditions reduces accumulation of mycoplasmas in the atmosphere. Lindqvist (1974), Aalund et al (1976) and Muirhead (1979) cite stocking density, group size, temperature and ventilation as being critical factors in the control of EPP. In winter it is important to provide a minimum level of ventilation, as house temperatures are often maintained at the expense of effective ventilation, allowing a build-up of aerosol bacteria and noxious gases.

(d) Control Schemes

Use was initially made of the observation that often litters from older sows were free of EPP. Sows were farrowed in isolation and siblings not required for restocking purposes were slaughtered and checked for EPP. If free, the remaining stock were assumed to be also free of infection and used for establishment of EPP-free herds. In recent years EPP-free pigs have been obtained by a number of techniques including hysterectomy, hysterotomy, "snatch" method at farrowing and medicated early weaning (Alexander et al 1980).

With the establishment of EPP-free herds certification schemes were established and lungs monitored at slaughter for presence of lesions of EPP. With the recent advances in specific diagnosis

such schemes became more scientifically based and are now operating in America, Britain, Canada, Denmark, Germany, Sweden and Switzerland. A standard (e.g. 10% of 120) number of lungs are monitored at 6 monthly intervals and suspect material submitted for definitive diagnosis.

A major problem, at least with the European schemes, has been the high breakdown rate. Between 1960-1966, 8.3% of herds broke down per annum in Britain. In the following 10 years to 1976 there was a 5.5% breakdown rate among an average register of 78 herds (Goodwin 1977). Most of these breakdowns occur without any known contact with unchecked pigs and can only be presumed to enter via aerosol or other unknown vectors. The breakdown rates of other schemes was reviewed by Whittlestone (1973) who quoted rates of 1 to 3% per year for the Swiss programme, 4% in the Bavarian scheme and 1% in the Nebraska scheme. By studying the epidemiology of possibly related breakdowns Goodwin (1965), Keller (1976) and Koch and Keller (1976) have revealed the presence of latent forms of M. hyopneumoniae infection. Transfer of stock have been associated with subsequent breakdowns in both the supplier and recipient herds implicating existing sub-clinical infections of up to 15 months.

An attempt to eliminate EPP from infected herds in the United States using the CF test has not been successful. CF-positive breeding stock were removed

during 1 year from 10 herds, however, subsequently lesions of EPP and CF reactors were found in 9 of the herds. The programme did, however, reduce the clinical signs in the herds.

1.9 Definition of unresolved problems and reasons for present investigations

There has been considerable discussion, in the absence of actual local data in the past, regarding the significance of sub-clinical EPP under Australian piggery conditions. For instance, Goodman (1973) "estimated" losses to be much lower than recorded in Britain, and Whittlestone (1973) observed that the prevalence of lesions at slaughter may have declined over the past 20 years; an observation based on results of single abattoir surveys conducted at different abattoirs in different climatic zones. However, Norton (1976) having found the prevalence of EPP lesions in pigs in an abattoir in Townsville to be 40%, double that found by Edwards et al (1971) in N.S.W. suggested the need for greater control of the disease. The debate surrounding the economic consequences of sub-clinical EPP is further confused by:

a) failure to detect reductions in growth rate and feed conversion due to inadequate recording

b) failure of producers to acknowledge the occurrence of losses resulting from subclinical infection, and to use farm records to detect them, and variation in the expression of the disease between herds due to dependence

on environmental and management conditions which vary between herds.

Furthermore, little use can be made of studies performed overseas to indicate the degree of losses occurring locally. Reports of reduced performance caused by EPP in the northern hemisphere have been obtained in experiments, often using pigs inoculated intranasally (unnaturally) and held under undefined conditions. For these reasons data obtained under experimental conditions is of questionable value when applied to the very different on-farm situation. Perhaps the most useful studies on the effect of EPP have been pre and post infection studies on commercial herds while other variables including the pigs, housing and environment, and nutrition held constant (Goodwin 1963; Braude and Plonka 1975). Such studies are almost impossible under Australian conditions due to the very small number of herds claiming freedom from EPP that may become infected, and lack of continual monitoring and specific diagnosis in those which appear to have become infected.

The aim of this study is to assess the significance of EPP to the South Australian pig industry. Investigation is also timely due to recent industry trends. Over the past 7 years average herd size has increased from 73 to 128 and the number of herds with greater than 1 000 pigs has doubled. In view of

findings of Aalund et al (1976), that prevalence and extent of lesions increase with increasing herd size, there exists the potential for EPP to be an emerging disease in the South Australian pig industry.

1.9 (i) Research Plan

a) To establish the prevalence and extent of lesions due to EPP and other types of lung pathology.

b) To identify environmental conditions and management practices commonly predisposing pigs to severe EPP in South Australia.

c) To evaluate the economic effects of EPP in relation to growth rate and feed conversion efficiency under environmental and management conditions recorded earlier.

1.9 (ii) Establishment and Verification of Diagnostic Techniques

Due to the lack of specific diagnostic techniques for EPP, tests for rapid diagnosis will be established and verified. These include:-

a) Classification of histological lesions as positive or suspect for enzootic pneumonia (McKean et al 1979).

b) Examination of giemsa stained touch preparations of pneumonic tissue (Whittlestone 1973).

c) Testing sera for antibodies to *M. hyopneumoniae* using the CF test (Etheridge and Lloyd 1980). Direct immunofluorescent staining of touch preparations (Giger *et al.* 1977) and lung sections (Meyling 1971).

CHAPTER 2

MATERIALS AND METHODS

2.1 Assessment of Lung Pathology

2.1 (i) Gross Examination

All lungs were placed on an examination table with the dorsal surface uppermost. Lobes were inspected and palpated to enable recording of the distribution of any pneumonic lesion, including pleurisy. Lungs were incised when lesions other than enzootic pneumonia were indicated. The lung was then placed with the ventral surface uppermost and the inspection procedure repeated, including the intermediate lobe.

(ii) Gross appearance of enzootic pneumonia lesions

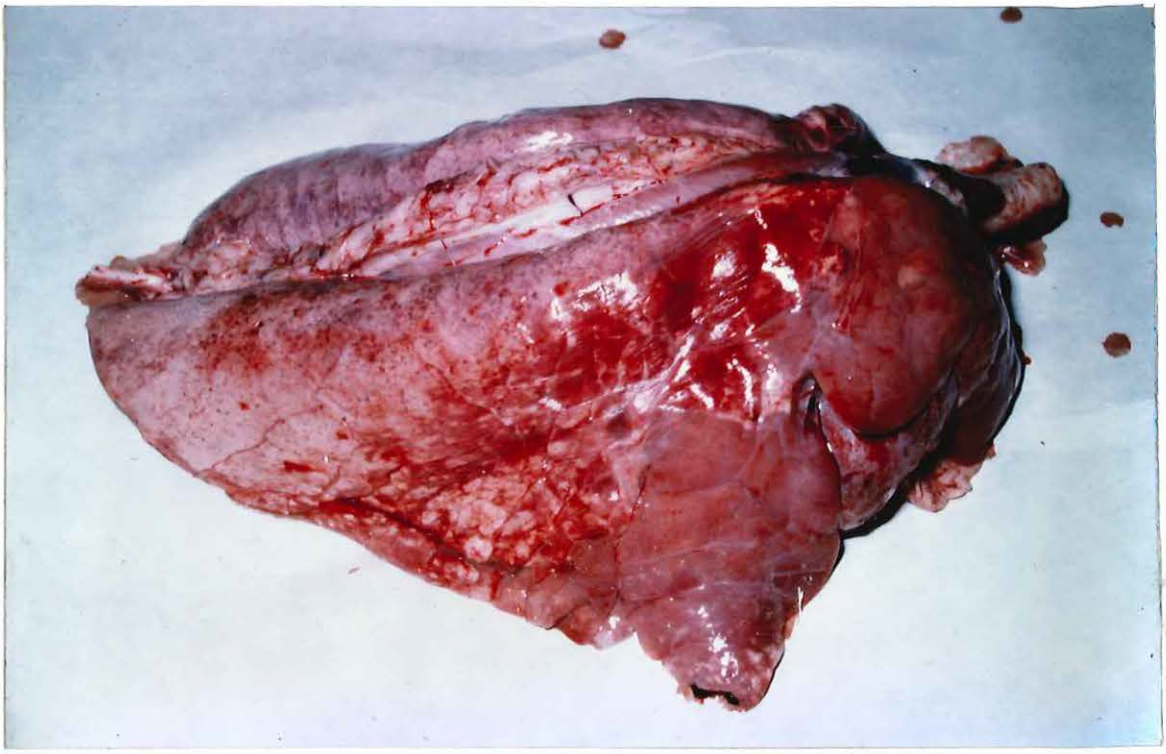
Lung lesions at slaughter are classified as being suspicious of EPP according to the following criteria.

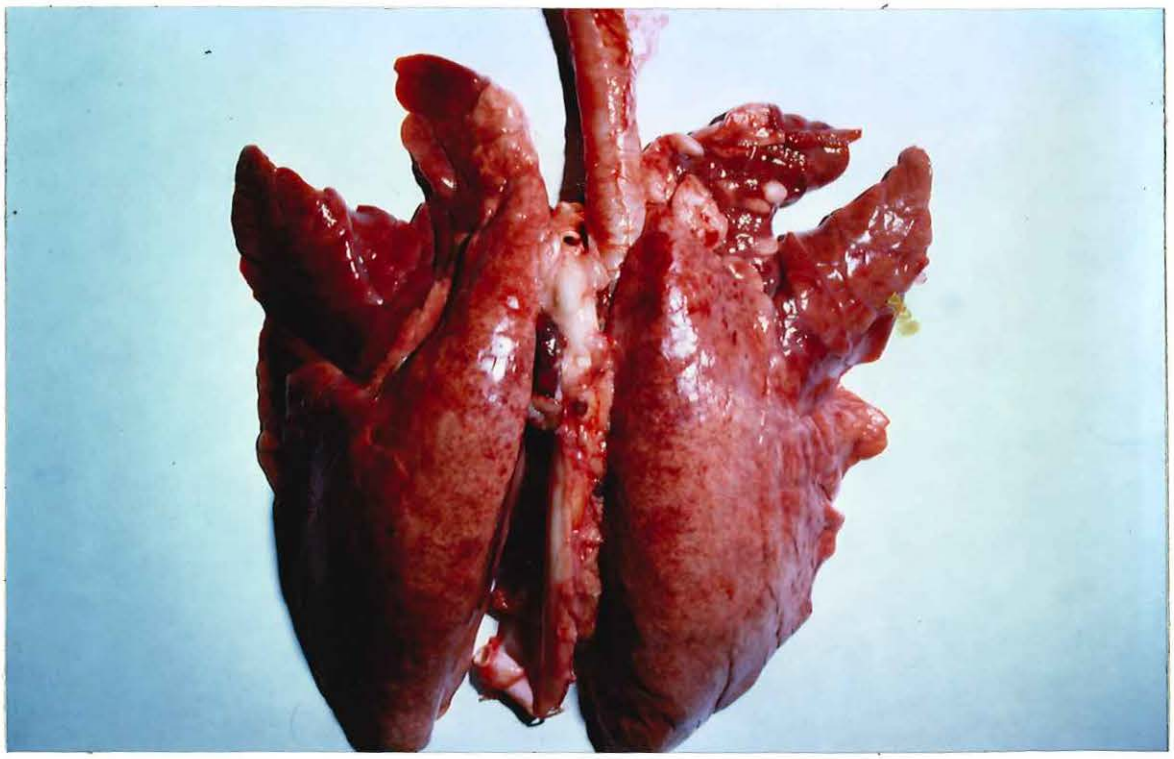
The pneumonic lesions are catarrhal in type and follow a lobular distribution. Lesions are well demarcated and found in the ventral aspects of the apical lobes. Early lesions are typified by greyish swelling of one or more lobules along the sharp borders of the lobes. Acutely affected lobules are grey or greyish-pink and are level with or slightly raised above the level of normal lobules (Fig. 2.1). Bronchioles exude a mucoid exudate and the cut surface is oedematous (Pullar 1948; Jubb and Kennedy 1963).

Chronic lesions are typified by shrunken reddened lobules (atelectatic) which are generally free of serocellular exudate (Fig. 2.2). Frequently active

Fig. 2.1. Acutely affected swollen lobules of the distal portion of the right cardiac lobe. Red tinge due to haemorrhage at slaughter.

Fig. 2.2. Chronic lesions showing atelactasis of lobules in the distal portion of the apical lobes.





and chronic lesions occur together in association with other emphysematous lobules (Fig. 2.3).

Secondary bacterial infection commonly occurs producing bronchopneumonia which may be complicated by pericarditis and pleuritis. In these cases bronchiolar exudate is yellowish and opaque, and necrosis and abcessation is apparent.

(iii) Calibration of lung lesions

In experiment 2 (Chapter 6) where EPP lesions were experimentally induced, the extent of lesions was scored by calculating the total area of lung tissue consolidated.(see Appendix I p269). This result was expressed in combination with the number of lobes in which lesions occurred in each lung. This presentation of gross pathology was considered to accurately quantify the extent and depict the distribution of actual lesions in a readily comprehensible form.

(iv) Histopathology

(a) Criteria used to assess experimental infection.

The histological changes in the lung are diffuse in the early stages and only in the chronic non-progressive stages do they more closely correspond to the gross lesions.

Early lesions occurring mainly between 7 and 28 days post infection are classified as second stage lesions (Whittlestone 1972).

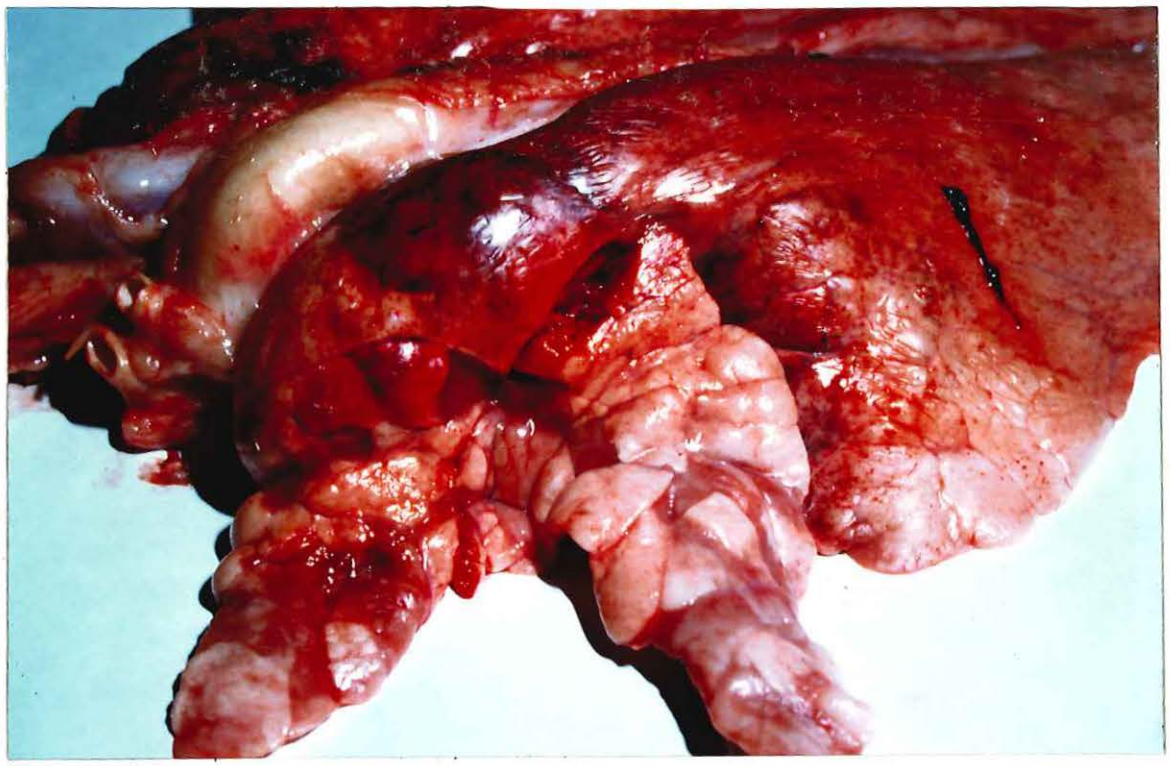


Fig. 2.3. Chronically affected lung with atelectic and emphysematous lobules interspread.

Initially lesions are characterised by migration of polymorphonuclear neutrophils, vacuolation, enlargement and proliferation of alveolar cells and accumulation of lymphocytes, macrophages and plasma cells in perivascular and peribronchiolar tissues. A serous or serocellular exudate develops in most lobules, often being copious in the anterior portions producing distension and grey consolidation (Fig. 2.4).

Third stage, established lesions may be found between 17 and 40 days post infection. These are characterised by progressive peribronchial and perivascular lymphoid hyperplasia, an increased accumulation of perivascular mononuclear cells and progressive development of alveolar pneumonia (Fig. 2.5).

Recovering lesions classified as fourth stage may be seen from 69 to 262 days post-infection. These are characterised by foci of peribronchial lymphoid hyperplasia which may compress associated bronchioles, and cellular exudates are progressively resolved (Fig. 2.6).

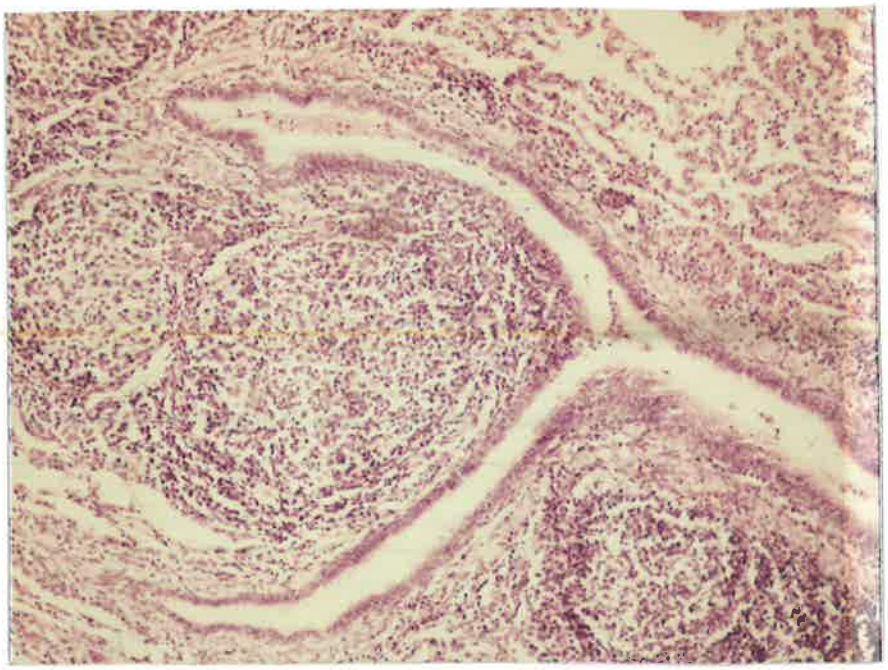
(b) Criteria used for field cases

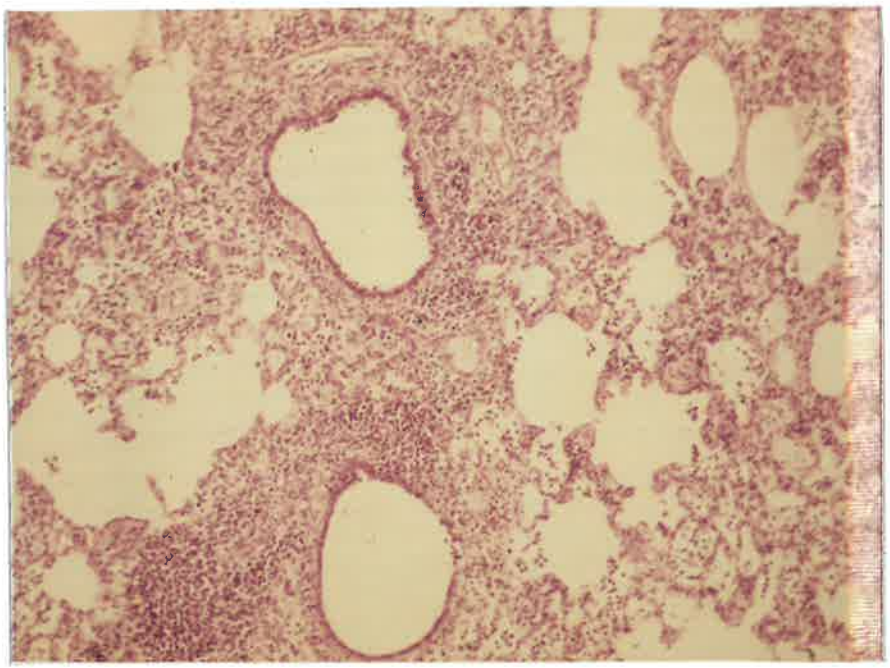
Although histological changes caused by enzootic pneumonia are non-specific, McKean et al (1979) showed strong correlation between

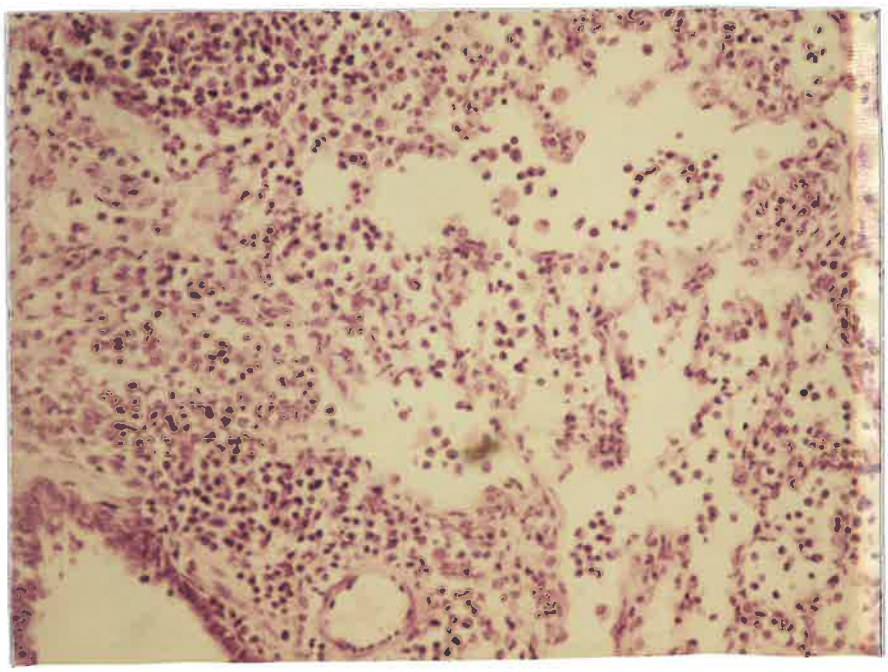
Fig. 2.4. Second stage EPP lesions. Note peribronchial and perivascular accumulation of lymphocytes and plasma cells together with commencement of alveolar pneumonia. H + E x 250

Fig. 2.5. Third stage EPP lesions. Note the development of peribronchial and perivascular lymphoid hyperplasia. H + E. x 40

Fig. 2.6. Fourth stage EPP lesions. Note extensive follicles of lymphoid hyperplasia compressing the adjacent airway. H + E. x 40







(1) CH1

positive and suspect histological changes and other specific diagnostic tests.

Those diagnosed as positive had large follicular peribronchiolar and perivascular lymphoid and mononuclear cell accumulations involving most bronchioles (Fig. 2.7). Those classified as suspicious had thin lymphoid and mononuclear cell accumulations involving most bronchioles and vessels, and only a few scattered peribronchial lymphoid nodules (Fig.2.8).

Lungs with accumulations of peribronchiolar and perivascular mononuclear cells affecting less than 10% of the bronchioles and vessels were classified as negative.

(c) Histological Methods

Where gross pneumonic lesions were present, tissue was dissected from the active front or border of the lesion, including both consolidated and grossly normal areas. In normal lungs, tissues were obtained from the distal portions of the apical lobes. These were fixed in 10% buffered formal saline and after paraffin embedding sections were cut at 5μ and stained with haematoxylin and eosin.

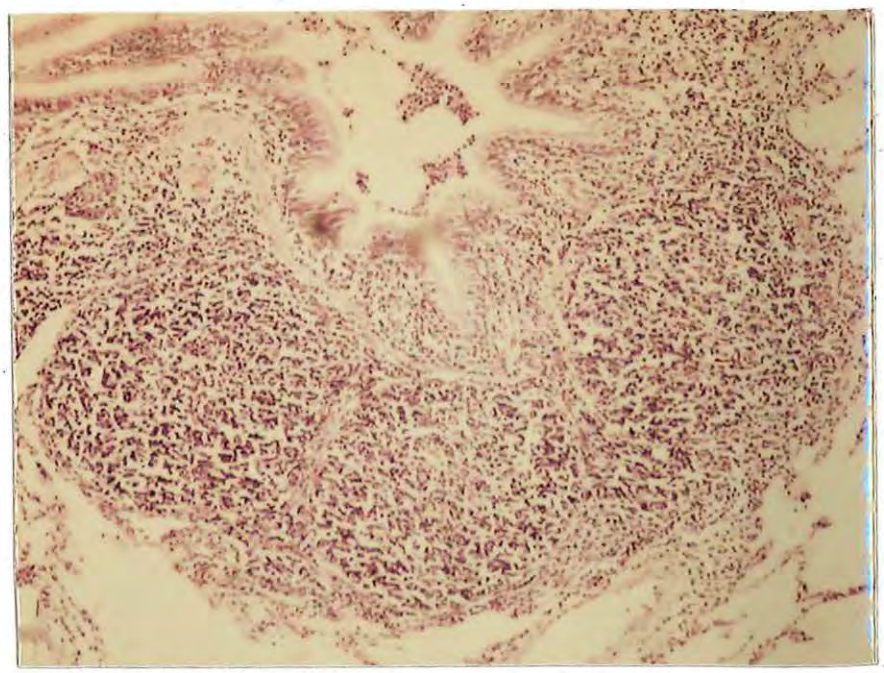


Fig. 2.7. EPP positive lung. Note the massive peribronchial follicles of lymphoid hyperplasia. H + E. x 40

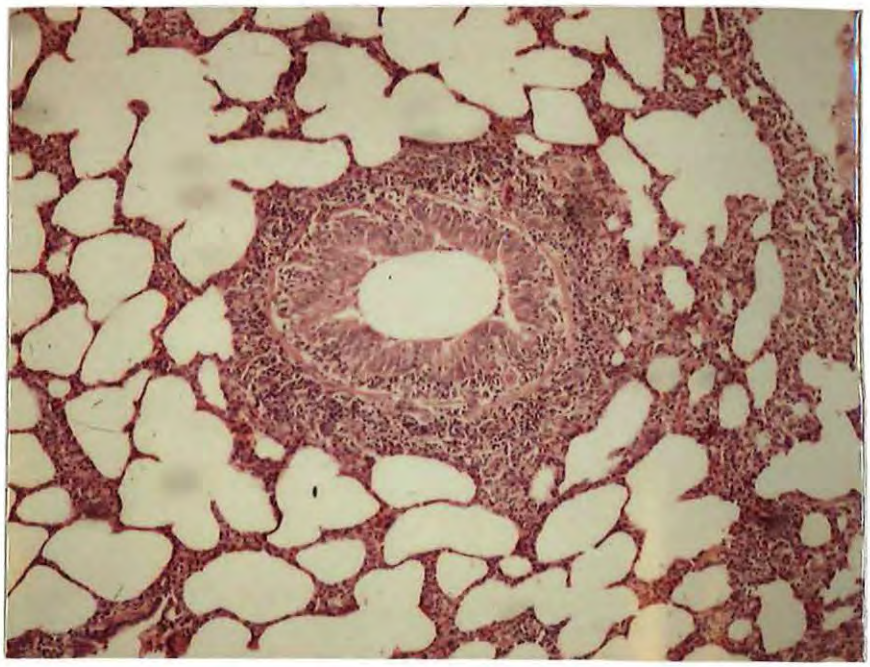


Fig. 2.8. Suspect EPP lung. Note peribronchial cuffing by mononuclear cells. H + E. x 40

2.2 Giemsa Staining of Mycoplasmas

2.2 (i) Touch preparations of pneumonic lung

Mycoplasmas were detected in Giemsa-stained touch preparations of pneumonic lung by the method described by Whittlestone (1973). The technique was developed for use as a rapid, non-specific test for detection of mycoplasmas.

Microscope slides, thoroughly cleaned with potassium dichromate in sulphuric acid and rinsed repeatedly in distilled water were used. Touch preparations were prepared from the cut surface of tissue collected for histopathology. If the cut surface of the tissue was moist, it was dried by dabbing onto clean blotting paper before making the impression on the slide. The cut section was placed lightly on the slide.

Preparations were then fixed in methyl alcohol for 3 - 5 minutes. Slides were then placed in Copland Jars containing Giemsa stain (Gurr's improved R66 stain) diluted 1 in 20 with citrate-phosphate buffer (pH 7.2) for 3 hours. The batch of stain was tested for its ability to stain mycoplasmas adequately in 3 hours. After staining excess stain was drained and washed in 2 changes of the same buffer. Slides were then dried by blotting and holding high above a bunsen flame. Finally they were rinsed vigorously in acetone for 8 seconds and blotted dry (Whittlestone pers. comm. 1979).

(ii) Smears of broth culture

Three millilitres of broth culture was spun at 10 000 g for 30 minutes. The supernatant was discarded and the pellet resuspended in 3 ml of PBS. After repeat centrifugation the supernatant was discarded and the pellet resuspended in a few drops of PBS (¼ - ½ ml). A small drop was then smeared onto an acid washed slide and heat fixed at 37°C. The smear was then fixed in methyl alcohol for 3 - 5 minutes. The staining procedure was as described in 2.2 (i).

2.3 Complement Fixation Test

The protocol for the complement consumption CF method developed by Etheridge et al (1979) is described in Appendix II. Minor modifications in the handling of normal pig serum (enhancing serum) are described.

The direct CF method of Slavik and Switzer (1972) was performed and is described in Appendix II. Using the same J strain antigen as supplied for the above method (Etheridge et al 1979) a checkerboard titration of antigen against complement was performed to determine the optimum antigen titre (1:7) and complement dilution (1/70) for the test proper. Enhancing serum was handled as described in Appendix II.

2.4 Immunofluorescence

M. hyopneumoniae and M. hyorhinis cultures grown in broth culture, and these organisms in touch preparations and cryostat sections of pneumonic lung were identified by direct staining with specific conjugated hyperimmune pig serum (Goodwin et al 1967; L'Ecuyer and Boulanger 1970; Meyling 1971; Livingston 1971).

The M. hyopneumoniae (J strain) and M. hyorhinis (BTS7 strain) hyperimmune pig serum was prepared (Etheridge et al 1979) and supplied by CSIRO Melbourne.

2.4 (i) Preparation of Immunoglobulin

The methods of Nairn (1964, 1969) were used in the preparation of the conjugates.

Cold saturated (40 - 45%) ammonium sulphate solution, 3.3 ml, was added drop by drop over 15 minutes to each of 5 ml aliquots of hyperimmune M. hyopneumoniae, M. hyorhinis and normal serum. During the addition the serum was slowly stirred without causing frothing. Stirring was continued for a further 15 minutes at 4°C. The precipitate was then centrifuged at 3000 g for 15 minutes at 4°C. The supernatant was discarded and the pellet (volume 0.5 ml) was resuspended to the original volume by the addition of 4.5 ml of PBS.

The redissolved globulin precipitate was transferred to dialysis tubing and immersed in 1 litre of PBS for 16 hours at 4°C. The dialysate was checked

for remaining SO_4 ions by adding equal volumes of Ba Cl_2 and dialysate. Absence of a precipitate of Ba SO_4 indicated that dialysis had removed SO_4 ions sufficiently. Globulin concentration was then estimated by the Biuret method.

(ii) Conjugation

This procedure was carried out at 4°C in a cold room. Protein concentrations of serum fractions were M. hyopneumoniae 24 gram/L, M. hyorhinis 28g/L and normal serum 13.5g/L. The former were adjusted to a concentration of 17.5g/L by addition of bicarbonate-carbonate buffer, pH 9, 0.5M (3.7g $NaHCO_3$, 0.6g Na_2CO_3, 100ml d.w.).

FITC Powder (BDH Isomer 1, Purity 80%) was added at a rate of 0.025 mg/mg of total protein (Meyling and Bitsch 1967). Initially it was dissolved in 0.5 ml of phosphate carbonate buffer and then added slowly to the globulin solution over a period of 15 minutes at 4°C. The solution was gently stirred during the addition and continued overnight.

(iii) Purification of conjugate

Unreacted fluorescent material was removed by placing each conjugate in dialysis tubing and immersing in 1 L of PBS at 4°C. Dialysis against PBS was continued for 72 h, the buffer being changed every 12 h.

The conjugates were further purified by absorption with pig liver homogenate. Fresh liver was washed free of blood with normal saline, diced with scissors and then rewashed. An equal volume of cold normal saline was then mixed with diced liver and homogenised without allowing excess warming. The homogenate was frozen at -80°C, then thawed, centrifuged at 4000 g for 10 minutes and washed twice in PBS.

Absorption was carried out by adding 0.5ml of thawed homogenate to 1 ml of conjugate and shaking for 2 hours at room temperature. Conjugate was recovered by centrifugation at 10 000 g for 20 minutes.

(iv) Storage

The conjugate was concentrated by addition of Carbowax flakes (21M, 21 000M.W.) to conjugate in dialysis tubing. The final volume of each conjugate was approximately 3 ml .

Merthiolate was added as a preservative (1:10 000) and then the conjugates were divided into 125 ul aliquots and stored in sealed capillary tubes at -80°C.

Before use conjugate was thawed and clarified by centrifugation at 10 000 g for 30 mins., then stored at 4°C when in use. During the latter stages of the project, dissociated aggregates of FITC molecules, to

which a slight degree of non-specific staining had been attributed, were successfully removed by a repeated absorption with pig liver homogenate.

(v) Staining Method

Touch preparations and culture smears were prepared as before (2.2(i) and (ii)). Preparations were rehydrated by immersing the slide in PBS for 5 minutes. Each slide was drained and the preparation dried by holding tissue paper at the edge of each impression, taking care not to directly touch the preparation. Preparations were stained for 30 minutes in a wet box at 37°C. Stain was removed as described and preparations were mounted in 90% glycerol saline pH 9.5. Coverslip edges were sealed with nail polish to prevent movement of the coverslip and drying of the preparation. Preparations were examined using an epi-fluorescent ultra violet Zeiss Standard microscope fitted with FITC specific filters.

(vi) Titration of conjugate

Two-fold dilutions of FITC conjugated hyperimmune antiserum, commencing at a concentration of 1/3 of the original volume in PBS were prepared.

Smears of broth cultures of M. hyopneumoniae and M. hyorhinis were stained with their respective stains at dilutions between 1/3 - 1/96 of the original volume. After this initial screening intervening dilutions were prepared to determine the specific dilution for use.

Preparations were evaluated for presence of maximum specific fluorescence in conjunction with minimum non-specific fluorescence.

(vii) Blocking test for conjugated antibodies

Specificity of conjugates was checked by the blocking test method (Nairn 1976).

Conjugates tested were M. hyopneumoniae antibody-FITC, M. hyorhinis and non-immune serum-FITC.

1. Each conjugate was used to stain smears of M. hyopneumoniae and M. hyorhinis cultures, and smears of uninoculated culture medium.

2. Culture smears were pretreated with specific immune serum and then stained with the appropriate conjugates.

Pretreatment of cultures with specific hyperimmune anti-serum was performed for 16 hours at $4^{o}C$. Staining time with FITC conjugates was reduced to 15 minutes to minimise the possibility of interchange of the conjugated and unconjugated antibody. In each case separate cultures were also pretreated with unconjugated non-immune serum and PBS.

(viii) Storage of tissue for IMF

Portions of affected and normal lung tissue (approximately 3cc volume) were wrapped in foil and snap frozen and stored at -196°C until sectioned on a cryostat. In cases where lungs were grossly normal, portions were selected from the tip of the right cardiac lobe.

When sections were embedded in paraffin prior to sectioning for IMF staining (Appendix IV) they were stored at -20°C for up to 6 months.

(ix) Preparation and staining of frozen sections

Lung sections were fixed on brass microtome blocks by embedding in frozen fixing compound (Tissue - Tek II, O.T.C. Compound, Lab-Tek Products). Lung sections were rectangular in shape with the edge aligned parallel to the knife-edge. Frozen sections of 5 µm thickness were then cut and placed on acid washed slides.

Sections were fixed on the slide by air drying at room temperatures. Water was absorbed from the section by immersion in cold saturated calcium sulphate, acetone solution. Sections were dried by holding tissue paper at the edge. Prior to staining sections were re-hydrated with PBS for 5 minutes, then dried as described and stained (2.5(v)).

(x) Paraffin sectioning for IMF staining

See Appendix IV

(xi) Staining of colonies

Agar blocks supporting colonial growth were cut from the plate and placed with the colonies touching a glass slide. Agar was then melted away by immersing the slide in dw at 80°C. Colonies were then stained with the FITC-conjugated antisera at the appropriate dilution for 30 minutes at 37°C. Colonies were then rinsed 4 times with PBS, each rinse being held for 30 minutes or longer and, frequently, the last rinse was held overnight at 4°C to remove non-specific staining caused by cross reaction with components of the agar possibly by conjugated antibody against media components used in the immunogen.

2.5 Culture Methods

Mediums used for the culture of M. hyopneumoniae and M. hyorhinis are described in Appendix V.

2.5 (i) Mycoplasmas

Mycoplasma hyopneumoniae, Beaufort strain (Etheridge et al 1979) was obtained from the CSIRO, Melbourne. Another MH medium adapted strain of M. hyopneumoniae (LKR) was also obtained from the same source.

A strain of Mycoplasma hyorhinis (CSIRO I) was obtained from CSIRO, Melbourne.

(ii) Sampling and preparation for culture

To culture an affected lung, 1cc was collected aseptically from the edge or active front of the lesion. This was placed in a stomacher bag with 9 ml of non-selective media (i.e. MH media without antibiotics) and pummelled in a Colworth Stomacher. Fluid from the stomacher (0.3 ml) was removed and placed in 3 ml of MH media. Two drops of lung suspension were inoculated onto PPLO agar plates routinely.

(iii) Culture methods

Cultures for M. hyopneumoniae were grown in 'Pyrex' glass tubes (1.5 cm diam. x 15 cm) containing 3 ml of selective MH medium. As recommended by Friis (1975) the tubes were sealed with rubber stoppers and placed on a tissue culture drum rotating at 1 revolution in 4 minutes during incubation at $4^{o}C$. Growth was accompanied by a change in colour of the phenol-red indicator. This became evident in 1 to 10 days depending on how well the strain had adapted to grow in the medium and the size of the inoculum. Cultures not showing colour change were passaged after 7 days incubation. These were cultured for 28 days before being discarded.

(iv) Routine bacterial isolation

All lung suspensions were routinely inoculated onto 10% sheep blood agar, McConkey agar, and incubated aerobically, and onto chocolate agar incubated under 10% CO_2. Bacteria were identified by routine morphologic and biochemical methods.

(v) Characterization of cultures

Acid production and very slight opacity in the selective MH medium were taken as evidence of growth of M. hyopneumoniae provided no growth was obtained on PPLO agar and routine bacterial isolation plates inoculated with the culture; this indicated the absence of less fastidious mycoplasmas and aerobic bacteria respectively. Sub-cultures were made in MH medium when a slight colour change occurred. This was detected by comparing the culture with a stoppered tube of the uninoculated medium which was incubated under the same conditions.

Cultures were then centrifuged, washed in PBS and resuspended in ¼ - ½ ml of PBS. These were identified by staining with M. hyopneumoniae, M. hyorhinis and EPP-antibody free FITC conjugates (2.4 (v)).

Colonies growing on PPLO agar were identified by IMF staining of colonies and smears of colonies (2.4 (xi)).

2.6 Inoculation of pigs

2.6 (i) Storage of M. hyopneumoniae for inoculation

Initially, frozen lung homogenate from a pig experimentally inoculated with M. hyopneumoniae (Beaufort strain) was supplied by CSIRO Melbourne, (M. hyopneumoniae had been recovered from this homogenate) This was stored at -196°C until use.

Further homogenate was prepared from transmitter pigs inoculated with this material during Experiment 1. A sample of lung tissue (weighed) showing active lesions was selected and placed in 8 ml of MH media. This was pummelled in a stomacher and sieved through a wire grid (mesh 0.4 mm) to separate M. hyopneumoniae lung suspension from the connective tissue. This suspension was diluted with MH medium to produce a 20% suspension. Aliquots of 10 ml were stored in autoclaved bottles at -196°C.

(ii) Sources of pigs

Enzootic pneumonia-free pigs were supplied by the Northfield Pig Research Unit, Department of Agriculture, Northfield, South Australia.

The unit was established in 1969 using hysterectomy derived, artifically reared piglets. The majority of subsequent introductions have included piglets obtained by hysterectomy, and the minority being boars from EPP free herds.

Groups of bacon-weight pigs have always been checked quarterly throughout the project and herd visits conducted frequently. Serum submitted from these pigs has routinely been CF negative.

(iii) Experimental challenge with M. hyopneumoniae

Aliquots of 20% lung suspension, (10 ml) were thawed at 37°C. This was diluted by addition of a

further 10 ml of MH to produce 20 ml of 10% lung suspension. This was stored on ice until inoculated.

Pigs to be inoculated were tranquilized with Stresnil (Ethnor Pty. Ltd., Sydney, N.S.W.) and laid sternally with the head arched back and 5 ml of 10% lung suspension was then syringed down each nostril.

CHAPTER 3

PRELIMINARY DEVELOPMENT OF LABORATORY TECHNIQUES

3.1 Introduction

The specific diagnosis of enzootic pneumonia has been advanced in recent years through the development of better techniques for the isolation of M. hyopneumoniae, its serological identification, detection of antibody response to infection and detection of the organism in affected tissues. Prior to these techniques, non-specific tests e.g. histopathology, gross pathology and detection of pleomorphic organisms in Giemsa-stained touch preparations, were routinely used (Goodwin and Whittlestone 1967; Whittlestone 1979).

The primary isolation of M. hyopneumoniae from pneumonic lungs, proven infectious by pig inoculation was successful in only a small percentage of cases (Goodwin et al 1967 ; L'Ecuyer 1969). Several reasons for this difficulty are the extreme lability of M. hyopneumoniae, its very fastidious growth requirements and the presence of M. hyorhinis in many infected lungs which because of its more rapid adaptation to artificial medium will generally overgrow M. hyopneumoniae. Despite these difficulties, improvements to the culture medium and techniques (Pijoan and Roberts 1973 ; Friis 1975 ; Goodwin 1976) have greatly improved the recovery rate (Etheridge et al 1979). However isolation remains difficult and laborious even under the best conditions. For these reasons and the need for rapid routine diagnosis required in enzootic pneumoniae control schemes,

the direct immunofluorescent staining technique was developed to demonstrate the presence of M. hyopneumoniae in pneumonic tissues.

M. hyopneumoniae was first demonstrated in frozen lung sections by L'Ecuyer and Boulanger (1970) using direct IMF. Further studies by Meyling (1971) proved the technique to be specific for M. hyorhinis also. Since this work the IMFT has been used widely as a simple and specific method for diagnosis. By allowing visualisation of mycoplasmas in pneumonic tissues (Livingston et al 1972 ; Holmgren 1974c ; Gois et al 1975 ; Giger et al 1977 ; Weng 1980 ; Amanfu et al 1980), IMF confirms the necropsy and serological diagnosis of enzootic pneumonia.

Antibodies to M. hyopneumoniae were first detected by Roberts (1968) using the CFT in experimentally infected pigs. Refinements to this method (Boulanger and L'Ecuyer 1968 ; Takatori et al 1968 ; Hodges and Betts 1969a ; Goodwin et al 1969a ; Wallis and Thompson 1969 ; Roberts and Little 1970 ; Eskildsen and Schjerning - Thiesen 1971 ; Slavik and Switzer 1972) have led to wide use of the CFT in enzootic pneumonia control schemes (Woods et al 1976 ; McKean et al 1979). However, demonstration of low levels of antibody in pig serum by CF methods is difficult because low dilutions of pig serum are usually very procomplementary (Eskildsen 1973). This problem is overcome by testing one low dilution of serum in the presence of a range of C' concentrations

thereby keeping the procomplementary affect for that serum constant, allowing the amount of C' fixed by the antibody-antigen complex to be calculated (Etheridge and Lloyd 1980).

This chapter describes the establishment and verification of non-specific and specific diagnostic techniques (Chapter 2) using specimens from experimentally infected pigs and field cases of EPP.

3.2 Materials and Methods

3.2 (i) Pathology

Pneumonic lesions were classified as being typical of enzootic pneumonia by the criteria outlined in 2.1.(i).

Lung tissues were then sampled and sectioned as described in 2.1.(iv)(c). Microscopic lesions in experimentally infected pigs were classified according to the description of lesions in 2.1(iv)(a) (Whittlestone 1972), whereas those from field cases followed the criteria detailed in 2.1(iv)(b) (McKean et al 1979).

(ii) Mycoplasmas

Cultures of Beaufort (wild type isolate) and LKR M. hyopneumoniae strains were grown in MH medium (Appendix V). A field strain of M. hyorhinis, isolated

and typed by CSIRO Melbourne, was cultured in MH broth and on PPLO agar. Preparations of these cultures were made for giemsa and IMF staining (2.2. and 2.4).

(iii) Giemsa staining

Preparations of mycoplasma cultures were stained with Gurr's R66 improved Giemsa for 3 hours (2.2(i)). Touch preparations for Giemsa and IMF staining were from the same cut tissue surface.

(iv) Complement Fixation

Serum samples were tested for presence of M. hyopneumoniae antibody using the C' consumption method described in Appendix II. Freeze dried positive and negative control serum samples were obtained from CSIRO Melbourne, to routinely check the sensitivity of the CFT. Later, control serum was obtained from pigs experimentally infected with M. hyopneumoniae which showed positive CF reactions when tested against the CSIRO control.

The direct CF method (Appendix III) was used to determine the titre of M. hyopneumoniae antibody in the hyperimmune serum supplied by the CSIRO. A further 194 serum samples collected at random from slaughtered pigs were tested by both methods to determine which CF method was most sensitive.

(v) Immunofluorescence

Determination of IMF titre

Dilutions of M. hyopneumoniae and M. hyorhinis FITC conjugates, ranging from 1/3 to 1/96 of the original volume of undiluted serum were prepared. Homologous culture preparations were then stained to ascertain the greatest dilution giving strong specific fluorescence with a minimum of non-specific fluorescence.

Specificity

The specificity of all conjugates, including FITC conjugated non-immune sera were checked by using each to stain cultures of M. hyopneumoniae, M. hyorhinis and smears of uninoculated MH medium. Blocking tests (2.4(vii)) using unlabelled homologous immune antiserum for pretreatment of smears, prior to staining with specific FITC conjugate were performed.

(vi) Lung sectioning for IMF

Initially fresh lung tissue was frozen and stored as described in 2.4(viii), then sectioned using a cryostat, 2.4(ix).

Due to processing of other highly infectious material on the same cryostat, and lack of a satisfactory alternative facility, an alternative sectioning method was used. Paraffin embedding of the thawed tissue blocks was adopted and the majority of specimens were processed by this method (2.4(x)).

(vii) Assessment of techniques

These procedures were assessed by initially using specimens from experimentally infected pigs and then on serum samples and typical lung lesions from field cases.

(a) Specimens obtained from experimentally infected pigs

Six pigs were inoculated intranasally with M. hyopneumoniae (Beaufort strain) and placed immediately in-contact with 24 other EPP free pigs. All pigs were bled prior to exposure and inoculated pigs were bled at weekly intervals until slaughter, between 4 to 10 weeks later. The in-contact pigs were slaughtered 11 weeks post-exposure at a local abattoir, whereas inoculated pigs were slaughtered on site to avoid contamination with other mycoplasmas and secondary bacteria.

A group of 6 placebo inoculated (MH medium) pigs were held in-contact with another 24 control pigs. All conditions described applied to these pigs also.

Lungs were obtained aseptically from the inoculated pigs, and in the case of the in-contact animals, from the viscera table at the abattoir. Each was placed in a separate

labelled plastic bag and taken immediately to the laboratory. Blood samples were collected from 6 pigs in-contact with the M. hyopneumoniae inoculated transmitters and from 6 similar control pigs, at 2 week intervals throughout the trial. All pigs were blood sampled at slaughter.

Specimens collected at slaughter were lung sections for histopathology and IMF, touch preparations for Giemsa staining and IMF and serum for CF testing.

(b) Specimens obtained from field cases

Serum samples were collected at slaughter from 412 pigs and correlated with presence or absence of gross lesions of EPP in the corresponding lung. Lungs were either evaluated on the viscera table at the abattoir (approximately a half) or removed for a more detailed examination later (Chapter 4). Serum samples were tested by one or both of the CF methods for presence of M. hyopneumoniae antibody.

Twenty two EPP affected lungs and 22 grossly normal lungs from this group of commercial pigs were submitted for laboratory examination. Lung sections were collected for histopathology, touch preparations prepared for Giemsa staining and serum samples collected for CF testing.

3.3 Results

3.3 (i) Mycoplasma culture

Beaufort and LKR strains of M. hyopneumoniae were both grown successfully in all batches of MH broth medium. Cultures of LKR (0.3 ml of stored culture thawed from -80°C, added to 3 ml of MH medium) produced a colour change from red to yellow (++++) in 48-72 hours, and a similar reaction in 24-36 hours when ++++ broth was subcultured. By comparison cultures of Beaufort strain grew at approximately half the rate of LKR.

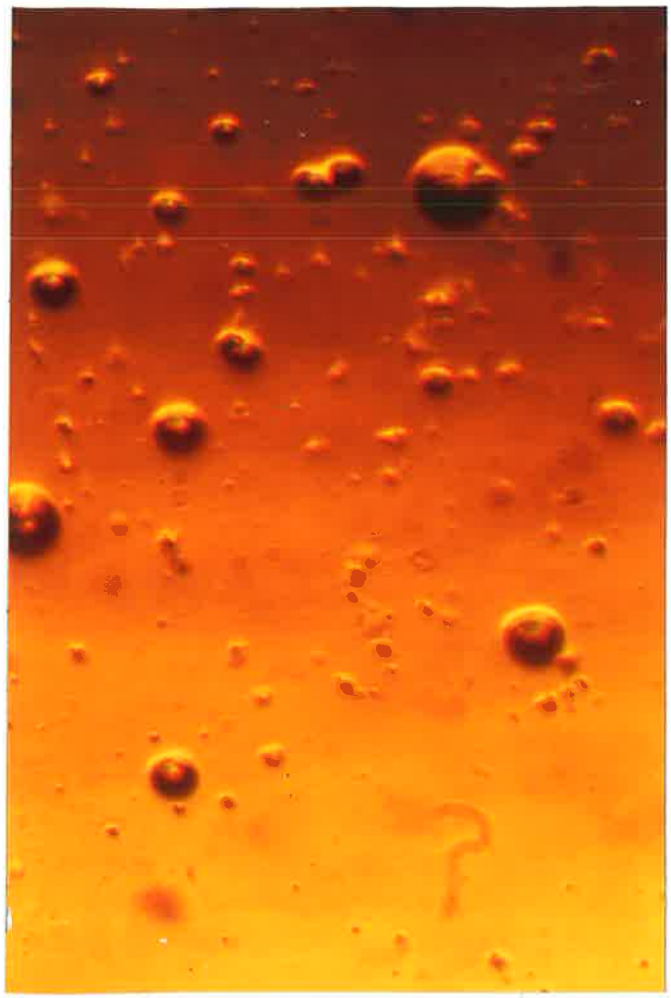
Primary cultures of M. hyorhinis in MH medium produced yellow (+++/++++) colour reactions within 24-36 hours. When this broth was inoculated onto PPLO agar plates colonies were visible within 2-3 days (Fig. 3.1). These were passaged by removing the block of agar containing cultures, inverting it and smearing over the surface of a fresh plate.

All lungs were cultured in MH medium, being passaged weekly for 4 weeks. Mycoplasmas were not recovered from any lung.

(ii) Giemsa staining

M. hyopneumoniae organisms appeared as small bipolar organisms and ring forms, often with thickenings at 1 or more points, in smears of broth culture (Fig. 3.2).

Fig. 3.1 M. hyorhinis colonies grown on agar. Note typical "fried egg" appearance as the central colony penetrates the agar.



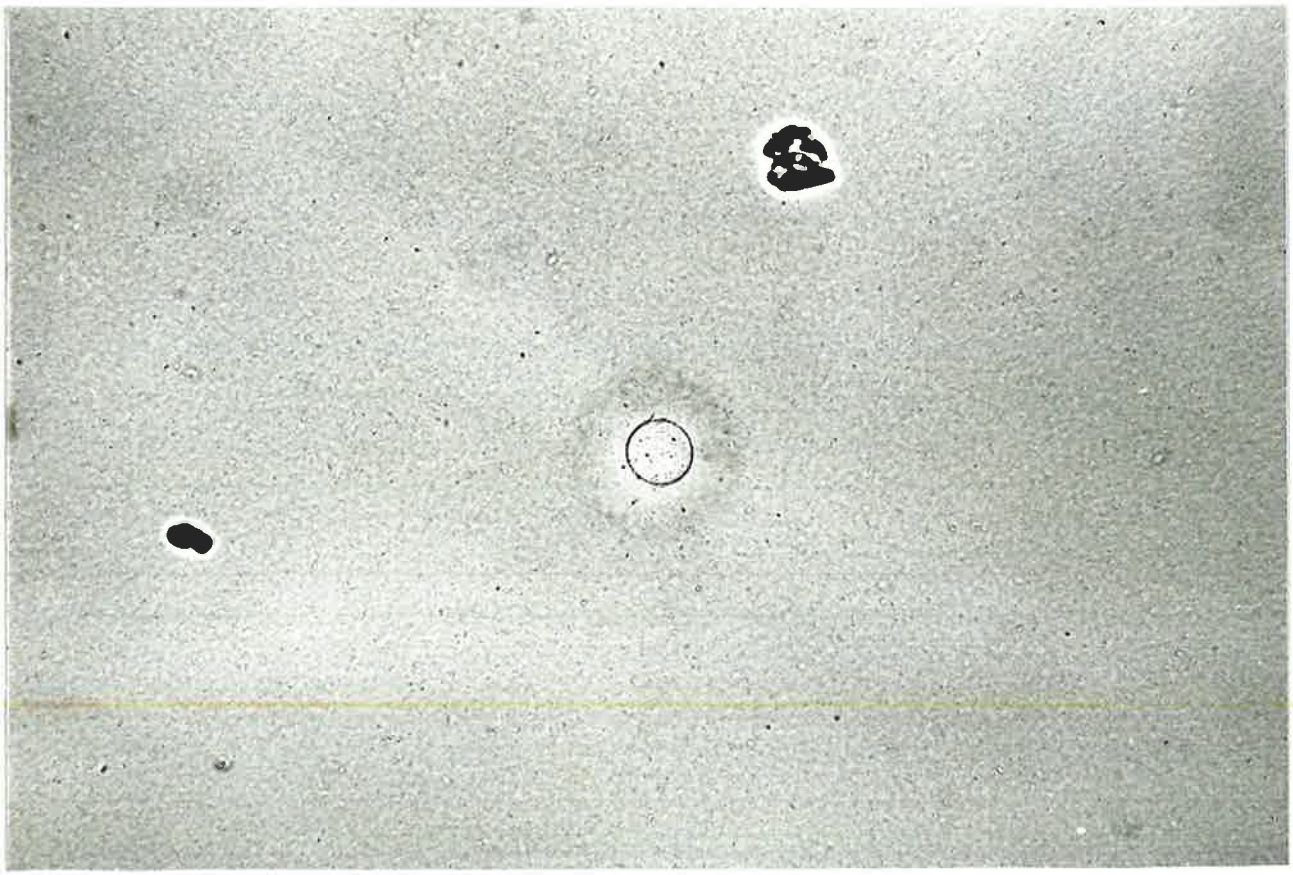


Fig. 3.2. Giemsa stained washed culture smear of M. hyopneumoniae featuring bipolar and "ring form" organisms. x 1000

Fig. 3.3. Giemsa stained smear of washed M. hyorhinis culture featuring comma shaped organisms (→) and cocci. (x 1000)

M. hyorhinis appears as comma-shaped organisms and cocci in culture smears (Fig. 3.3).

(iii) CFT

C' consumption titres on positive control serums were of the same order as titres recorded on the samples by CSIRO. Serum samples from EPP free-pigs were used as negative controls.

The working dilution of some batches of M. hyopneumoniae antigen were adjusted to between 1/10 to 1/20 in order to maintain the same positive control titre and sensitivity.

The CF antibody titre of the M. hyopneumoniae hyperimmune antibody was >31 $C'H_{50}$ units for the C' consumption method and 1/1024 for the direct method.

Both CF methods had the same average probability in detecting positive and negative titres, which was confirmed by the Wald test ; in 87% of cases the results of the 2 tests agreed (Table 3.1(1)).

The C' consumption method detected antibody in 61 serum samples compared with 53 by the direct method. The increased number detected by the C' consumption method was due to more serum samples with low antibody levels being detected by this method only (Table 3.1(2)).

Table 3.1(1). Relationship between C' consumption CF and direct CF methods on 194 serum samples collected from slaughtered pigs.

C' consumption $C'H_{50}$ units	Direct CF titre (inverse of dilutions)		TOTAL
	$< 1/8$ (-)	$> 1/8$ (+)	
<4.6	124	9	133
>4.6	17	44	61
TOTAL	141	53	194

Wald test for repeated measurements on the same sample.

H_0 = both tests have the same average probability of classifying positives and negatives.

$$\chi^2_1 = 2.46 \quad NS$$

$\therefore$ Retain H_0. (in 87% of cases the results agree).

Table 3.1 (2) Actual antibody titres recorded by both methods on the same serum samples.

C' consumption $C'H_{50}$ units	Direct CFT (inverse of dilutions)									
	0	*8	16	32	64	128	256	512	1024	
0	48	-	-	-	-	-	-	-	-	48
0.4	41	-	-	-	-	-	-	-	-	41
1	23	-	3	1	-	-	-	-	-	27
1.8	10	-	-	-	-	-	-	-	-	10
3	2	1	-	3	1	-	-	-	-	7
*4.6	9	1	3	2	1	-	1	-	-	17
7	6	-	3	-	2	-	-	-	-	11
10	-	-	4	2	-	1	-	-	-	7
15	1	-	-	1	-	-	2	-	-	4
22	1	-	2	5	2	1	-	-	-	11
31	-	3	1	3	2	-	-	1	1	1
TOTAL	141	5	16	17	8	2	3	1	1	194

*More serum samples with lowest level of antibody detectable by each test, were identified by the C' consumption method than by the direct CF method.

(iv) IMF

M. hyopneumoniae - FITC conjugate produced specific fluorescence at a dilution of 1/48 of the original volume but was employed at a dilution of 1/12 to give maximum specific fluorescence with minimal non-specific staining.

Similarly M. hyorhinis - FITC conjugate produced specific fluorescence at a dilution of 1/128 of the original volume but was employed at a dilution of 1/32 to give maximum specific fluorescence with minimal non-specific staining.

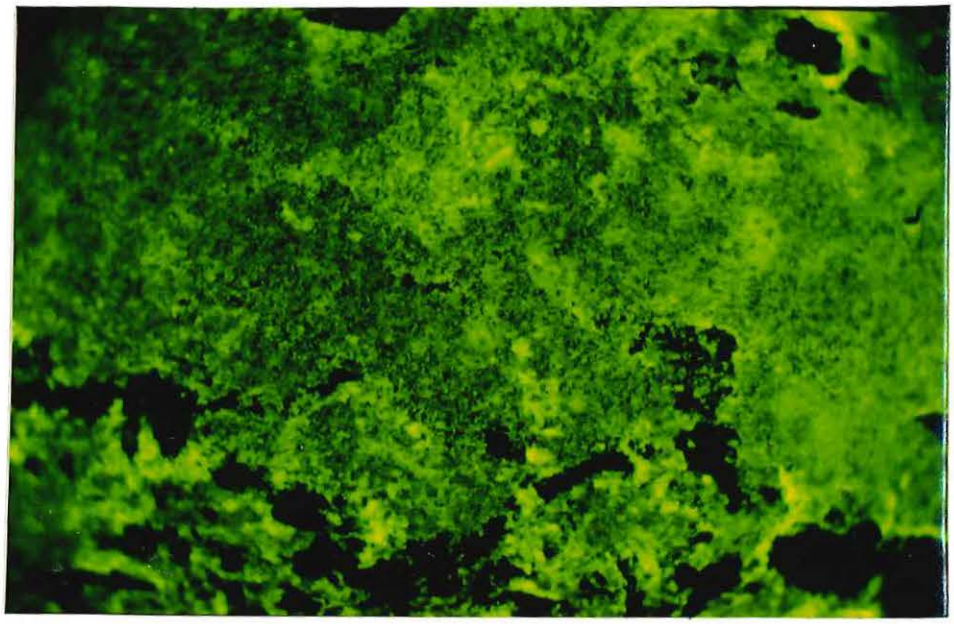
Each conjugate produced specific fluorescence only on homologous cultures. Non-immune - FITC conjugate failed to produce specific fluorescence on either strains of mycoplasmas and when used at a dilution of 1/12 of the original volume produced no non-specific fluorescence.

Smears of mycoplasma culture sediments stained with homologous immune pig conjugates appeared as intensely fluorescent, greenish-yellow bodies, or clumps of bodies evenly distributed over the glass surface (Fig. 3.4).

Blocking tests showed that fluorescence was reduced only when culture smears were pretreated with homologous antiserum. Figure 3.5 shows reduction in fluorescence of M. hyopneumoniae culture smears after pretreatment with M. hyopneumoniae antiserum.

Fig. 3.4. (1) M. hyopneumoniae broth culture smear stained with M. hyopneumoniae - FITC conjugated antiserum. Note fluorescence of individual organisms and broth colonies. (x400).

(2) M. hyorhinis broth culture smear stained with M. hyorhinis - FITC conjugated antiserum. Note fluorescence of individual organisms and broth colonies. (x400)



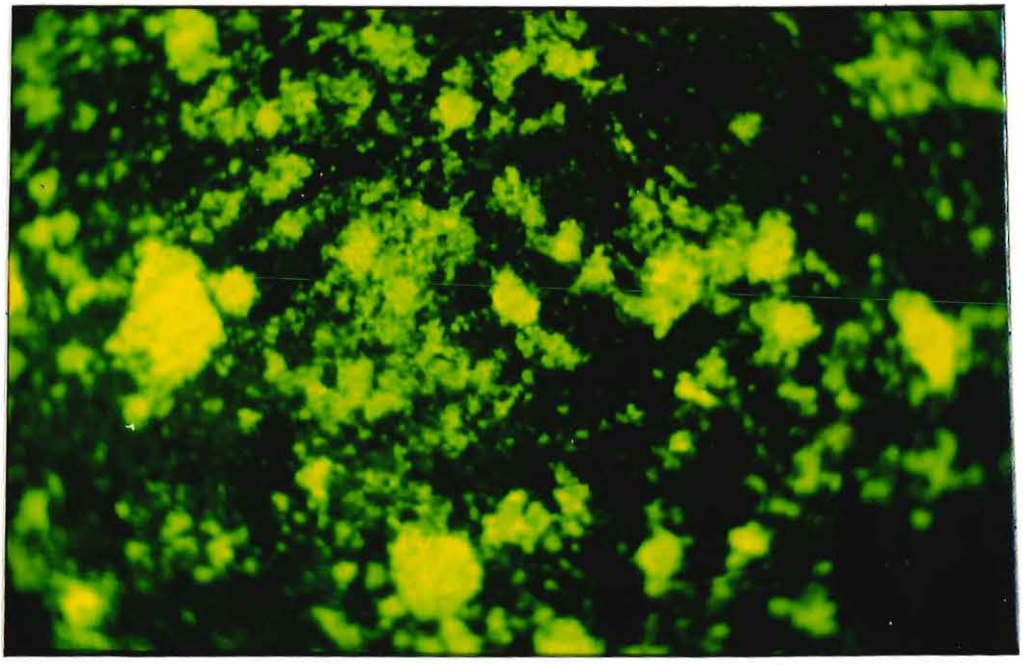
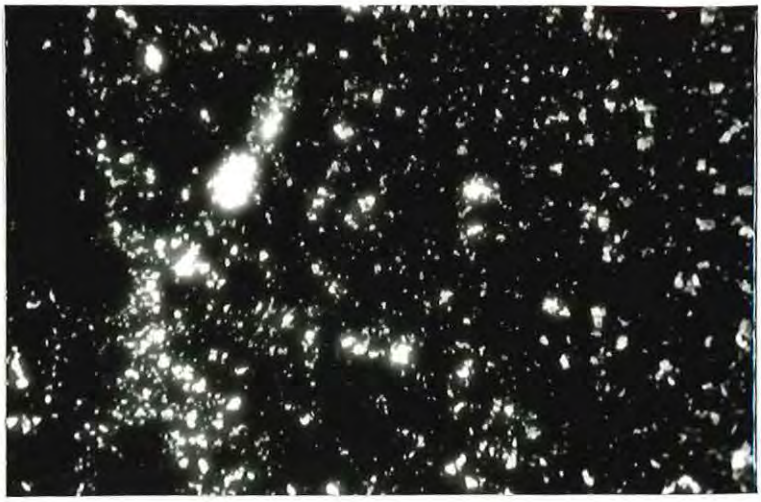


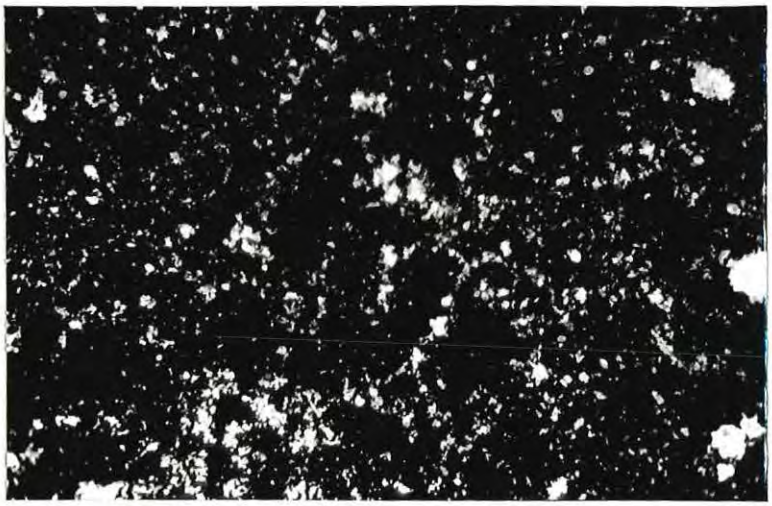
Fig. 3.5. Blocking test for M. hyopneumoniae - FITC conjugated antiserum. All M. hyopneumoniae broth culture smears were stained with homologous conjugate for 15 mins. after pretreatment.

(1) Pretreatment with PBS for 16 hrs. at 4°C. (x400)

(2) Pretreatment with normal serum for 16 hrs. at 4°C. (x400).

(3) Pretreatment with M. hyopneumoniae antiserum for 16 hrs. at 4°C. (x400).





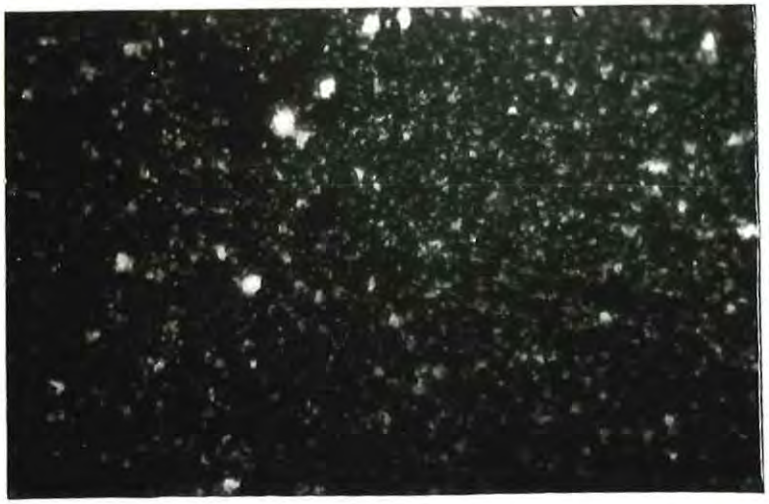
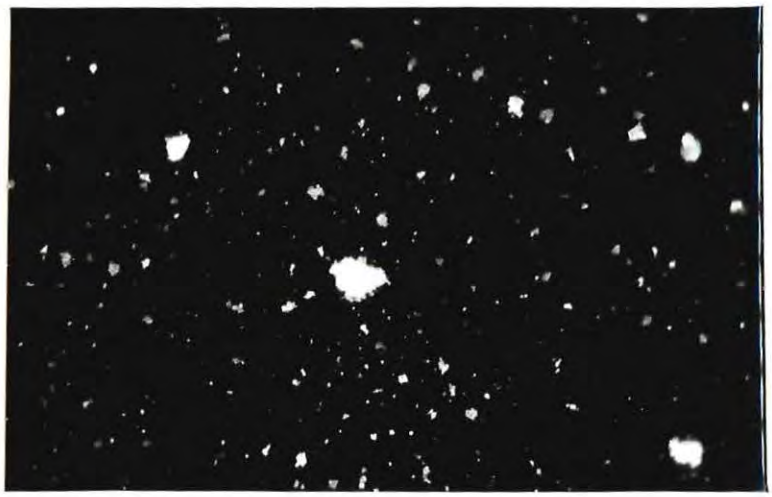


Figure 3.6 Blocking test for M. hyorhinis-FITC conjugated antiserum. All M. hyorhinis broth culture smears were stained with homologous conjugate for 15 mins. after pretreatment.

(1) Pretreatment with PBS for 16 hours at 4°C. x400

(2) Pretreatment with normal serum for 16 hours at 4°C. x400

(3) Pretreatment with M. hyorhinis antiserum for 16 hours at 4°C. x400



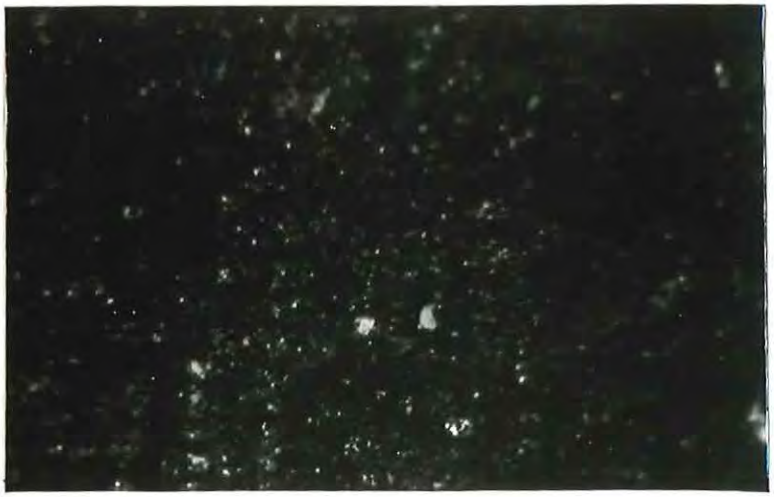


Figure 3.6 shows a similar homologous reaction for M. hyorhinis - FITC conjugate.

Staining of duplicate culture smears with non-immune FITC conjugate did not at any time produce specific fluorescence.

Slight non-specific fluorescence was observed on some tissue sections with all 3 conjugated antisera. This may have been due to staining by dissociated aggregates of fluorescein molecules which were removed by absorption on pig liver homogenate after thawing.

(v) Assessment of diagnostic procedures

Diagnostic procedures were evaluated on specimens from both experimentally infected and natural field cases of enzootic pneumonia.

(a) Clinical and post mortem findings in infected pigs

Pigs inoculated intranasally with suspension of pneumonic lung

All 6 inoculated (transmitter) pigs developed a harsh hacking cough which was first observed 6 days p.i. (mean 8.2 days).

Five of these had gross EPP lesions in 1 or more lobes when slaughtered 4 to 10 weeks p.i.

Pigs held in-contact with inoculated pigs

Twenty two of 24 in-contact (naturally challenged) pigs were observed to cough. The first was observed to cough 10 days after the transmitter pigs were challenged (mean 30.8 days).

Seventeen of 24 (71%) pigs had gross EPP lesions when killed 11 weeks after initial exposure. Acute lesions were present in 36.4% and chronic lesions in 63.6% of lungs. Lesions were present in 1 or more lobes with the right cardiac being the most frequently affected ; 76% of affected lungs had lesions in this lobe.

Gross pneumonic lesions were not observed in any of the control pigs.

(b) Results of diagnostic procedures on specimens from experimentally infected pigs

The results of the diagnostic procedures on the 6 inoculated and 24 naturally challenged pigs are shown in Tables 3.2 and 3.3.

All of the 30 pigs experimentally infected with M. hyopneumoniae developed typical histopathologic changes. Twelve had third stage lesions (Fig. 2.5) while 11 had fourth stage lesions (Fig. 2.6).

Table 3.2 Results of diagnostic procedures on pigs inoculated and naturally challenged with *Mycoplasma hyopneumoniae* and on control pigs.

Enzootic Pneumonia	Gross Evaluation of lungs	Number of lungs examined	Histopathology				Touch preparations T.P.		Complement fixation test		Immunofluorescence			
											T.P.		Lung Sections	
			4	3	2	-	+	-	+	-	+	-	+	-
Inoculated	Lesions present	5	4	1	0	0	3	2	5	0	1	4	4	1
	Lesions absent	1	1	0	0	0	1	0	1	0	0	1	0	1
	Total	6	5	1	0	0	4	2	6	0	1	5	4	2
Exposed to infected pigs	Lesions present	17	14	3	0	0	9	8	9	8	8	9	9	8
	Lesions absent	7	1	6	0	0	2	5	3	4	0	7	1	6
	Total	24	15	9	0	0	11	13	12	12	8	16	10	14
Placebo Inoculated Controls	Lesions present	0	0	0	0	0	0	0	0	0	0	0	0	0
	Lesions absent	6	0	0	0	6	0	6	0	6	0	6	0	6
	Total	6	0	0	0	6	0	6	0	6	0	6	0	6
Exposed to placebo inoculated pigs	Lesions present	0	0	0	0	0	0	0	0	0	0	0	0	0
	Lesions absent	24	0	0	0	24	0	24	0	24	0	24	0	24
	Total	24	0	0	0	24	0	24	0	24	0	24	0	24

+=Positive -=Negative 4=Fourth stage lesion 3=Third stage lesion 2=Second stage lesion

175

TABLE 3.3

Results of diagnostic techniques on experimentally infected pigs

Identity	Challenge Method	Gross Lesions	Histo-Pathology	CFT†	Touch preps	Immunofluorescence	
						T.P.	Sections
Y_3	C.N.	Present	4	++	+++	+++	+++
W_1			4	++	++	+++	++
G_1			4	+	+	++	+++
W_3			4	++	+	+	++
Y_1			4	+++	-	++	+
BW_1			4	++	+	-	++
R_5			4	+++	-	-	+
P_5			4	-	-	-	+
W_5			3	-	-	-	+
P_1			4	+++	+	+	-
B_3			4	-	+	+	-
O_3			3	-	-	+	-
R_1			3	-	+	-	-
Y_5			4	-	+	-	-
O_5			4	+++	-	-	-
BW_5			4	-	-	-	-
R_3			4	-	-	-	-
B	C.N.	Absent	3	++	-	-	+
P			3	-	+	-	-
G			3	-	+	-	-
BW			3	+	-	-	-
B_1			3	+	-	-	-
O_5			3	-	-	-	-
G_5			4	-	-	-	-
1-12	I.N.	Present	4	++	+	+	+
5-7			4	+++	++	-	+
5-4			4	+++	+	-	+
10-1			3	+	-	-	+
3-1			4	+++	-	-	-
5-5	I.N.	Absent	4	+	+	-	-

I.N. = Inoculated intranasally 4 = Fourth stage lesions + = weak positive

C.N. = Challenged naturally 3 = Third stage lesions ++ = moderate

† CFT at slaughter +++ = strong

Results of isolation attempts on infected, in-contact and control animals appear in Table 3.4. Pasteurella multocida and Haemophilus parainfluenza were recovered from 2 pneumonic lungs only, while α-haemolytic Streptococci were isolated from challenged pigs only.

Mycoplasmas with morphology typical of M. hyopneumoniae, bipolar or ring form, were observed in giemsa stained touch preparations in 50% of 30 cases (Fig. 3.8 (1)).

All pigs had negative CF titres prior to exposure, with positive titres then being detected in all inoculated pigs 11 weeks after infection was introduced. Pig B_3 (Table 3.3), one of 6 in-contact pigs blood sampled through out the trial, first developed a positive CF titre 6 weeks after inoculation of the transmitters, but recorded a negative CF titre 5 weeks later at slaughter.

Table 3.4 Bacteria isolated from lungs of infected and control pigs.

EPP Infection Status	Number of pigs	*M. hyopneumoniae*	*M. hyorhinis*	*Bordetella bronchiseptica*	*P. multocida*	*H. para-influenza*	α-haemolytic Streptococci
Inoculated *M. hyopneumoniae*	6	NC	-	-	-	-	2
In-contact with above	24	NC	-	-	1	1	4
Placebo inoculated	6	NC	-	-	-	-	-
In-contact with above	24	NC	-	-	-	-	-

NC = Not cultured

Fluorescence observed in IMF stained touch preparations appeared as spots or clumps concentrated around cells and debris (Fig. 3.7). The fluorescent points matched the giemsa stained touch preparations in order of frequency and distribution (Fig. 3.8). These individually fluorescing organisms could also be demonstrated in washed M. hyopneumoniae cultures (Fig. 3.5).

Difficulty was encountered as granulocytes stained non-specifically. Intracellular granules or granules released from ruptured cells were yellow and appeared larger than mycoplasmas. The granules stained with both immune conjugates and non-immune control conjugate.

In lung sections, fluorescence was typically limited to the surface of bronchi and bronchioles (Fig. 3.9) and to the exudate contained (Fig. 3.10). The antigen appeared as granular, intensely fluorescent yellow-green aggregates and small colonies which closely followed the epithelial folds in the large airways.

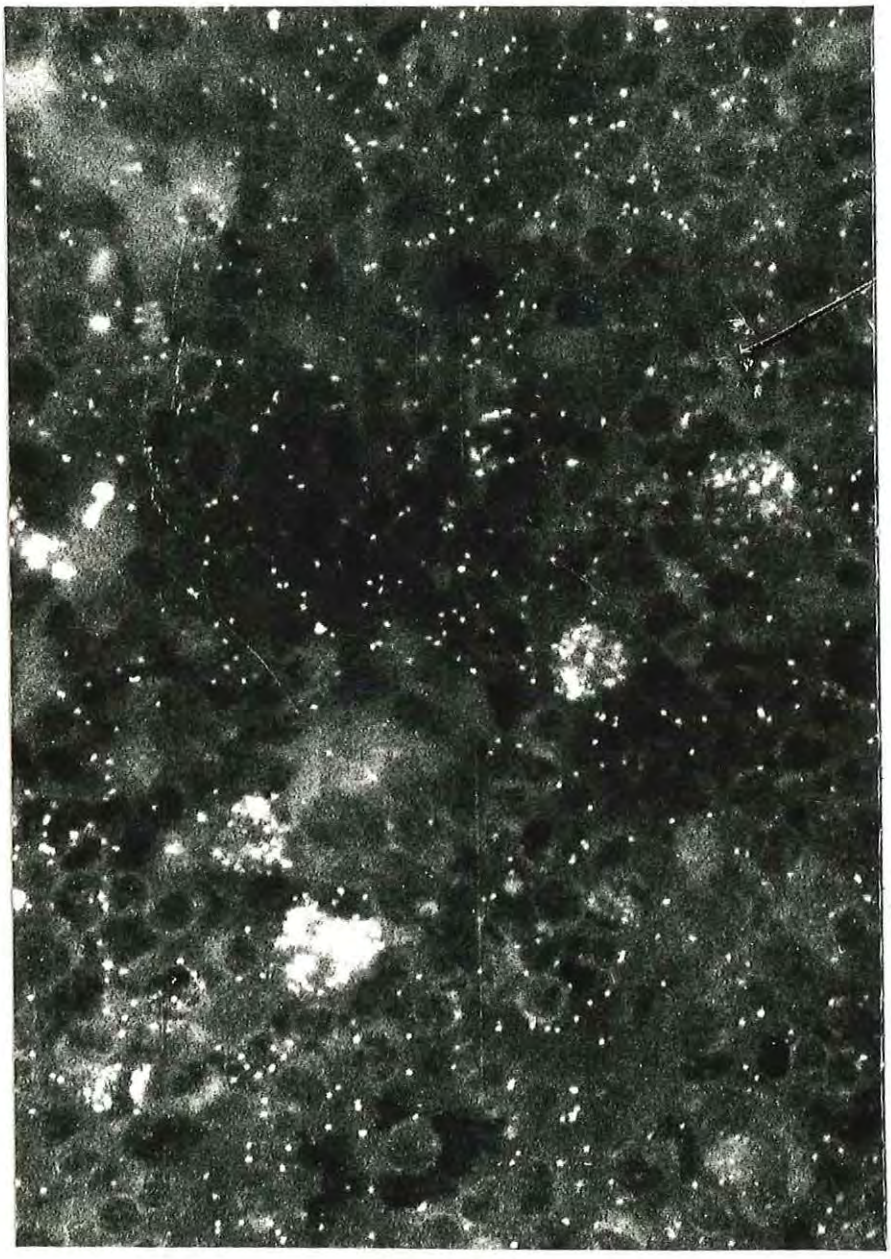
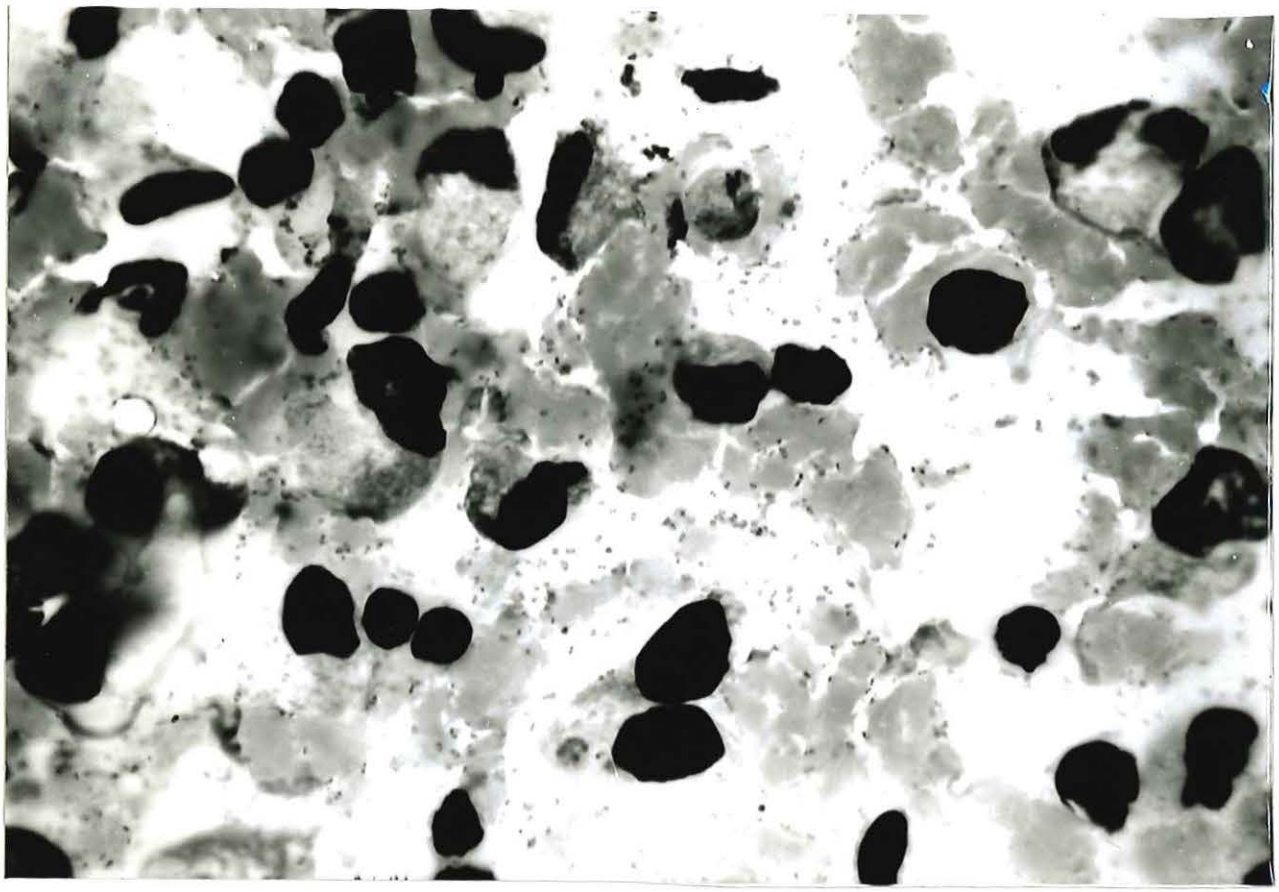


Fig. 3.7. M. hyopneumoniae organisms stained by M. hyopneumoniae - FITC conjugated antiserum in a touch preparation prepared from pneumonic lung. (x400).

Fig. 3.8. Comparison of touch preparations from pneumonic tissue of pig Y_3 stained by Giemsa and IMF methods.

(1) Presence of plentiful bipolar stained organisms with morphology typical of M. hyopneumoniae between dark staining nuclei of mononuclear inflammatory cells. (x1000)

(2) Specific fluorescence of M. hyopneumoniae organisms in a duplicate touch preparation of above. (x400)



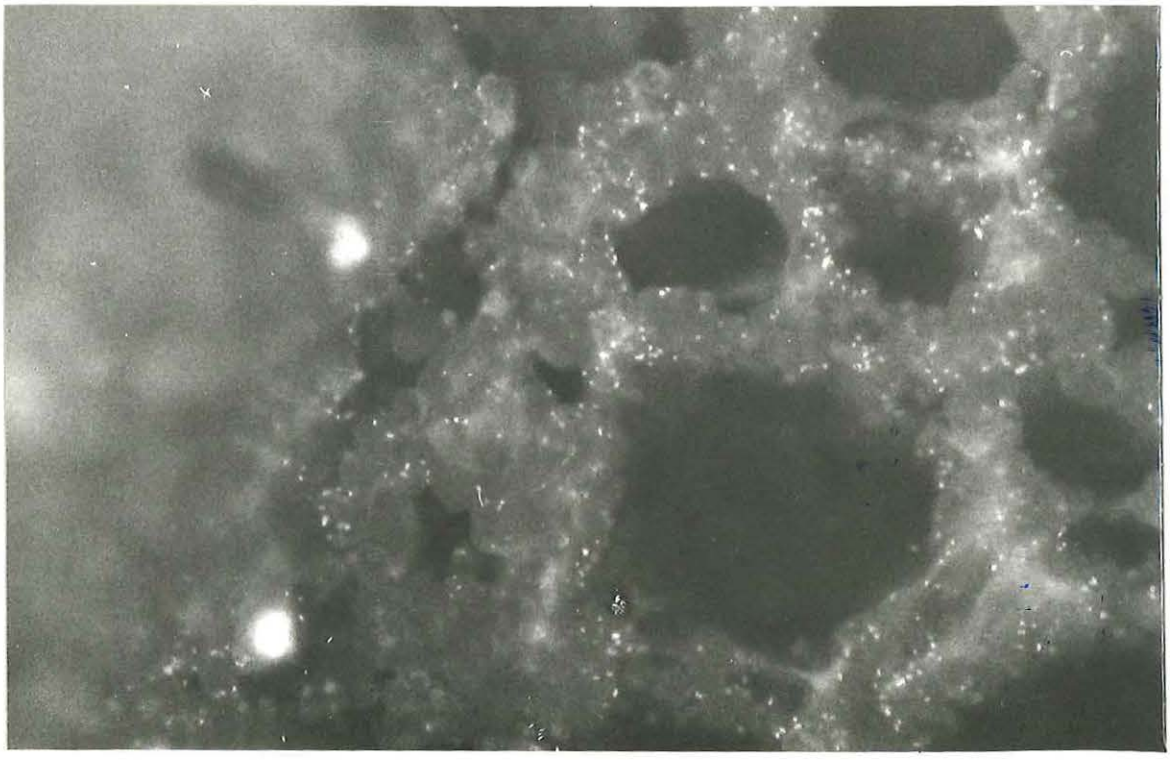
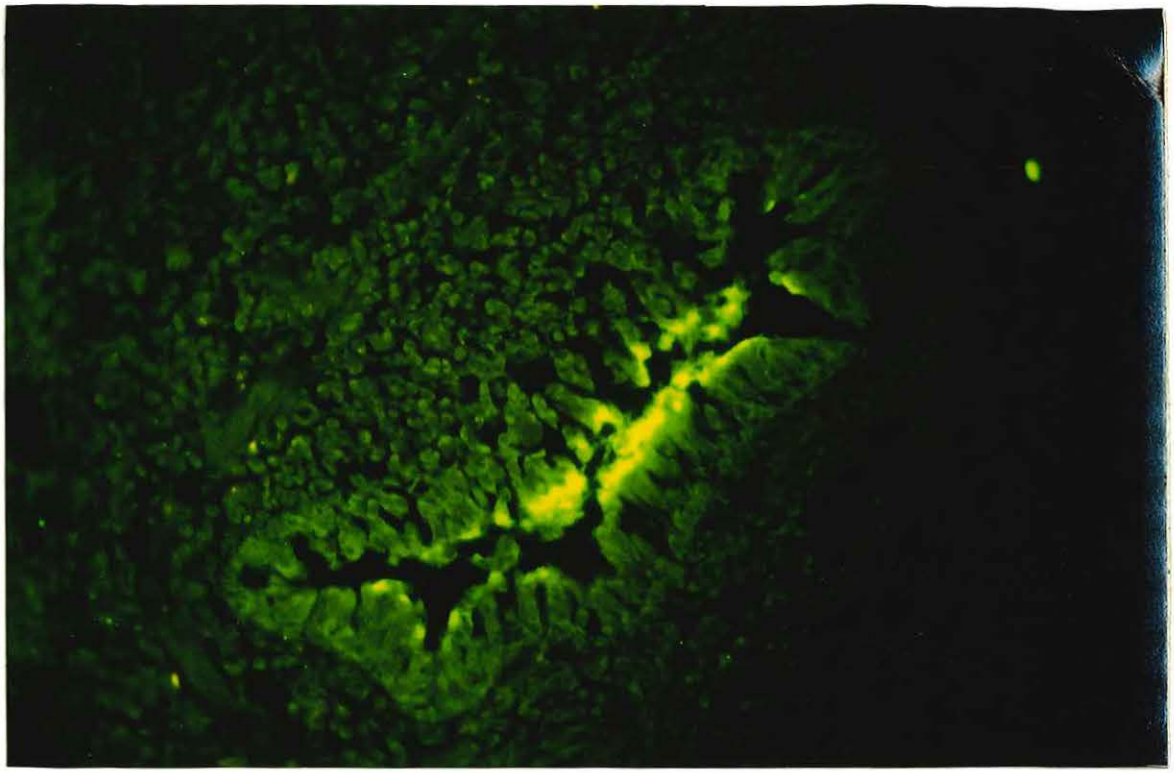
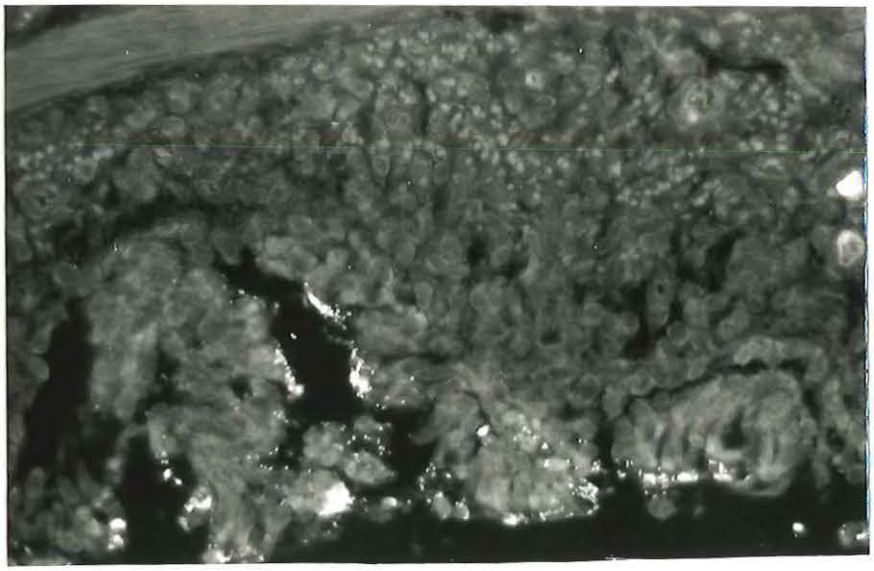


Fig. 3.9. (1) Large numbers of M. hyopneumoniae organisms fluorescing on the surface of a bronchiole. Lung section from pig Y_3 x 250.

(2) Individual M. hyopneumoniae organisms visible on respiratory surface. Section from pig Y_3 x 400.





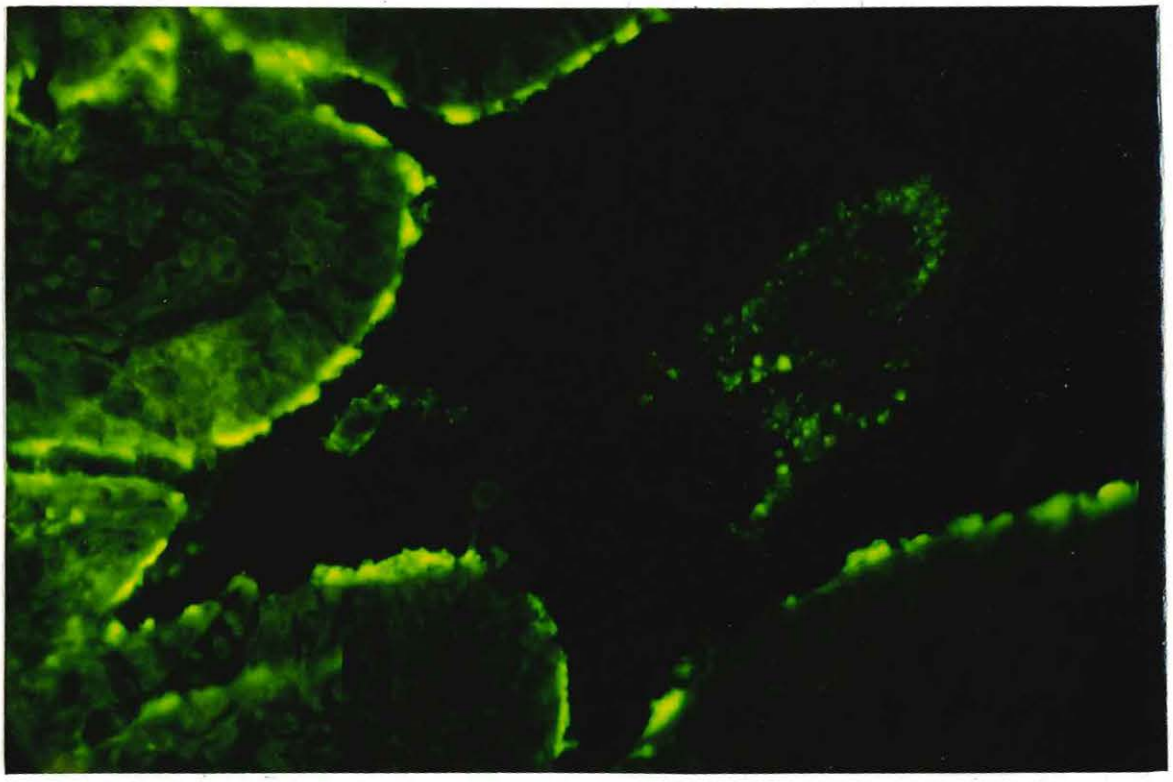


Fig. 3.10. Exudate contained within an airway showing M. hyopneumoniae organisms stained with M. hyopneumoniae - FITC conjugated antiserum - lung Y_3. (x400).

The results of each test on specimens from the 30 M. hyopneumoniae challenged pigs is shown in Table 3.3. The strength of test reaction is also compared.

Results of tests on the 22 lungs with gross E.P. lesions, indicated that giemsa and IMF staining of touch preparations were dependent ($p<0.1$). Figure 3.8 shows results of both methods on touch preparations of lung Y_3. IMF staining of touch preparations was positive in 41% of cases whereas 59% of lung sections were IMF positive.

The diagnosis of EPP, based on presence of typical gross lesions in 22 lungs (Table 3.3) was confirmed either by CFT or IMF on tissue sections in all but 5 cases. All 22 cases showed histological evidence of EPP, 14 had detectable CF antibody at slaughter, lung sections from 13 fluoresced specifically to M. hyopneumoniae FITC conjugate, mycoplasmas were observed in giemsa stained T.P. from 12 and 9 T.P. were IMF positive.

In the 8 challenged cases where lungs were grossly normal at slaughter, 4 had detectable CF titres, mycoplasmas were

observed in Giemsa stained T.P. of 3 and 1 lung section was IMF positive. All had histological evidence of enzootic pneumonia.

(b) Results of diagnostic procedures on field cases of enzootic pneumonia.

The presence of a positive CF titre to Mycoplasma hyopneumoniae in serum in association with gross lesions typical of enzootic pneumonia was highly significant ($X^2_1 = 55.32$, $p<0.001$). In cases where serum samples were positive 69.8% of corresponding lungs had EPP lesions, whereas with CF negative serum samples 27.7% of lungs had EPP lesions (Table 3.5).

Where lungs were grossly affected with EPP, 44.9% of 156 corresponding serum samples were CF positive. In 256 normal lungs 12.1% of corresponding serum samples were CF positive.

In commercially reared pigs (Table 3.6) with pneumonic lesions, 59.1% had histological lesions positive for enzootic pneumonia, 27.3% had suspect lesions, while the remaining 13.6% had lesions of

Table 3.5. Presence and absence of CF titres to M. hyopneumoniae in 412 sera and EPP lesions in corresponding lungs

E.P. Lesions	C' Consumption Method Reaction		TOTAL
	Positive	Negative	
Present	70	86	156
Absent	31	225	256
TOTAL	101	311	412

*** $p<0.001$

H_0 : EPP lesions and complement fixation reaction are independent

df	X^2	
1	55.32	***

$\therefore$ reject H_0

Table 3.6. Result of diagnostic procedures on field specimens.

Lesions of enzootic pneumonia	Number Examined	Microscopic evaluation			Touch preparations		Complement fixation test	
		+	±	-	+	-	+	-
Present	22	13	6	3	10	12	16	6
Absent	22	4	6	12	2	20	1	21

+ = positive ± = suspect - = negative

necrotising bronchopneumonia. Mycoplasmas were observed in 45.5% of giemsa stained touch preparations and CF antibodies detected in 72.7% of serum samples.

In the 22 grossly normal lungs 18.2% had histological lesions positive for EPP, while 27.3% were suspect. Mycoplasma organisms were observed in 9.1% of touch preparations and 4.5% had detectable CF titres. Mycoplasma organisms and the positive CF titre were present in cases in which histological examination indicated EPP.

3.4 Discussion

The investigation verified the specificity and indicated the sensitivity of the diagnostic procedures established and when used on experimental and field cases of EPP proved accurate in diagnosis. From the results it is recommended that each case be subjected to a combination of diagnostic methods to obtain the maximum number of diagnoses.

Establishment and verification of diagnostic techniques

All batches of MH medium supported growth of Beaufort and LKR strains of M. hyopneumoniae and also M. hyorhinis when tested. However, when these batches were used for isolation of M. hyopneumoniae from experimentally infected pigs, mycoplasmas were not isolated. During these attempts the MH medium adapted strains continued to grow. The reason for this lack of sensitivity of the medium was not determined despite checking of medium production techniques. Further investigation was not conducted due to emphasis of the project being on rapid diagnostic methods and mycoplasmas were successfully cultured for testing of IMF stains.

While the direct CF method (Slavik and Switzer 1972) was simpler and quicker to perform, it was shown to be less sensitive than the C' consumption CF method (Etheridge and Lloyd 1979). The latter method was more sensitive in detecting low levels antibody, which was facilitated by elimination of the variable procomplementary activity of pig serum which makes interpretation of direct CF antibody titres $< {}^{1}/_{8}$ difficult.
Due to this advantage the C' consumption CF method was adopted for routine use in all further studies.

The specificity of the immunofluorescent staining technique was ascertained by cross-staining of washed mycoplasma organisms grown in broth. Mycoplasma

organisms were stained only by homologous antibody conjugates and appeared as intensely fluorescent bodies or clumps of bodies. Non-specific fluorescence was minimal in cross-stained mycoplasma smears when conjugates were used at the highest dilution giving strong specific fluorescence on homologous cultures. No fluorescence was detected in smears of M. hyopneumoniae or M. hyorhinis organisms stained with non-immune conjugates. Staining could be effectively blocked by pre-incubation with homologous unlabelled immune pig serum.

Mycoplasma organisms typical of M. hyopneumoniae as described by Whittlestone (1973) were observed in Giemsa stained touch preparations from experimentally infected pigs (Fig. 3.8(1)). Staining of similar touch preparations with M. hyopneumoniae - FITC conjugates produced specific fluorescence with a similar distribution to that seen on Giemsa staining (Fig. 3.8(2)). IMF staining of touch preparations proved to be a simple, rapid and successful technique in the diagnosis of EPP considering that lesions were chronic with fewer organisms present. That Giemsa staining of touch preparations detected organisms in 8 cases when IMF was negative may also relate to the presence of only few organisms ; whose typical morphology on Giemsa staining allowed positive recognition, while their scarcity did not allow classification as IMF positive.

IMF staining of lung sections had the added advantage of allowing visualisation of the causative agent in situ in the affected tissue. Fluorescence appeared as previously reported(L'Ecuyer and Boulanger 1970 ; Meyling 1971 ; Livingston et al 1972 ; Holmgren 1974c ; Gois et al 1977 ; Giger et al 1977 ; Weng 1980, Amanfu et al 1980), as being limited to the epithelium of the bronchi and bronchioles and to the exudate filling the smaller airways (Figs. 3.9 and 3.10).

The hyperimmune M. hyopneumoniae antiserum used for conjugation had a direct CF titre of 1/1024, and the FITC conjugate was still active at dilution of 1/48, but was employed for staining at a dilution of 1/12. These working titres of the conjugates are consistent with those of L'Ecuyer and Boulanger (1970) who found that serum with a direct CF titre of 1/320 produced specific fluorescence at a titre of 1/20 and a working titre of 1/5. By comparison Weng (1980) found with a similar pig antiserum, with an IHA titre of 1/2048, conjugated with FITC was still active at a dilution of 1/32, but was employed at a dilution of 1/ 8.

Diagnosis of EPP in experimentally infected pigs

Gross lesions of enzootic pneumonia were present in 17 of 24 (71%) naturally challenged pigs at slaughter, 11 weeks after inoculation of transmitters. These results are similar to those of Etheridge and Lloyd (1979) who reported EPP lesions in 76.7% of 43 pigs slaughtered after

being held in-contact with transmitters (inoculated with Beaufort strain) for 4 to 11 weeks. The diagnosis of EPP based on appearance of gross lesions was confirmed at slaughter in 13 of these cases, by either CFT or IMF. Where naturally challenged pigs had grossly normal lungs, EPP was diagnosed in 3 of 7 cases by these methods, which was indicated in all cases by typical histologic lesions and mycoplasmas present in T.P. of 2 cases. These results support the observations of Slavik and Switzer (1972) and McKean et al (1979) which suggest that gross lesion resolution may have occurred before CF titre disappearance. However, the converse also appears to occur as pig B_3 developed a positive CF titre 6 weeks post-exposure which was undetectable 5 weeks later when gross lesions of EPP were found at slaughter. The results are also in accord with a previous observation (McKean et al 1979) that a greater number of pigs had histological lesions than CF titres, suggesting that CF may disappear before resolution of histologic lesions of EPP.

Slightly fewer lungs were positive by IMF staining of touch preparations when compared to IMF staining of sections. However, had material from bronchi been scraped out and a smear stained with IMF conjugate (Giger et al 1977) an increased number of lungs may have been diagnosed positive by the method.

Although the majority of affected lungs had chronic resolving lesions, reported to give reduced

fluorescence (L'Ecuyer and Boulanger 1970 ; Amanfu et al 1980), 59% of 22 with gross lesions and 12.5% of 8 normal lungs were diagnosed positive by this method. By comparison, in other studies using IMF staining of pneumonic lung sections (L'Ecuyer and Boulanger 1970 ; Meyling 1971 ; Holmgren 1974c ; Gois et al 1975) 66% of 12 lungs, 70% of 50 lungs, 52% of 25 lungs and 54% of 39 lungs were positive.

A good correlation was found between results of Giemsa and IMF stained touch preparations, and IMF staining of lung sections from 5 lungs, Y_3, W_1, G_1, W_3, 1-12 (Table 3.3). Reduced correlation seen in other lungs may be attributable to the low number of organisms present in chronic or resolved cases and the use of tissue sections for IMF from a different site than used for touch preparations. The strength of test reactions on these 5 lungs was found to compare well, as found by Giger et al (1977) on a group of 10 selected lungs. All tests were negative on specimens from the 30 control pigs.

A combination of specific and non-specific diagnostic tests were found to produce the highest frequency of EPP diagnosis. All 22 lungs (experimental) with gross lesions of EPP had typical histological lesions and in 16 of these the diagnosis was confirmed. Three were positive by CFT alone, 2 were positive only by IMF, and 11 were positive by both procedures.

Diagnosis of EPP in field cases

Results of the diagnostic procedures on field cases demonstrated a high degree of accuracy. The non-specific diagnosis of EPP based on gross appearance of pneumonic lesions at slaughter was confirmed by the highly significant association between detectable CF titres and presence of gross pneumonic lesions in corresponding samples from 412 pigs at slaughter ($p<0.001$) (Table 3.5). These results, using the complement consumption method (Etheridge and Lloyd 1980) compare favourably with those of McKean et al (1179) using the conventional complement fixation method (Slavik and Switzer 1972), who also found a significant association ($p<0.005$). Positive CF titres were recorded in 44.9% of serum samples corresponding with lungs with EPP lesions indicating that CF titres frequently disappear before lesions resolve. Conversely 12.1% of 256 serum samples corresponding with normal lungs had positive CF titres. These may be due to persistence of titres beyond resolution of lesions (Slavik and Switzer 1972) or be non-specific CF reactions which were found to occur in 9.4% of serum samples from EPP free herds (Woods et al 1976). The presence of positive CF titres in the absence of gross lesions observed in field cases may be due to resolution of lesions in some cases ; this explanation is supported by similar results recorded in 4 experimentally infected pigs (Table 3.2).

Results of diagnostic tests on field cases supported the diagnosis of EPP based on appearance of gross lesions. Of the 22 lungs with lesions (Table 3.6) only 3 had histological lesions other than being positive or suspect for enzootic pneumonia. These 3 showed extensive necrotising bronchopneumonia , evidence of secondary bacterial involvement. Mycoplasma organisms were observed in touch preparations from 45.5% of lungs and positive CF titres were detected in 72.7% of corresponding serum samples. While this positive CF reactor rate is substantially higher than recorded for the 412 serum samples of 44.9%, it probably results from testing a small sample , which is not representative. A small portion of grossly normal lungs showed evidence of EPP. Non-specific evidence of EPP in these was suggested by histologic lesions in 4 of 22 (18.2%), which was supported by the detection of mycoplasma organisms in T.P. of 2, and a positive CF titre in 1 of these 4 cases.

On the basis of these results an accurate diagnosis of EPP at slaughter can be based on a combination of gross evaluation, histopathology, examination of Giemsa stained touch preparations and CF testing of corresponding serum samples. This can rapidly be confirmed by IMF staining of either touch preparations or sections of pneumonic lung. The best way to achieve a positive diagnosis therefore appears to be to use a battery of tests to cover all stages of infection when antibody or organisms may or may not be present.

CHAPTER 4

PREVALENCE OF LESIONS TYPICAL OF ENZOOTIC PNEUMONIA IN SOUTH AUSTRALIA - AN ABATTOIR SURVEY

4.1 Introduction

Abattoir surveys of enzootic pneumonia of pigs in Australia have shown the prevalence to range from 19-67% (Pullar 1949a ; Edwards et al 1971 ; Norton 1976 ; Mercy 1981). Whittlestone (1973) suggested that there were indications that prevalence of pneumonia had decreased in Australia as Edwards et al (1971) had found only 19% of lungs affected, a prevalence much lower than 20 years earlier. This apparent reduction had occurred despite the trend toward increasing herd size, but had occurred in association with improved husbandry standards and disease awareness. However, a further survey by Norton (1976) found 40.4% of lungs from porkers affected, indicating that either prevelence was higher in northern Queensland or confirming that porkers have a higher prevalence as indicated by Pullar (1949a).

An abattoir survey of pigs in Denmark (Aalund et al 1976) demonstrated that prevalence and extent of lesions of enzootic pneumonia increase as herd size increases. The trend toward larger herds has been apparent in the pig industry for some years; in South Australia the mean herd size has increased from 73 to 128 pigs and the number of herds with over 1 000 animals has doubled over the past 7 years. If the association of prevalence with herd size occurs in Australia then enzootic pneumonia may be an emerging disease in the local pig industry.

4.2 Materials and Methods

4.2 (i) Selection of lungs

Lungs examined were from 3 major abattoirs (1, 2 and 3), each slaughtering between 105 000 - 150 000 pigs annually. Approximately 60 lungs, predominantly from bacon-weight pigs, were sampled at 1 of these abattoirs each fortnight i.e. each abattoir being sampled twice per season. The sample consisted of 30 lungs which were held for later examination (group A), while the remaining 30 were examined on the viscera table (group B). To maximise the number of lines of pigs monitored, every second lung was sampled. Each abattoir was visited at least once on each day pigs were routinely slaughtered. This was done to avoid bias produced by larger units which slaughter on the same day each week. A total of 1 430 lungs were examined; 712 in group A and 718 in group B. Lungs were examined between January 1st to December 22nd, 1980.

(ii) Examination of lungs

Group A lungs were examined either at the abattoir of collection or at a nearby laboratory on the day of collection. Lungs were examined by the method described in section 2.1.

In the examination of group B lungs there was only sufficient time to record the presence and the type of pneumonic lesions. Both dorsal and ventral aspects of all lobes were examined as in group A.

(iii) Classification of lesions

The classification of pneumonic lesions as being typical of enzootic pneumonia was based on the description provided in 2.1(ii).

(iv) Microbiology and Histopathology

Tissues were sampled from 22 affected and 22 normal lungs and processed as described in sections 2.1 (iv)(c). Results are reported and discussed in sections 3.3 and 3.4.

(v) Serology

At each of the 14 abattoir visits 10 ml. of blood was collected from 30 pigs at the point of slaughter. These pigs were then tattooed, allowing collection of the corresponding lungs (group A) for examination. Serum samples were tested for presence of M. hyopneumoniae antibody with the CFT (Appendix II).

4.3 Results

4.3 (i) Prevalence of Pneumonic Lesions

Lesions typical of enzootic pneumonia in groups A and B were observed in 645 of 1 430 (45.1%) lungs examined during the 12 month survey (Table 4.1). Seasonal prevalence varied ($X^2_3 = 7.751$, $p<0.1$) with the highest prevalence being recorded in summer with 51.1% and winter the lowest with 41.7%.

Table 4.1

Seasonal prevalence of Enzootic Pneumonia - like lesions over 12 months (%) (Groups A & B)

EPP Status	Spring	Summer	Autumn	Winter	Total
EPP.- like Lesions	152 * (42.6)	186 * (51.1)	158 * (44.8)	149 * (41.7)	645 (45.1)
Lungs Normal	214	178	195	208	785
Total	356	364	353	357	1 430

* $p<0.1$

The prevalence of lesions at abattoir 1, 2 and 3 varied significantly (X^2_2 = 243.8, p<0.001) with 22.2%, 41.9% and 71.2% of lungs affected respectively (Table 4.2). The seasonal prevalence varied significantly (X^2_3 = 17.47, p<0.001) at abattoir 2 where the highest prevalence was recorded in summer with 55.6% of lungs affected and the lowest in winter with 29.7%.

In group A (Table 4.3) the prevalence of enzootic pneumonia was 43.3% and other types of pneumonia were observed in 1.3% of 712 lungs examined. The seasonal prevalence varied significantly (X^2_3 = 14.53, p<0.01) with the highest prevalence being recorded in summer of 53.4% of lungs affected and spring the lowest with 34.4%. Twenty eight of the lungs in this group were from porkers, of which 42.9% had lesions typical of enzootic pneumonia.

Lesions were observed most frequently in 1 or 2 lobes per lung (Table 4.3) with 12.6% and 10.1% of the total prevalence of 43.3%. This trend was consistent for all seasons.

The distribution of enzootic pneumonia lesions (Table 4.4) in the 7 lung lobes varied significantly (X^2_6 = 474.29, p<.001), with the right cardiac lobe recording the highest prevalence - 83.8% of affected lungs having lesions in this lobe, followed by the left cardiac, right apical, intermediate, left apical, right diaphragmatic and left diaphragmatic. Significantly more lobes were

Table 4.2

Seasonal prevalence of E.P.P., Lesions in Lungs at each abattoir (Group A & B)

Abattoir	Total lungs examined	Prevalence of lesions of enzootic pneumonia %				
		Spring	Summer	Autumn	Winter	Total
1	477	19.7%	24.0%	20.5%	24.0%	22.2%***
2	477	42.9%***	55.6%***	39.0%***	29.7%***	41.9%***
3	476	65.0%	73.9%	74.6%	71.4%	71.2%***

*** $p < 0.001$

Table 4.3

Seasonal prevalence and distribution of Enzootic Pneumonia and other Lung Lesions in 712 examined Lungs (Group A)

Season	Lungs examined	No. of lung lobes affected with Enzootic Pneumonia %							% Prevalence of		
		1	2	3	4	5	6	7	Enzootic Pneumonia	Other Pneumonias	Pleurisy
Spring	180	11.1	6.7	5.0	4.4	2.8	2.3	2.2	34.4**	-	13.9
Summer	176	15.3	10.8	9.7	7.4	2.3	4.0	4.0	53.4**	-	11.4
Autumn	179	11.7	9.5	6.7	3.9	2.8	1.1	3.9	39.7**	-	16.8
Winter	177	12.4	13.6	5.1	4.0	5.6	1.1	4.0	45.8**	-	15.8
Total	712	12.6	10.1	6.6	4.9	3.4	2.1	3.5	43.3	1.3	14.5

** $p<0.01$

Table 4.4

Prevalence of Lesions of Enzootic Pneumonia in the 7 Lung Lobes %
(Group A)

R. Apical	R. Cardiac	R. Diaphragmatic	Intermediate	L. Apical	L. Cardiac	L. Diaphragmatic
44.8***	83.8***	20.8***	28.9***	27.9***	70.1***	15.9***

*** $p<0.001$

Table 4.5

Association of Pleurisy with Enzootic Pneumonia in 712 examined Lungs (%) (Group A)

E.P. Status	Spring	Summer	Autumn	Winter	Total with Pleurisy	Total Examined
E.P. + Pleurisy	11	17	23	17	*** 68	308
Normal Lung + Pleurisy	14	3	7	11	35	404
Total	25 (13.9)	20 (11.4)	30 (16.8)	28 (15.8)	103 (14.5)	712

*** $p<0.001$

affected on the right side of the lung (X^2_1 = 26.20, p<0.001). The intermediate lobe was the only lobe affected in 4 of 308 (1.3%) lungs with lesions of enzootic pneumonia.

The association between pleurisy and cases of pneumonia was highly significant (X^2_1 = 25.31, p<0.001) with pleurisy present in 68 of 308 lungs with pneumonia and in 35 of 404 normal lungs (Table 4.5). The prevalence of pleurisy for group A lungs was 14.5%. (Table 4.3) which did not vary significantly between seasons. The prevalence of pleurisy varied significantly between abattoir (X^2_1 = 22.42, p<0.001) with a prevalence at abattoir 1 of 6.3%, at 2 of 16.8%, and 3 of 20.2%.

(ii) Laboratory diagnosis

Serology, microbiology and histopathology results of the field specimens are presented in Tables 3.5 and 3.6.

4.4 Discussion

The prevalence of enzootic pneumonia at 3 major abattoirs in South Australia of 45.1% was higher than in recent surveys (Edwards et al 1971 ; Mercy 1981). The estimate is considered to be representative of the State's pig herd as 85% of the annual number of pig slaughterings occur at these abattoirs. After allowing for the number of pigs slaughtered annually at each abattoir, an estimated 47% are affected.

The survey showed that where only single abattoirs are sampled biased estimates of prevalence may be produced if results are interpreted as statewide or national estimates. In this study, prevalence of lesions of EPP varied significantly ($p<0.001$) among 3 abattoirs sampled. If only abattoir 1 had been sampled, a similar low estimate (22.2%) to that of Edwards et al (1971) would have been recorded, significantly underestimating the prevalence of the disease in the local industry. By citing results of single abattoir surveys, Whittlestone (1973) suggested that the prevalence of enzootic pneumonia in Australia had declined over the preceeding 21 years.

These South Australian results indicate that the disease is widespread and may not have declined due to improved husbandry standards and greater disease awareness (Whittlestone 1973) developed over recent years.

The highest seasonal prevalence of enzootic pneumonia was recorded in summer, when 51.1% of lungs were affected. This is consistent with previous reports of Pullar (1948) and Edwards et al (1971) who concluded that due to "Australian environmental influence, the disease spreads more rapidly amongst suckers and weaners in the autumn and winter months" causing a peak in prevalence in summer when these pigs are sold. During these winter months sheds are often closed in an effort to maintain temperatures at the expense of ventilation, increasing bacterial challenge and levels of noxious gases which combine to increase the incidence of infection.

The seasonal prevalence of enzootic pneumonia varied significantly ($p<0.001$) at abattoir 2, indicating that seasonal factors predisposed these pigs to enzootic pneumonia. At this abattoir the highest prevalence was again recorded in summer with 55.6% of lungs affected and the lowest in winter of 29.7%. The reason for this trend may be that a large number of pigs slaughtered at this particular abattoir are purchased at auction, these sales being mainly supplied with pigs from semi-intensive and extensive herds where pigs would be more exposed to the direct effects of seasonal conditions. Contrasting with this is the observation that there was no seasonal effect at abattoir 3. The majority of pigs supplied to this abattoir were from 4 large fully intensive units where major efforts are taken to buffer the effects of seasonal changes, and the high level of endemic infection is self-perpetuating.

The seasonal prevalence among group A lungs was significant ($p<0.01$) with the highest prevalence being recorded in summer with 53.4% of lungs affected. The reduced significance of seasonal prevalence variation, from group A ($p<0.01$) to group A plus B ($p<0.1$) was due to an increased prevalence of lesions occurring in winter at only abattoir 2 in group B lungs. No difference in sampling or examination method occurred at that time.

The prevalence of pleurisy in 712 group A lungs was 14.5%, also higher than that reported by Edwards et al (1971) of 3.8%. This would be expected because of the highly significant ($p<0.001$) association found between the presence of pleurisy together with lesions of enzootic pneumonia (Table 4.5). Predictably the prevalence of pleurisy followed the pattern set by prevalence of enzootic pneumonia at abattoir 1, 2 and 3, the abattoir prevalence of pleurisy being 6.3%, 16.8% and 20.2% respectively*. The combination of pleurisy with lesions of enzootic pneumonia has been shown to cause a reduction in growth rate of 29% in a commerical herd in Western Australia (Mercy 1981). These results indicate that with a prevalence of pleurisy of 14.5% in South Australia, extensive losses may be occurring. Given the strong correlation between pleurisy and pneumonia found in this study ($p<0.001$), efforts to control pneumonia might reduce the prevalence of pleurisy.

The distribution of lesions found between lung lobes (Table 4.3, 4.4) supports previous observations (Pullar 1949b ; Edwards et al 1971) which have attributed this phenomenon to the anatomical structure of the major airways. Significantly more lesions occurred in the right lung ($p<0.001$) and the prevalence of lesions in each lobe varied substantially ($p<0.001$) with the right cardiac being the lobe most commonly affected.

* but it is not yet known if this pleurisy is a direct manifestation of M hyopneumoniae infection in the field, or whether other agents are involved.

The classification of lung lesions as enzootic pneumonia based on gross appearance at slaughter was supported by laboratory testing. Results and discussion are presented in Chapter 3.

The high prevalence of lesions of EPP and pleurisy at slaughter indicate widespread infection throughout the S.A. pig industry. Considerable bias could have been introduced if only 1 abattoir had been surveyed and the prevalence accepted as the prevalence of the state or country. The high prevalence of pleurisy found in association with lesions of enzootic pneumonia indicates that losses may be occurring (see p. 205) which will necessitate the need for better disease control.

CHAPTER 5

EPIDEMIOLOGY OF ENZOOTIC PNEUMONIA IN SOUTH AUSTRALIAN PIGGERIES

5.1 Introduction

The aetiological or predisposing role played by environmental and management factors in complex respiratory disease syndromes of pigs are well documented (Gordon, 1963 ; Backstrom 1973 ; Lindqvist 1974 ; Aalund *et al* 1976 ; Whittlestone 1976b ; Flesja 1978 ; Backstrom and Bremer 1978 ; Whittlestone 1979 ; Muirhead 1979). Enzootic pneumonia is a common disease where pigs are housed together in large numbers within a confined airspace, with the severity of the condition being highly dependent on environmental conditions. Its worldwide importance (Ross 1982) lies not only in the direct losses in performance but also in reducing the ability of pigs to utilise their full genetic potential. For these reasons a comprehensive understanding of the epidemiology of the condition is essential to implement effective control measures.

Many studies have related the effect of environment and management of pig production to performance: temperature and humidity of housing (Morrison and Mount 1971 ; Tonks *et al* 1972), ammonia concentration (Stombaugh *et al* 1969 ; Kalich and Schuh 1979), number and density of pigs per pen (Standel and Lynch 1963 ; Jensen 1964 ; Gehlbach *et al* 1966 ; Diggs and Baker 1967 ; Bryant and Ewbank 1972, 1974). Generally the health status of the animals in relation to these factors was not widely considered until more recently, with the exception of Gordon (1963). In this latter study the prevalence and

extent of lesions of enzootic pneumonia were associated with pig house temperature and relative humidity in 2 herds. More recent studies have utilised abattoir post mortem observations from many herds to investigate associations between a variety of factors and the herd prevalence of EPP (Lindqvist 1974 ; Aalund et al 1976 ; Backstrom and Bremmer 1976). Apart from factors already mentioned others of importance are ammonia levels (Kovacs et al 1967 ; Mickwitz et al 1975 ; Curtis 1980 - cited by Backstrom and Curtis 1982) shed numbers and stocking density (Lindqvist 1974 ; Backstrom and Bremer 1978 ; Muirhead 1979) herd size (Aalund et al 1976), continuity of stocking (Lindqvist 1974 ; Muirhead 1979) and purchase of stock for finishing (Fogedby 1967 - cited by Lindqvist 1974 ; Aalund et al 1976).

This investigation into the epidemiology of enzootic pneumonia in South Australia was motivated by two recent developments. Firstly, as mentioned in 4.1, there appears to be potential for EPP to be an emerging disease in expanding herds in South Australia (see Chapter 4). Secondly, as a large proportion (47%) of pigs had lesions of enzootic pneumonia at slaughter in 1980 it became important to determine reasons for the high prevalence to enable formulation of control procedures. Assertions regarding the importance of the many predisposing factors, determined largely in Europe are commonplace, however no extensive study of the importance of these factors under Australian conditions has been reported.

The purpose of this study was to determine which factors were commonly involved in predisposing pigs to enzootic pneumonia in South Australia.

5.2 Materials and Methods

5.2 (i) Selection of piggeries

Piggeries were selected on the basis of the prevalence of EPP obtained by monitoring lines of lungs at 3 abattoirs, during the month prior to the herd visits. Prevalence of lesions typical of EPP was recorded for each line of pigs slaughtered during several days at each abattoir. Monitoring was concluded when sufficient herds which fulfilled the selection criteria were identified. In the case of the smaller herds which generally marketed insufficient pigs at one time to obtain an accurate prevalence of EPP, at least one subsequent line of pigs was monitored.

Two herd categories were established, based on criteria defined by Muirhead (1979). High-prevalence herds were those with a prevalence of lesions in 70% of pigs or greater, while low-prevalence herds were those with 30% or less affected (Table 5.1). Herds within these pneumonia categories were stratified on the basis of production emphasis, with a division into fully intensive units being herds with >100 sows and sideline units with between 20-70 sows. These methods

Table 5.1

Abattoir Prevalence (%) of Lesions of EPP for Each Herd Visited

(31 herds)

Season	Herd Size (Sows)	High Prevalence (> 70%)		Low Prevalence (< 30%)	
Summer	20-70	79	(17)	8	(10)
		84	(13)	7	(7)
		72	(18)	6	(8)
	> 100	75	(13)	28	(12)
		93	(8)	25	(13)
		72	(18)	13	(9)
		81	(13)	10	(8)
		72	(18)		
Winter	20-70	75	(18)	28	(16)
		71	(13)	4	(8)
		71	(18)	14	(14)
		71	(16)	6	(11)
				22	(14)
	> 100	85	(5)	19	(10)
		95	(7)	22	(12)
		71	(13)	30	(8)
				27	(13)

Confidence interval ± ( )

were adopted to allow for any influence that increasing herd size could exert on environment or management within the herds. By choosing herd groups with a large difference in the prevalence of EPP lesions, greatest potential was created for the detection of difference in each factor between high and low-prevalence herd groups. To assess the importance of factors which are influenced by seasonal conditions, a similar number of piggeries were visited in late summer and mid winter of 1981. Herds visited in late summer were excluded from the winter sample due to implementation of management changes aimed at controlling EPP.

Production in all herds was of a continuous nature, with all growing pigs in the large herds being intensively housed, while in all smaller piggeries the growing phase included an intensive housing period of at least 8 weeks.

(ii) Evaluation of piggeries

A questionnaire was designed to be answered by the piggery owner/manager in conjunction with one of the researchers when in the piggery. Criteria used for assessing each factor were standardized between the two researchers on several herds prior to the study. Factors assessed appear in Table 5.2. About 60 variables in housing environment and management were recorded, however after preliminary study only a limited number of these were selected for detailed analysis.

Table 5.2

Information Collected by the On-farm Questionnaire and Measurements

Management Data	• Age of herd (since establishment)	
	• Annual sow culling rate	
	• Purchase of pigs for finishing	
	• Use of anti-dust agents in feed	
	• Presence of antibiotics in feed	
	• Average market age	
	• Average market weight	
Age Group Data - Weaners (W), Growers (G), Finishers (F)		
	• Total pigs in section	- W, G, F
	• Group size	- W, G, F
	• Pen area stocking density	- W, G, F
	• Airspace stocking density	- W, G, F
	• Pen design	- W
	• Potential for drafts	- W, Farrowing Unit
	• Type of ventilation	- W, G, F
Measurements - for 1 week	• Maximum and minimum temperatures	- W, G
	• Relative humidity (at 3.00 p.m.)	- W, G
	• Ammonia - 3 sites/herd	- F

The number of sows culled during the previous twelve months was recorded and annual sow culling rate calculated.

The criteria for pigs purchased for finishing were all pigs introduced to the property from markets or from other breeders at an age from "slips" (weaners from 5-10 weeks old) to unfinished bacon for the sole purpose of finishing for slaughter.

Herds using measures to reduce the amount of dust generated from the feed included those which added tallow or oils to the feed ration and those which used pelleted feed for growing pigs.

In-feed antibiotics which were considered to be of importance were those commonly used to control EPP and its secondary bacterial invaders and included the tetracyclines. tylosin, lincomycin and sulphadimidine.

The average market age and weight of pigs were obtained from herd records where possible. In the herds in which individual pigs were not identified the age at marketing was estimated from basic herd records. To partially overcome this inaccuracy, the data was stratified (Fig. 5.1).

The total number of pigs in a shed section (e.g. undivided shed or air space completly separated from other sections) for any age group included all pigs of other age groups if necessary. Group size was taken as the number of pigs per pen.

Pen area stocking density was calculated by dividing the total weight of pigs of a particular age group (weaners, growers or finishers) by the total area which they occupied at that time. Air space stocking density was calculated similarly, but used the total mass of pigs in the section divided by its volume (i.e. if weaners, growers and finishers were present in the same shed, then the air space stocking density was the same for each age group).

Weaner pen design was assessed under four categories, being pens on the floor with or without slats/mesh, pens with slats/mesh above growers/finishers and multi-tiered weaner crates.

Potential for draughts was assessed by presence of obvious faults e.g. holes in walls, incomplete walls, poorly fitted shutters and absence of flaps at the end of dung channels.

Ventilation methods were classified as either fan forced or natural with side shutters.

The siting of all maximum-minimum thermometers and hygrometers in piggeries prior to the designated recording periods ensured all piggeries recorded conditions in weaner and grower areas during the same two weeks in summer and winter. The equipment was suspended 0.5 metres above weaner pens and 1 metre above grower pens in all piggeries. All equipment was tested and calibrated prior to each recording period and again upon siting in each new piggery.

Ammonia levels were recorded as close as practical to normal morning shutter opening time, allowing estimation of overnight accumulation within the finisher unit. Three readings were obtained using a multigas detector*

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*Drager (Aust.) Pty. Ltd., Adelaide, South Australia

2cm above the floor surface at the junction of the sleeping and dunging area. The temperature and relative humidity at each recording site was also recorded.

(iii) Statistical analysis of data

The categorical nature of the management data and some of the environmental data lend themselves to contingency table analysis (Bishop et al 1975 ; Plackett 1981). For each management or environment factor the hypothesis tested is of independence between the factor and the prevalence of enzootic pneumonia.

The Mantel-Haenszel test simultaneously tests for independence between at t-level treatment factor (e.g. pen space stocking density) and or r-level response factor (e.g. severe or subclinical), in each of S- strata (e.g. weaners, growers, finishers). The first papers on this subject were by Cochran (1954) and Mantel and Haenszel (1959). A recent review is Landis et al (1978). The independence of the factors in Tables 5.4a, b and 5.5 were tested with the Mantel-Haenszel test.

Temperatures, relative humidity and ammonia levels were analysed with analysis of variance or linear regression.

5.3 Results

Comparison of management data between high and low-prevalence herd groups is presented in Table 5.3. Significantly more high-prevalence small herds had higher sow culling rates ($p<0.1$) with 7 of 8 herds (85.5%) culling at a rate of >15% while 4 of 8 low-prevalence herds culled at <15%. Similarly, more high-prevalence small herds purchased pigs for finishing ($p<0.07$). Pigs were purchased equally from markets or direct from breeders. Other factors were not significantly different between herd groups.

Table 5.3

Comparison of Management Data between High- and Low-Prevalence Herd Groups

Factors	Herd Size	Significance of Difference Between Herd Groups
Age of herd	All	NS
Annual sow culling rate	20-70	*
	> 100	NS
Purchase of pigs for finishing	20-70	**
	> 100	NS
Use of anti-dust agents in feed	20-70	NS
	> 100	NS
Presence of anti-biotics in feed	20-70	NS
	> 100	NS

NS Not Significant * $P < 0.1$ ** $P < 0.07$

Analysis of the average market age and bodyweight of pigs from all herds (Fig. 5.1) revealed that more high-prevalence herds had pigs which were marketed at an older age ($p<0.06$) and lower liveweight ($p<0.06$) than pigs from low-prevalence herds.

Analysis of housing data (Table 5.4a) for weaners, growers and finishers for the 2 herd groups revealed that significantly more high-prevalence small herds had more pigs per section ($p<0.001$) and more pigs per group ($p<0.01$) than low-prevalence herds. Significantly more high-prevalence large herds stocked pens ($p<0.05$) and airspace more densely ($p<0.05$) than low-prevalence herds (Table 5.4b).

Pen design for weaners did not vary significantly between high and low prevalence herd groups. Weaners were most frequently housed in pens on the floor, with 14 using partial or full slats or mesh and 7 using totally solid floors. In 7 herds weaners were housed in partially meshed pens over growers and in 3 herds multi-tiered weaner crates were used.

Significantly more high-prevalence small herds had potentially draughty weaner and farrowing units ($p<0.01$) (Table 5.5).

Mechanical ventilation was used in only 1 weaner unit, with all other age groups in all herds being naturally ventilated.

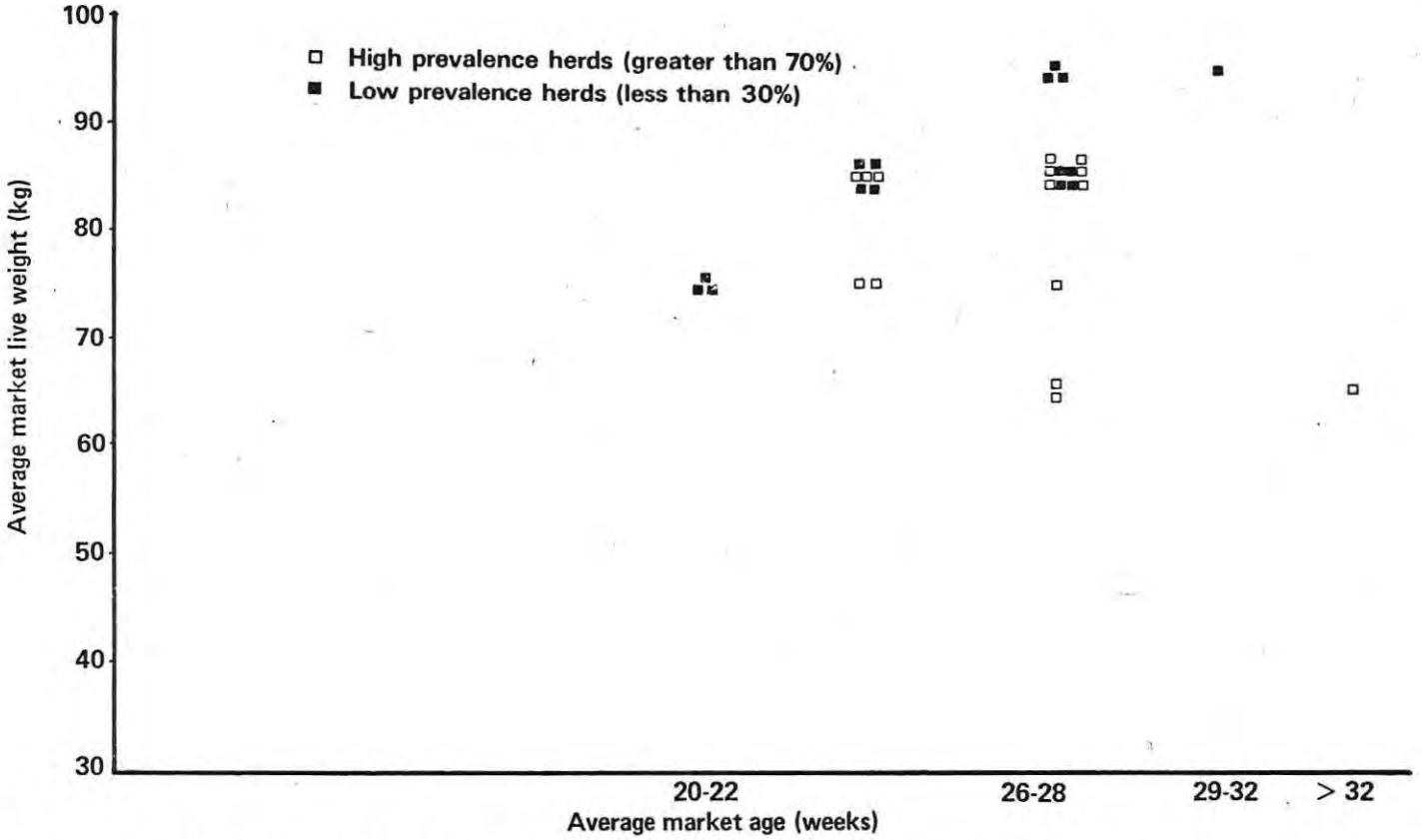


FIGURE 5.1: AVERAGE MARKET AGE AND LIVEWEIGHT OF 31 PIG HERDS IN SOUTH AUSTRALIA.

Table 5.4(a)

Statistical Difference in Housing Data between the Herd Groups

Total Pigs per Section

Herd Size	Herd Pneumonia Status	Weaner Unit			Grower Unit				Finisher Unit			Significance of Difference
		<100	100-199	≥200	<100	100-199	200-399	≥400	<400	400-799	≥800	
20-70	High-Prevalence	1	2	4	0	2	3	2	0	5	2	***
	Low-Prevalence	3	4	1	3	4	1	0	6	1	0	
		<300	300-499	≥500	<400	400-799	≥800		<400	400-799	≥800	
> 100	High-Prevalence	2	3	3	1	4	3		2	3	3	NS
	Low-Prevalence	2	4	2	3	3	2		3	3	2	

Average Group Size

		Weaner Unit			Grower Unit			Finisher Unit			
		<15	15-24	≥25	<11	11-16	≥17	<10	10-11	≥12	
20-70	High-Prevalence	4	0	3	4	0	3	3	1	3	**
	Low-Prevalence	6	2	0	4	4	0	3	4	1	
		<15	15-29	≥30	<12	12-17	≥18	<11	11-12	≥13	
> 100	High-Prevalence	4	2	2	3	2	3	3	3	2	NS
	Low-Prevalence	2	4	3	3	3	2	3	4	1	

** $P < 0.01$ *** $P < 0.001$

Table 5.4(b)

Statistical Difference in Housing Data between the Herd Groups

Pen Space Stocking Density (kg/m^2)

Herd Size	Herd Pneumonia Status	Weaner Unit			Grower Unit			Finisher Unit			Significance of Difference
		<25	25-39	>40	<40	40-82	>83	<67	67-124	>125	
20-70	High-Prevalence	3	2	2	3	1	3	2	3	2	NS
	Low-Prevalence	2	2	4	3	4	1	4	2	2	
		<40	40-49	>50	<50	50-82	>83	<100	100-124	>125	
> 100	High-Prevalence	0	2	5	0	4	4	0	4	4	*
	Low-Prevalence	2	1	4	2	1	5	3	3	2	

Airspace Stocking Density (kg/m^3)

		Weaner Unit		Grower Unit		Finisher Unit		
		<10	>10	<20	>20	<20	>20	
20-70	High-Prevalence	2	3	4	1	4	1	NS
	Low-Prevalence	4	2	2	3	1	4	
		<10	10-19	>20	<20	20-29	>30	<20	20-29	>30	
> 100	High-Prevalence	0	4	4	2	4	2	1	7	0	*
	Low-Prevalence	2	4	1	4	2	1	3	2	2	

* $P < 0.05$

Table 5.5

Potential for Draughts when Shutters Closed

Herd Size	Penumonia Status	Farrowing House		Weaner Unit		Significance of Difference
		Present	Absent	Present	Absent	
20-70	High-Prevalence	6	1	6	1	**
	Low-Prevalence	3	5	2	6	
> 100	High-Prevalence	6	1	0	8	NS
	Low-Prevalence	3	5	2	6	

** $p < 0.01$

Average minimum daily temperatures and diurnal temperature fluctuation for each herd group in summer and winter did not vary significantly between herd groups (Table 5.6). The average minimum temperature in all small and large herds in winter was 10.7°C and 14.4°C respectively. The diurnal fluctuation was significantly greater in the small low-prevalence herds in summer ($p<0.1$) and in the large high-prevalence herds in winter ($p<0.1$) for growers only. The average diurnal fluctuation for weaners in winter in small herds was 7.8°C and in large herds was 7.9°C.

Minimum temperatures in weaner houses in winter were highly dependent on the potential for draughts in both small ($p<0.01$) and large herds ($p<0.05$) (Table 5.7).

The average afternoon relative humidity (3.00p.m.) for each of the herd groups in summer and winter are presented in Table 5.8. Significantly more high-prevalence small herds had higher relative humidities in weaner sections in summer ($p<0.1$) while significantly more low-prevalence large herds had higher relative humidities in weaner sections in winter ($p<0.05$).

The average ammonia concentration was higher in significantly more high-prevalence large herds in summer ($p<0.1$). The average ammonia concentration in summer of all the large high-prevalence herds was 11.3ppm

Table 5.6

Temperatures

(Average of 7 Consecutive Days)

Age Group	Season	20-70 Sows		> 100 Sows	
		High-Prevalence	Low-Prevalence	High-Prevalence	Low-Prevalence
Average Minimum Daily Temperature					
Weaners	Summer	18.4	16.2	17.9	18.4
	Winter	9.1 (10.7)	11.8	14.0 (14.4)	14.8
Growers	Summer	19.4	16.7	18.6	18.4
	Winter	8.7	10.5	12.5	14.5
Average Diurnal Temperature Range					
Weaners	Summer	8.9	13.9	10.7	10.1
	Winter	7.9	7.1	8.7	8.1
Growers	Summer	8.6	12.5	9.8	9.3
	Winter	7.6	7.7	10.3	6.9

( ) Average for all small and large herds respectively

Table 5.7

Association between Minimum Weaner House Temperatures and Potential for Draughts

Season	Weaner House Minimum Temperatures (Average of 7 consecutive days)	
	Herd Size	
	20-70 Sows	> 100 Sows
Summer	NS	NS
Winter	**	*

NS Not Significant * P < 0.05 ** P < 0.01

Raw data presented in tables 6 and 7

Table 5.8

Average Relative Humidity (3.00 p.m.)

Age Group	Season	20-70 Sow Herds		> 100 Sow Herds	
		High-Prevalence	Low-Prevalence	High-Prevalence	Low-Prevalence
Weaners	Summer	53	44	53	52
	Winter	78	73	63	70
Growers	Summer	53	43	50	54
	Winter	73	67	72	69

Table 5.9

Average Overnight Ammonia Accumulation (ppm)
on Each Herd Group in Each Season

Herd Size	Season	High-Prevalence	Low-Prevalence
20-70	Summer	7.7 (3-20)	9.8 (1-22)
	Winter	4.0 (1-10)	7.1 (3-21)
> 100	Summer	11.3 (2-35)	5.9 (3-22)
	Winter	5.8 (1-30)	5.4 (1-18)

( ) range in ppm

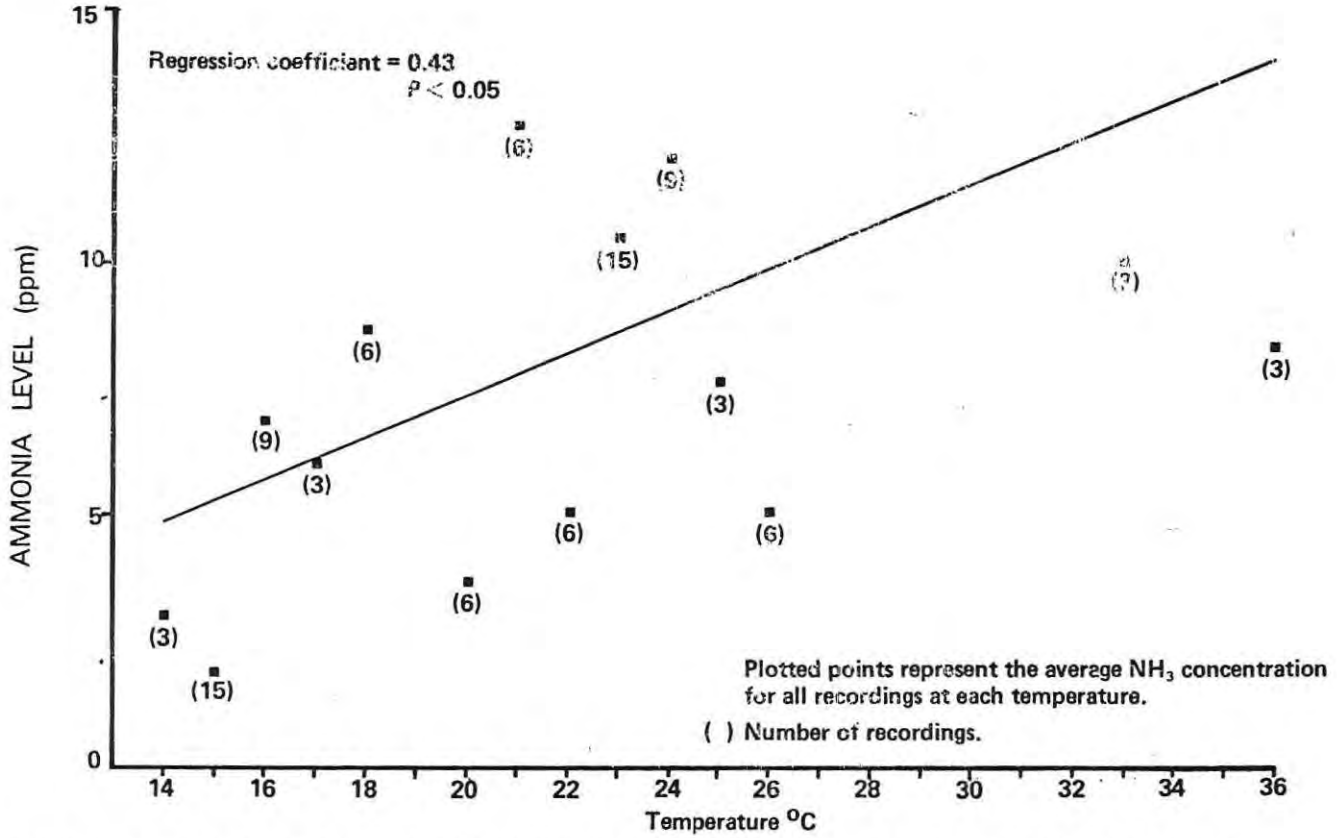


FIGURE 5.2: ASSOCIATION BETWEEN ATMOSPHERIC AMMONIA LEVELS AND SHED TEMPERATURES.

compared with 5.9ppm in large low-prevalence herds (Table 5.9). Ammonia concentration of all individual recordings (93) was found to be dependent on the temperature at the recording site ($r = 0.43$, $p<0.05$) (Fig. 5.2).

5.4 Discussion

The study demonstrated substantial differences in environmental and management factors between high and low-prevalence herd groups, within each herd size category. In the small herds a high prevalence of enzootic pneumonia at slaughter was associated with a high sow culling rate, purchase of pigs for finishing, large numbers of pigs per shed section, larger group sizes and draughty farrowing and weaner accommodation. In large herds, factors associated with a high prevalence of EPP were higher stocking densities of pen space and air space and high atmospheric ammonia levels in summer. These factors commonly associated with a high-prevalence of EPP in South Australia are the same as those determined to be of importance in similar studies in the northern hemisphere (Lindqvist 1974 ; Aalund et al 1976 ; Backstrom and Bremer 1978).

The finding that different factors were important in the difference herd size groups clearly justifies the stratification of herds according to size in the analysis. This finding may be explained by

changes in emphasis in environmental and management conditions which occur as herd size increases. For example, larger herds were found to depend entirely upon the production of their own piglets, while the practice of purchasing pigs for finishing was a common practice among the smaller piggeries. Similarly with the increased financial pressure of a high total cost of construction of larger piggeries, shed population and stocking density is increased (Table 5.4) in an effort to reduce the cost per unit produced.

It is of particular interest to note that no factor in common was determined to be of importance for both small and large herd groups. However, while shed section and group numbers were significant factors for the smaller herds, this trend was also supported in the larger herds in most instances, where they may have assumed significance if more herds had been studied. The emergence of pen and airspace stocking densities as being significant factors in the low number of large herds studied, may indicate that stocking rates exert a greater effect than pig populations in large herds. The possibility of factor dominance is supported by results of Lindqvist (1974) which indicated that volume may be more dominant than pen lying space.

The study indicates that production in high-prevalence herds was significantly decreased due to depressed growth rate (Fig. 5.1). More high-prevalence

herds had pigs which were marketed at an older age and at a lower liveweight when compared with low-prevalence herds. Estimates of quantitative losses are unavailable from this study as the effect of EPP varied between herds, probably because of the combined effects of other diseases, and environment and management differences between herds. However despite these other uncontrolled variables influencing production, a significant depression was recorded in high-prevalence herds. Previous studies (Lindqvist 1974 ; Muirhead 1979) equate performance achieved in environments which control EPP with that experienced in EPP-free herds. Therefore from this result it is estimated that due to decreased performance in severely affected herds and widespread infection throughout industry indicated by the abattoir survey (Chapter 4), that losses due to EPP are high in the South Australian pig industry.

The aetiological significance of each factor is considered in the following discussion.

Annual sow culling rate. The importance of this factor is supported by observations of Goodwin (1965) who demonstrated a reduced prevalence of EPP lesions with age. The consequence of this is that infection is most frequently transmitted from gilts and second litter sows to piglets (Muirhead 1979). Therefore by decreasing the average age of sows (a common practice to speed genetic improvement), breeding herd disease is increased along with increased morbidity among suckling piglets.

The reasons for this factor to be of apparent influence in the small herds but not large herds may be due to the spasmodic nature of culling in smaller herds leading to differences, as compared to the routine culling programme operating on most large herds.

Pen space and air space stocking density. In substantially more large high-prevalence herds stocking rates were higher in all age groups of growing pigs. Lindqvist (1974) and Backstrom and Bremer (1978) also found a similar relationship but could not determine if pen space or air volume was the major factor. In this study both factors, when treated individually, were associated with a high prevalence of EPP. Reduced pen space per pig would increase the potential for contact spread and reduced air space would cause an increase in atmospheric bacterial contamination. This in turn would increase the challenge dose to susceptible pigs continually added to the population. It was observed that in all high-prevalence large herds, finisher pen stocking rate was >100 kg/m^2, whereas 37% of the low-prevalence herds stock at <100 kg/m^2. Similarly an airspace stocking density of 20 kg/m^3 for finisher in large high-prevalence herds appears to be a significant or threshold level, beyond which pigs are more likely to develop severe pneumonia. While these values were chosen arbitarily for the purpose of statistical analysis they serve as a guide to establishing maximum stocking densities under current housing standards.

For the control of EPP a reduction in stocking density is recommended, however if done in isolation from adjustment of other predisposing factors possibly operating, and antibiotic therapy, the effect may be swamped.

Potential for draughts. Potentially draughty farrowing and weaner units were more common among the high-prevalence small herds ($p<0.01$) (Table 5.5). When analysed against the minimum weaner temperatures (Table 5.6) it was found that these were dependent upon the potential for draughts in both small ($p<0.01$) and large herds ($p<0.05$) (Table 5.7). The combination of low temperatures with draughty conditions would lead to a "chill" situation, causing reduced resistance to infection through cilial damage. This would lead to widespread infection among weaners in winter, which would cause an increased prevalence of lesions at abattoirs in summer when these pigs are marketed (Pullar 1948 ; Edwards et al 1971).

Atmospheric ammonia. More high-prevalence large herds had higher levels of ammonia in summer than low-prevalence herds ($p<0.1$). The average level for large high-prevalence herds in summer was 11.3ppm compared with 5.9ppm in low-prevalence herds. These results are supported by those of Mickwitz et al (1975), Kovacs et al (1967) and Curtis (1980 - cited by Backstrom and Curtis 1982) who reported higher levels of ammonia in association with pigs with clinical pneumonia. From these former studies it was concluded that ammonia levels should be kept below 10ppm.

Several controlled experimental studies have demonstrated the role of atmospheric ammonia in respiratory infections of poultry (Sato et al 1973 ; Quarles and Kling 1974), rats (Brodersen et al 1976) and pigs(Drummond et al 1978 ; Drummond et al 1981). The effect observed has been reduced clearance of inhaled bacteria and greater multiplication of respiratory pathogens. The prevalence of pneumonia in rats was directly correlated with an increase in environmental ammonia concentrations when exposed to levels commonly encountered in piggeries (20ppm).

As higher levels of ammonia were more frequently recorded in summer it was postulated that levels may be dependent upon temperature within the pig unit. Further analysis (Fig. 5.2) revealed that this was the case ($r = 0.43$, $p<0.05$). As house temperatures in winter are often maintained at the expense of ventilation (Muirhead 1979), this latter association between factors assumes greater significance. It therefore appears that ventilation in summer in many herds is currently inadequate A high potential for problems also exists in winter when minimum ventilation is not provided as units are closed to conserve temperatures.

Minimum daily and diurnal temperatures. The average minimum daily temperatures (Table 5.6) for weaners in winter in both small (10.7°C) and large herds (14.4°C) were well below current recommended levels of 25°C for 3 week old weaners, down to 21°C for 6 week old weaners

(Wright 1981). While they were not significantly different between herd groups, it is likely that low minimum temperatures are important determinants of EPP when combined with draughty conditions.

The diurnal temperature range (Table 5.6) was also larger than the recommended limits of 5°C (Baxter 1969) which predisposes to enteric as well as pneumonic problems (Cargill 1981). The diurnal temperature fluctuation was significantly higher in the large high-prevalence herds in winter for growers. This result for growers and not weaners may reflect a decreased emphasis placed on temperature control for the older pigs.

Relative humidity RH. Levels of relative humidity recorded in this study at 3 p.m., when temperatures were near the daily maxima were well below those known to control EPP. Gordon (1963) reported that R.H. >95% in conjunction with temperatures >27°C were found to be associated with lower levels of EPP. Tonks et al (1972) and Whittlestone (1976b) record that in these conditions atmospheric droplets in the range of 1-3µm sediment at a faster rate, decreasing atmospheric bacterial contamination.

In a review of the effect of R.H., Whittlestone (1976b) stated that midrange R.H. was more detrimental to the survival of mycoplasma organisms than a R.H. of either 25% of 95%. In this study the average R.H. for

each herd group in each season was within mid-range values. Obviously, organisms were excreted at a continually high rate in the high-prevalence herds to maintain the high incidence of infection.

The study highlighted many environmental and management factors commonly involved in predisposing pigs to EPP in South Australia. As these factors are the same as those identified in similar studies in the northern hemisphere, measures developed there should be evaluated in the control of EPP in South Australia.

CHAPTER 6

INVESTIGATION INTO THE EFFECT OF EPP ON GROWTH RATE AND FEED CONVERSION EFFICIENCY

6.1 Introduction

Reduction of performance of growing pigs by EPP has been investigated in many studies in the northern hemisphere (Betts and Beveridge 1953 ; Betts et al 1955 ; Goodwin 1963 ; Eikmeier and Mayr - cited by Huhn 1970a ; Huhn 1970b ; Braude and Plonka 1975) with results varying from no effect to a reduction in growth rate and feed conversion efficiency of up to 20%. This variability may be explained in part by possible differences in pathogenicity of strains of M. hyopneumoniae used, but is also likely to depend largely on experimental methods and trial conditions used. Early trials of Betts et al (1955) used EPP-free pigs which were inoculated intranasally with pneumonic lung suspension. Pigs inoculated this way were severely affected, however it was thought that the effect of EPP in the field was as great. Further experimental work by Huhn (1970b) used pigs which were naturally challenged in several endemically infected herds and found that depression in performance was related to the extent of gross lesions at slaughter. Extrapolation of results of these trials to the commercial situation is difficult, as in the former case pigs were challenged by an unnatural route by an unknown challenge dose. In the latter case commercial pigs with extensive lesions may be those most recently infected, thereby being those on which the disease has had minimal time to act. Attempts to obtain a more dependable estimate used herds

which were known to have become infected, and compared performance data of the pre and post-infection periods (Goodwin 1963 ; Braude and Plonka 1975). These probably provide a more valid estimate if other variables can be held static.

The effect of EPP on performance under Australian conditions is unknown. Due to the subclinical nature of EPP and the milder environmental conditions in Australia when compared to northern hemisphere countries where the effects of EPP have been documented, it has been argued that the disease in its uncomplicated form is of little significance. As a result, few studies have been performed with the exception of those by Pullar (1948, 1949a,b,c 1958) which however, did not evaluate the effect on performance. The option of performing pre and post-infection field studies is difficult under Australian conditions due to the paucity of units known to be free of EPP which may "breakdown".

Consequently, it appears that the best way of estimating the effect of EPP under Australian conditions is to naturally challenge genetically related pigs free of other disease capable of affecting performance, with a known local strain under environmental and management conditions operating commercially. This investigation studies the effect of EPP on performance in two parts. Experiment 1 assesses the effect of initial "breakdown" of EPP-free pigs and Experiment 2 assesses the on-going losses caused by endemic EPP infection. Both trials use

naturally challenged pigs held under environmental and management conditions operating commercially (Chapter 5), with the latter trial covering the entire growth period, allowing for the expression of compensatory growth.

EXPERIMENT 1

6.2 Materials and Methods

6.2 (i) Pigs

Forty eight large White EPP free pigs were stratified on the basis of weight, age, sex and genetic background into 3 pairs of matched groups of 8 pigs.

(ii) Feed

All pigs were fed pellets ad lib from 10 weeks of age until slaughter. N.P.R.U. grower ration G_{13} (D.E. 14.1 mega J/kg, C.P. 17.5%, Lysine 0.73%) was fed until the average liveweight of the control and infected groups respectively reached 50kg, when it was changed to N.P.R.U. finisher ration F_{10} (D.E. 14.2 mega J/kg, C.P. 15.8%, Lysine 0.73%).

(iii) Inocula and their administration

Transmitter pigs (6) were inoculated intranasally with the Beaufort strain of M. hyopneumoniae by methods described in 2.6. These pigs were then placed immediately in the pens containing trial pigs. Placebo controls (6) were inoculated intranasally with MH medium.

(iv) Monitoring of infection

Pigs were observed daily for a minimum of 30 minutes to determine the onset of coughing for each pig.

Animals showing severe clinical signs i.e. a persistently elevated temperature, abdominal breathing ("thumps") and persistent anorexia (greater than 2 days) were treated with Streptopen for a minimum of 3 days.

(v) Experimental conditions

Environment and management conditions in the 2 identical grower units 1 and 2 were matched. Due to the close proximity (200 metres) of the EPP free breeder unit, it was necessary to seal and mechanically ventilate the units, with air from unit 1 being exhausted through a HEPA filter. Fan speeds were adjusted twice daily to balance conditions between the units and provide daily maximum and minimum temperatures similar to those recorded in commercial piggeries Table 5.6.

Stocking density was kept within values commonly found commercially. A pen size adjustment was made to simulate movement from grower to finisher accommodation.

Pigs were housed in pens with totally solid floors which were cleaned daily, keeping sleeping areas dry.

Control and infected groups were slaughtered separately when the average liveweight for each group reached 85kgs respectively.

(vi) Samples from experimental animals

All pigs were blood sampled pre-inoculation at 10 weeks of age, and again at slaughter. Throughout the trial blood samples were taken weekly from 2 animals in each control and infected group to monitor development of infection and check for continuing freedom from infection in the control pigs (Unit 2).

Pigs used as transmitters or placebos in units 1 and 2 were slaughtered on site to avoid bacterial contamination at slaughter.

All trial pigs were sent for slaughter at a local abattoir. Samples were collected and processed as described in Chapter 3.2.

(vii) Statistical analysis of pig performance data

Results were analysed using analysis of variance carried out on growth rates and feed conversion efficiency.

(viii) Experimental design

One of each of the 3 paired groups was allotted to each of units 1 and 2 giving each a total population of 24 trial pigs.

The groups housed in unit 1 were naturally challenged with M. hyopneumoniae in mid June 1982, by the addition of 2 inoculated transmitter pigs to each group immediately after inoculation. After 4 weeks the transmitter pigs were removed and held in adjacent pens separated by wire mesh panels to allow further contact and transmission while prohibiting access to feeders. An equal number of placebo inoculated control pigs were managed identically in unit 2.

All trial pigs were weighed weekly from 10 weeks of age until slaughter at 85kgs liveweight. To determine the amount of feed consumed per group each fortnight, any remaining feed was "weighed-back" at the end of each period.

6.3 Results

Coughing was first noted among the inoculated transmitters in unit 1, 6 days post-challenge with the onset averaging 8 days. Coughing was first observed among in-contact pigs after 10 days of exposure to inoculated pigs and averaged 30.8 days. Two naturally challenged pigs were not observed to cough. Figure 6.1 shows the cumulative frequency of when pigs were first observed to cough. Ten of 22 pigs were first observed to cough during the third and fourth week post-exposure to transmitters.

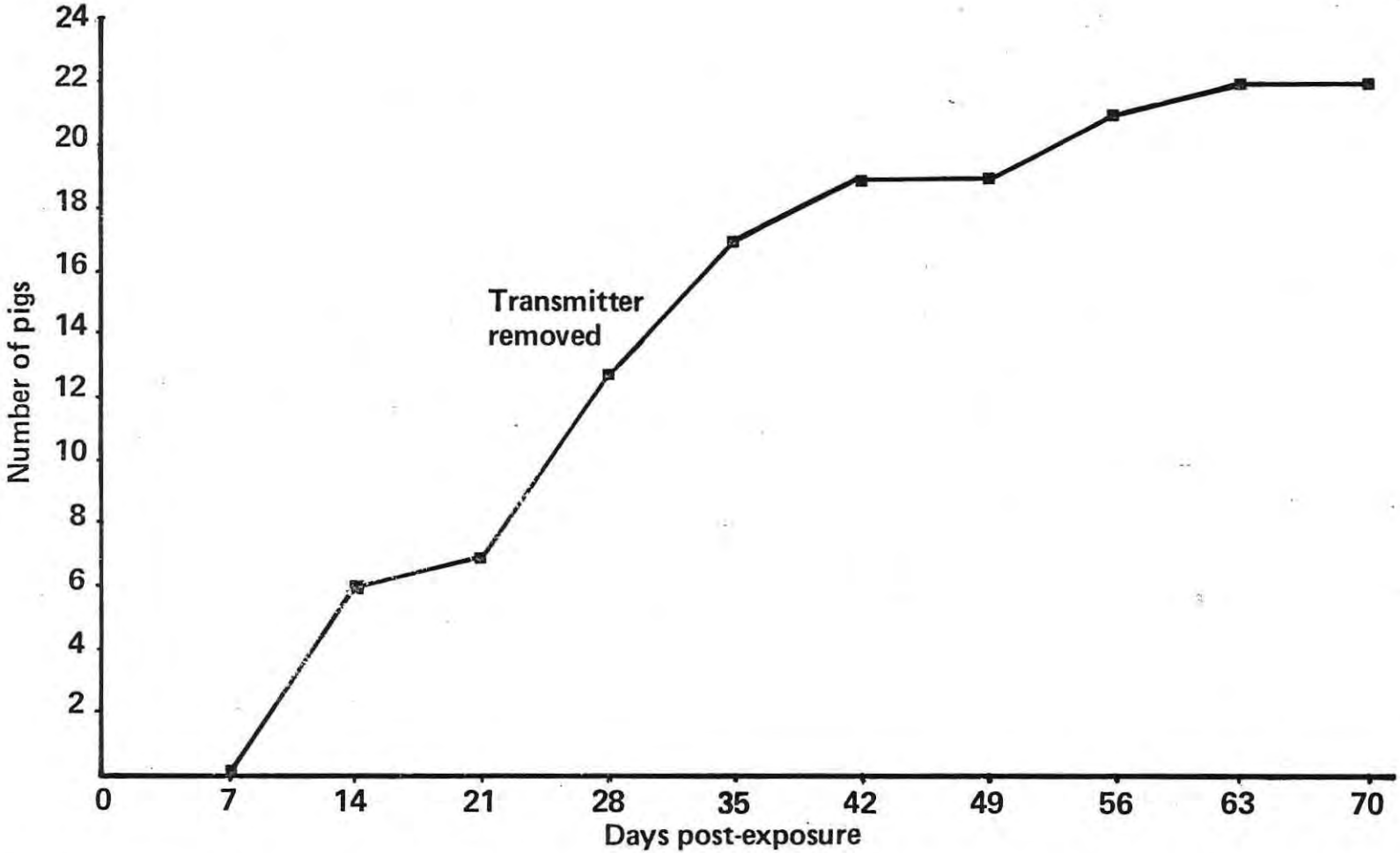


FIGURE 6.1: EXPERIMENT 1. CUMULATIVE FREQUENCY OF WHEN PIGS FIRST OBSERVED COUGHING.

Trial environment and management conditions are shown in Table 6.1.

Gross lesions of EPP were present in 17 of 24 (71%) of naturally challenged pigs at slaughter (Table 3.2). An average of 1.6 lobes per lung contained gross lesions and the left and right cardiac lobes were the lobes most frequently affected. The percentage of total lung area affected with gross lesions was not recorded.

Results of laboratory diagnostic procedures on collected specimens are presented in Tables 3.2 and 3.3.

The growth curves for infected and control groups are shown in Figure 6.2. Table 6.2 shows the mean growth rate and feed conversion efficiency for each group with the standard deviation of the mean for growing and finishing periods as well as the entire trial period. From 25-50kg liveweight performance was unaffected, but from 50-80kg the growth rate of infected pigs was depressed by 12.7% ($p<0.01$) causing a delay in marketing at 85kg of 7 days.

The difference between weekly mean body weight gains of infected and control groups are shown in Figure 6.3. From the fourth week post-exposure mean

TABLE 6.1.

Experiment 1. Environment and management conditions under which pigs were held.

VARIABLES	UNIT 1	UNIT 2
Average minimum temperature (^{o}C)	15 ±1	15 ±1
Average maximum temperature	19 ±1	18 ±2
Diurnal temperature fluctuation	4± 2	3.5 ±3
Average minimum relative humidity(%)	60± 5	59 ±12
Average maximum relative humidity	73 ±4	84 ±6
Maximum grower stocking density-kg/m^2	60-65	60-65
Maximum finisher stocking density	85-90	85-90
Average group size	8	8

± Standard Deviation.

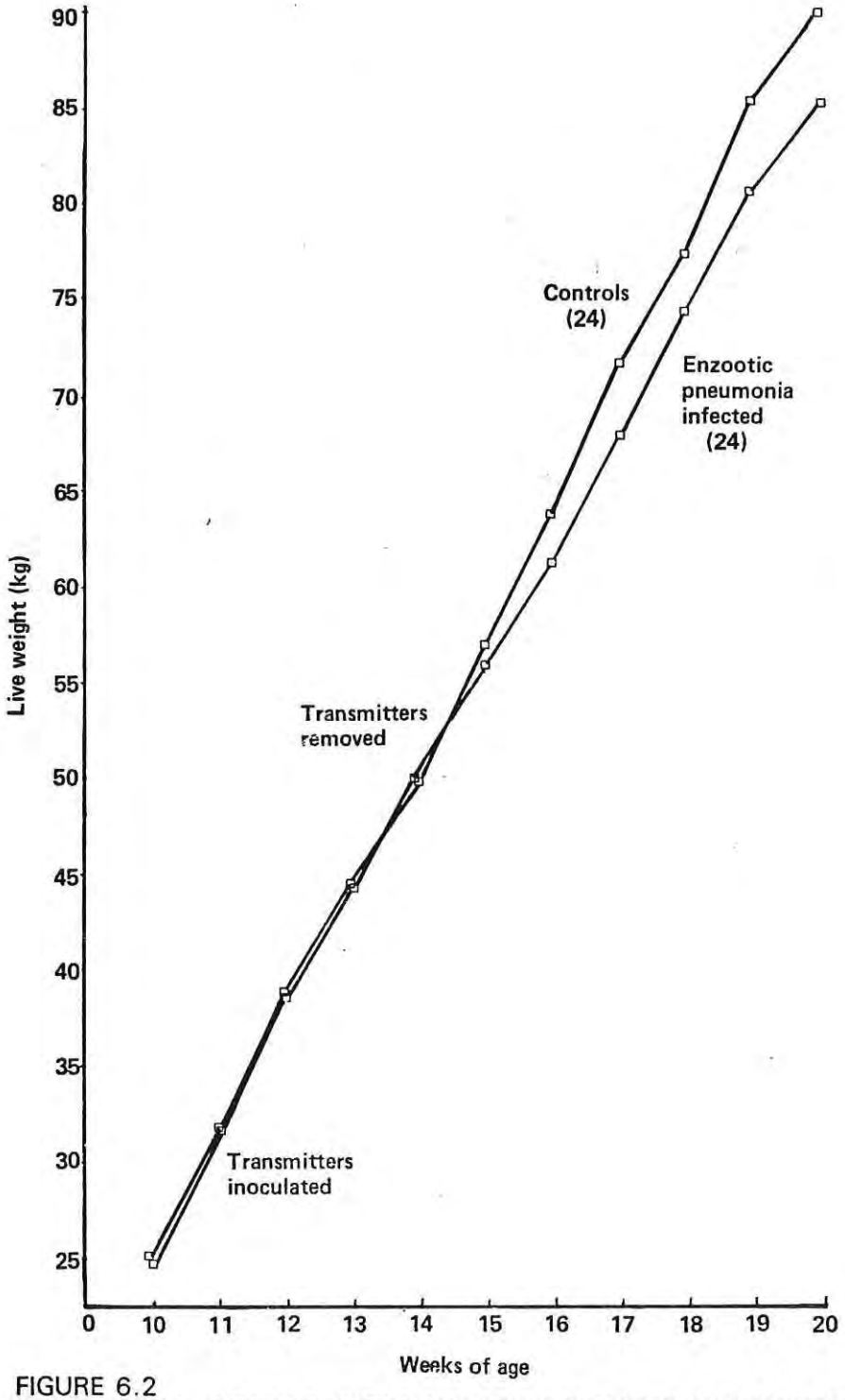


FIGURE 6.2
EXPERIMENT 1. AVERAGE LIVEWEIGHT OF EPP-INFECTED AND CONTROL PIGS DURING THE HERD "BREAKDOWN" PERIOD OF INFECTION.

TABLE 6.2.

Experiment 1 - Effect of EPP on growth rate and feed conversion efficiency.

	INFECTED GROUP	CONTROL GROUP
Number of animals	24	24
First exposure to EPP	10 wks	10 wks
Type of housing	Intensive	Intensive
Growth rate (grams/day)		
25-50kg		
Mean	910	885.4
S.D.	±91	±142
Range	660-1035	536-1089
Decrease in G.R. (%)	-	2.8
50-85kg		
Mean	827.9**	947.9
S.D.	±154	±134
Range	600-1275	763-1237
Decrease in G.R. (%)	12.7	-
25-85kg		
Mean	861.6	901.9
S.D.	±95	±103
Range	750-1161	728-1125
Decrease in G.R. (%)	4.5	-
Feed conversion ratio †		
25-50kg		
Mean	2.65	2.50
S.D.	NA	NA
Range	NA	NA
Decreased efficiency (%)	6.1	-
50-85kg		
Mean	3.12	3.18
S.D.	NA	NA
Range	NA	NA
Decreased efficiency (%)	-	1.8
25-85kg		
Mean	2.93	3.00
S.D.	NA	NA
Range	NA	NA
Decreased efficiency (%)	-	2.3

*p<0.05 **p<0.01 ***p<0.001 †Daily feed intake/daily liveweight gain
NA: Not available - individual pig FCE not recorded

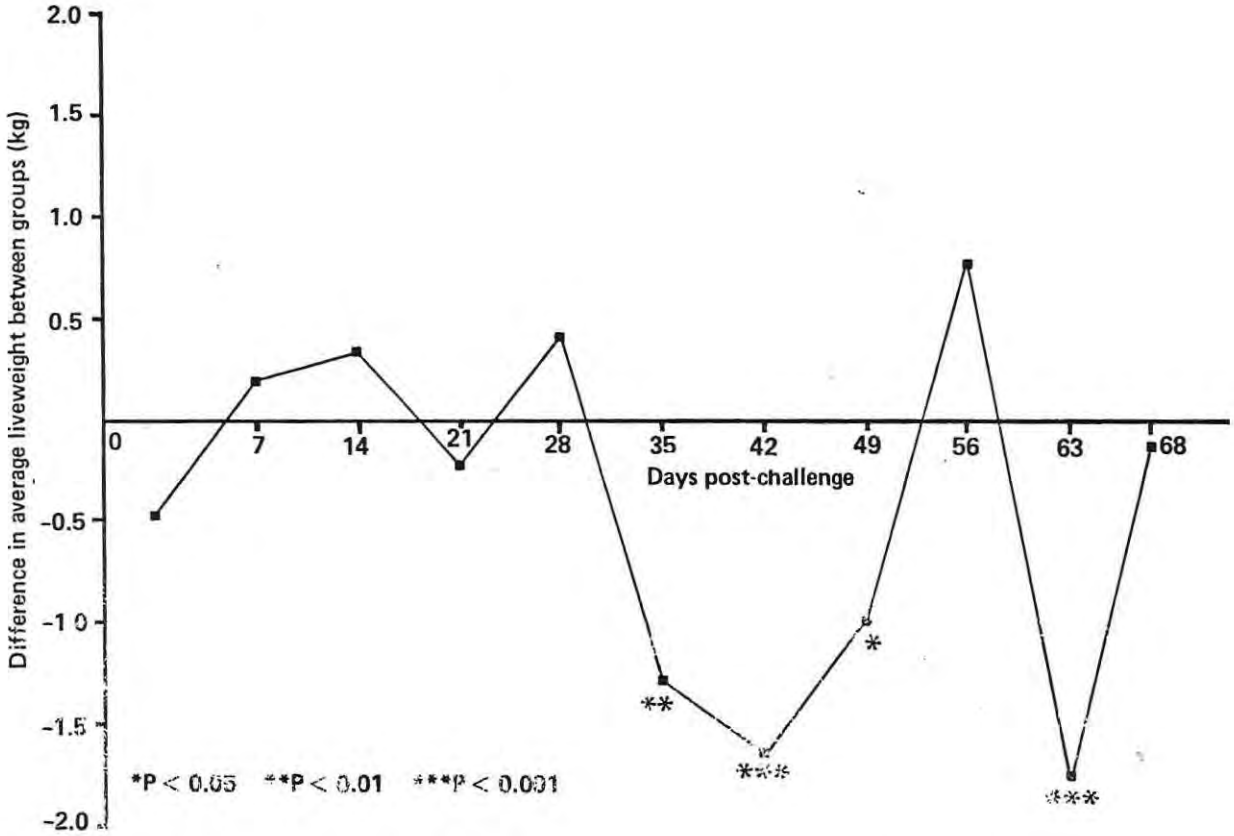


FIGURE 6.3

EXPERIMENT 1. DIFFERENCE BETWEEN WEEKLY AVERAGE LIVEWEIGHT GAINS OF EPP-INFECTED AND CONTROL GROUPS OF PIGS DURING THE HERD "BREAKDOWN" OF INFECTION (INFECTED-CONTROL).

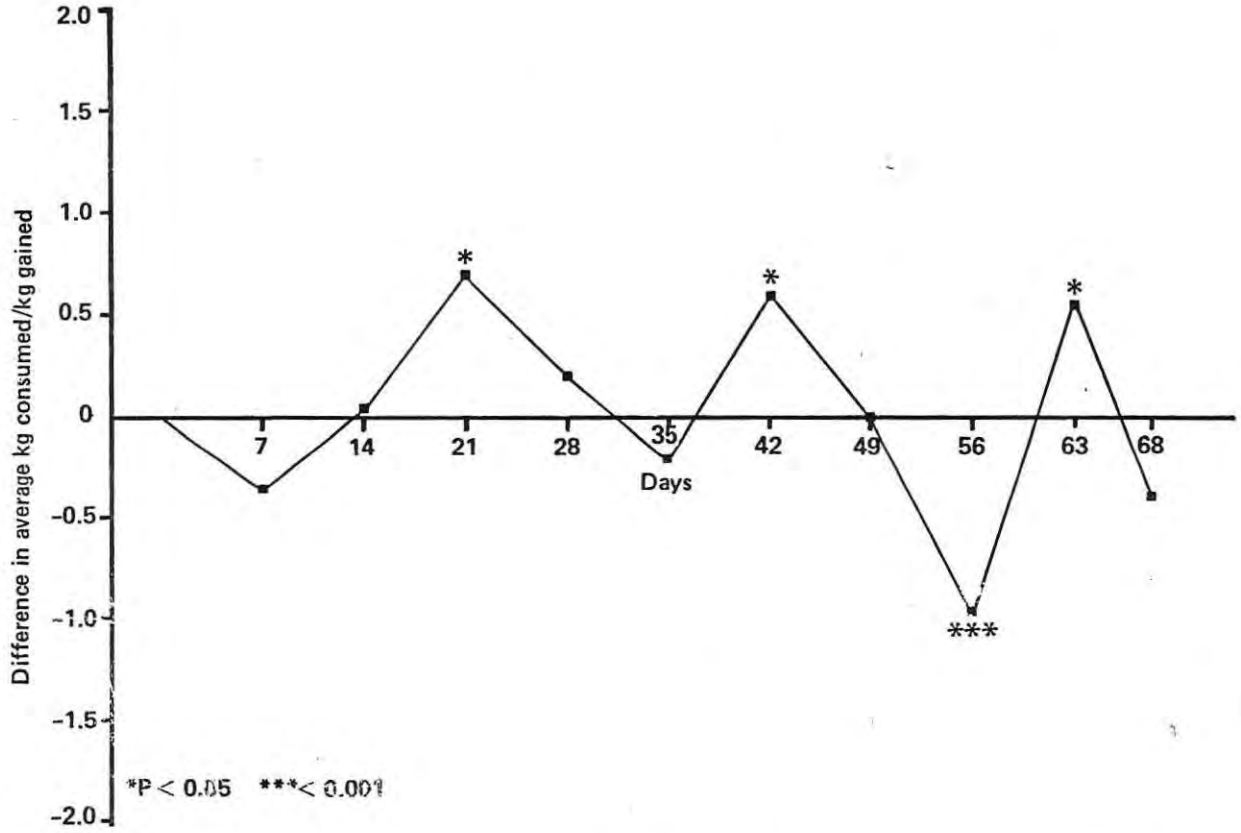


FIGURE 6.4:
EXPERIMENT 1. DIFFERENCE BETWEEN AVERAGE WEEKLY FEED CONVERSION EFFICIENCIES OF EPP-INFECTED AND CONTROL GROUPS OF PIGS DURING THE HERD "BREAKDOWN" TO INFECTION (INFECTED-CONTROL).

body weight gains of the infected group was significantly less in 4 of the following 6 weeks. The difference between fortnightly FCE of infected and control groups are shown in Figure 6.4. During the second last recording period the FCE of infected pigs was significantly better than that of control pigs due to decreased FCE among control pigs, however this trend was not sustained.

6.4 Discussion

The effect of EPP infection on performance of pigs during the "breakdown phase" of infection was significant in terms of reduced growth rate. Widespread coughing became apparent among the in-contact pigs during the third and fourth week post-inoculation of the transmitters (Fig. 6.1). It was at that time when the effect of the disease was first manifest, causing a significant reduction in the mean growth rate over all pigs during the following 3 weeks (Figure 6.3). Growth rate during the finishing period (50-85kg) was depressed by 12.7% while feed conversion efficiency was not significantly altered (Table 6.2). However, over the second last recording period, the feed conversion efficiency of the infected pigs was substantially better than that of controls (Figure 3.3). This occurred not because of better performance of infected pigs, but rather due to poorer performance of the control pigs during this period. The reason for this is unknown as there was

no variation in environmental and management conditions affecting the control pigs at that time. Overall, the growth rate was reduced by 4.5% between 25kg to 85kg, resulting in a further 7 days being required by the infected pigs to reach market weight. At a current feed cost of $200 per tonne for finishing rations and a daily feed consumption of 2kg per pig, this would add an extra $2.80 to the cost of production of each pig. Further costs include those of additional housing, labour, water, electricity etc. which amounts to approximately 10¢/pig/day at current costs and rates of depreciation. The total cost caused by introduction of M. hyopneumoniae to EPP-free pigs at 10 weeks of age recorded in experiment 1 was $3.50 per pig, irrespective of lung pneumonia status at slaughter.

By comparison with other estimates of the effect of EPP, the reduction in growth rate of 12.7% falls midway between the estimates of Betts and Beveridge (1953) of 25%, Betts et al (1955) of 16% where EPP-free pigs were intranasally inoculated, and estimates of Huhn (1970b) of 7% and Braude and Plonka (1975) of 5.5% using naturally infected commercial pigs. Previous studies which examined the effect of FCE found reductions of 22% (Betts et al 1955), 4.6% (Braude and Plonka 1975) and no effect at all (Eikmeier and Mayr 1965 - cited by Huhn 1970a). In this trial reduction in growth rate was due to reduced feed intake as reduction in FCE played no part. It is possible therefore that the use of a diet with a higher energy content may have overcome the effect of a depressed appetite, however this

practice under commercial conditions may be impractical due to the increased proportion of down-graded fat carcasses which would result.

The pathological condition produced in this experiment was similar to that recorded by Etheridge et al (1979) and Etheridge and Lloyd (1980). Although lesions were recorded in 71% of lungs at slaughter, a level considered to represent severe herd infection (Muirhead 1979) lesions were generally mild and healing, being confined to the tips of 1 or 2 lobes per lung. The extent of lesions produced was less than those produced by Etheridge and Lloyd (1980) who found a mean of 5.1 lobes affected in pigs killed between 4 and 10 weeks after exposure to transmitters inoculated with the Beaufort strain of M. hyopneumoniae. However the healing recorded at 10 weeks post-exposure in our trial was similar to that observed in a previous trial with this strain (Etheridge et al 1979) where gross lesions were absent in all 6 pigs killed 10 weeks post-exposure. Confirmation of M. hyopneumoniae infection among these pigs is reported and discussed in Chapter 3. * See back

Whether compensatory growth would have occurred is not known as pigs were only held for a short period post-challenge. However, it seems unlikely that it would have occurred, as by the end of the trial the obvious resolution of the condition had not been

accompanied by a sustained improvement in performance. Further studies are required to fully investigate this phenomenon, which requires holding pigs over their normal growing period after initial natural challenge by infected dams and by other infected weaners.

Of particular interest was the relative absence of significant secondary bacterial contamination of existing pneumonic lesions. Only Pasteurella multocida and Haemophilus parainfluenza were isolated separately from 2 of 24 lungs (Table 3.4). These lesions may have been far worse had there been widespread secondary bacterial infection with P. multocida (Smith et al 1973). In studies overseas (Little 1975 ; Gois et al 1975 ; Bolske et al 1980 ; Gois et al 1980) P. multocida is a common secondary invader of M. hyopneumoniae infection and is commonly isolated from severe field cases in South Australia (unpublished data). The significant reduction in performance recorded in this trial was primarily due to mycoplasma infection in isolation from secondary bacteria.

The relative absence of significant secondary invaders presents the opportunity to further study the effect of M. hyopneumoniae infection in isolation. While industry acknowledges that production losses may be severe with complicated EPP, there is considerable argument regarding the effect of uncomplicated EPP under local environmental conditions. By combining

aspects of this trial with natural infection of piglets by their dam, in an additional experiment, it may be possible to study the effect of endemic EPP in isolation from secondary complicating bacteria and thereby clarify the situation.

EXPERIMENT 2

6.5 Materials and Methods

6.5 (i) Pigs

Twenty four Large White EPP-free gilts were batch mated at NPRU. From those confirmed pregnant 14 were selected on the basis of the genetic background of their foetuses and an estimated farrowing date of mid-May 1982. These gilts were then paired according to the above criteria and then like pairs were randomly allotted to either unit 1 or 2.

(ii) Feed

All litters received pelletted NPRU creep-weaner ration C_5 (D.E. 14.4 mega J/kg, C.P. 22.2%, Lysine 1.2%) from 2 weeks of age until the average body weight in each unit respectively reached 25kgs. From then until slaughter pigs were fed ad lib NPRU grower-finisher ration G_{13} (D.E. 14.1 mega J/kg, C.P. 17.3%, Lysine 0.85%).

(iii) Inocula and their administration

Each of the 7 gilts housed in unit 1 were intranasally inoculated with lung homogenate prepared from transmitter pigs used in Experiment 1, according to protocols detailed in 2.6. Control gilts in unit 2 were inoculated with MH medium.

(iv) Monitoring of infection

All trial pigs were monitored for 30 minutes daily and all pigs observed coughing recorded to determine the day of onset of coughing for each pig. Pigs showing severe clinical signs of pneumonia were assessed and treated as described in Experiment 1.

(v) Environmental conditions

The trial was conducted under conditions outlined in Experiment 1 which were set by results of the field study (Chapter 5).

Gilts were farrowed in crates and piglets were provided with a creep lamp from birth. All piglets received 2 ml of injectable iron (Ferrelan 100, Lienert Aust.) on their third day and were checked for scours daily. At weaning litters were mixed and checked for scours and other illness twice daily.

Ammonia levels were also recorded in this experiment, once during the growing stage and once during the finishing stage according to the methods detailed in Chapter 5.2.

(vi) Samples from experimental animals

All trial gilts were blood sampled prior to inoculation and again at slaughter 9 weeks later. All piglets were blood sampled at 10 weeks of age and again at slaughter (15 weeks of age for transmitters and at 21-23 weeks for trial groups).

Lungs were collected at slaughter from all gilts, transmitters and trial pigs. Gross lesions of EPP were mapped on calibrated graph paper, and specimens collected for histopathology and IMFT as described in Chapter 2.

(vii) Statistical analysis of pig performance data

Results were analysed using analysis of variance carried out on growth rates and feed conversion.

(viii) Experimental design

Infection was introduced by the following method to reproduce natural infection of piglets as it occurs in endemically infected herds. Gilts in unit 1 were inoculated with pneumonic lung suspension 4 weeks prior to farrowing to provide challenge for piglets during suckling. The inoculation of the runt piglet of each litter ensured transmission between piglets in all litters prior to weaning.

At weaning at 30 days of age, all uninoculated piglets (43) in unit 1 were stratified on the basis of weight and sex into 2 matched groups of 16 piglets. Four transmitters were then added to each group, producing 2 weaner groups of 20 piglets. This procedure was repeated in unit 2 where there were 47 piglets available for selection.

Transmitter piglets were removed from the trial groups two weeks post-weaning, when they were held in adjacent pens, separated by a wire mesh panel which allowed contact with trial pigs for a further 8 weeks.

When the weaners stocking density reached 45kg/m^2 in each unit the pens were expanded using moveable panels. When the grower stocking density reached 72.5kg/m^2 the two trial groups were halved.

All trial pigs were weighed at weekly intervals from weaning until slaughter at 85kgs liveweight. Feed was "weighed back" after each fortnightly period to enable FCE calculations.

6.6 Results

All gilts in unit 1 challenged with M. hyopneumoniae in pneumonic lung suspension were observed to cough at an average of 9 days post-

inoculation. In the post-weaning period all in-contact trial piglets were observed to cough, at an average of 9 days post weaning.

Details of pigs requiring antibiotic treatment are shown in Table 6.3. All infected and control pigs were treated for scours commencing 5 days post-weaning with antibiotic and electrolytes. Four of 24 infected and 2 of 24 control pigs were treated for poly-arthritis while 4 of 24 infected pigs were treated for "thumps", 3 of which required a second course. One pig (T_4) died after prolonged antibiotic therapy.

Environment and management conditions under which the experiment was conducted are shown in Table 6.4.

Details of post-weaning mortalities are shown in Table 6.5. In both trial groups there was a 21% post-weaning mortality rate, attributable to coli-bacillosis and gastric torsion. Pig D_6 was euthanized due to symptoms suggestive of oesophageal achalasia, which was confirmed at necropsy. Pig D_7 (T_4 in Table 6.4) died due to secondary bacterial complication of existing EPP lesions. Gross lesions were extensive in the infected pigs which died, with lesions being present in an average of 4.5 lobes per lung, covering an average of 18.4% of the total lung area.

TABLE 6.3

Experiment 2. Details of antibiotic treatments during the trial

EPP Infection Status	Identity	Age (weeks)	Drug	Duration of Treatment (days)	Reason	Presence of pleurisy at slaughter
Infected	T_1	4.5	Streptopen	3	arthritis	-
	All	5	Neoampho	"	scours	-
	T_2	5	Streptopen	"	"thumps"	-
	T_3	7	"	"	arthritis	-
	T_4	7	"	"	arthirits	-
	T_5	8	"	"	"thumps"	yes
	T_6	8	"	"	"thumps"	-
	T_7	8	"	"	arthritis	-
	T_8	9	"	"	"thumps"	-
	T_5*	9	"	"	"thumps	-
	T_7*	9	"	"	"thumps"	-
	T_4*†	13	"	7	"thumps"	yes
Uninfected	All	5	Neoampho	3	scours	-
	T_9	6	Streptopen	3	arthritis	-
	T_{10}	11	"	3	arthritis	-

* repeat course

† died 9 days after last treatment

TABLE 6.4.

Experiment 2. Environment and management conditions under which pigs were held.

VARIABLES	UNIT 1			UNIT 2		
	8-25kg	25-50kg	50-85kg	8-25kg	25-50kg	50-85kg
Average minimum temperature (^{o}C)	12±2	15±2	17±2	12±1	15±	16±3
Average maximum temperature	16±1	20±3	23±3	16±2	21±3	22±3
Diurnal temperature fluctuation	4±1			4±1		
Average minimum relative humidity (%)	62±5	55±9	52±7	60±11	51±15	49±11
Average maximum relative humidity	73±4	66±7	66±7	79±9	69±14	72±11
Maximum stocking density (kg/m^2)	42-47	70-75	75-80	42-47	70-75	75-80
Group Size	20	14	6	20	14	7
Ammonia level - 2 sites (ppm)	- -	7 5	8 10	- -	7 7	8 11

± Standard Deviation

TABLE 6.5

Experiment 2. Details of post-weaning mortalities recorded during the trial

EPP Infection Status	Identity	Age at death (weeks)	Percentage lung affected area with EPP	Number of lobes with lesions	Cause of death
Infected	D_1	5	0	0	Coli bacillosis
	D_2	5	15.2	4	Coli bacillosis
	D_3	5	35	7	Coli bacillosis
	D_4	13	11.3	5	Gastric torsion
	D_5	13	22.2	6	Gastric torsion
	D_6	15	6.7	3	Oesophageal achalasia
	D_7*	16	38	7	Pneumonia, pleurisy, pericarditis, peritonitis and α-haemolytic Strep. isolated.
Uninfected controls	D_8	5	0	0	Coli bacillosis
	D_9	5	0	0	Coli bacillosis
	D_{10}	5	0	0	Coli bacillosis
	D_{11}	10	0	0	Coli bacillosis
	D_{12}	12	0	0	Gastric torsion

* Pig D_7 previously represented as pig T_4 in Table 6.3.

Details of lung lesions of EPP in transmitter and in-contact pigs are shown in Table 6.6. Eleven of 25 (44%) in-contact pigs had gross lesions at slaughter in an average of 1.7 lobes per lung, covering an average of 0.78% of the total lung area. No gross pneumonic lesions were observed in control pigs.

Results of diagnostic tests on specimens to confirm specific infection with M. hyopneumoniae are shown in Table 6.7. All but one of the lungs from animals challenged by either method in this trial showed histologic lesions typical of third and fourth stage EPP lesions. All gilts were CF negative prior to inoculation. Positive CF titres were detected in 93% of 15 transmitters between 9 and 15 weeks post-inoculation and in 90% of 29 in-contact pigs at 10 weeks of age. At slaughter 32% of 25 in-contact pigs had positive CF titres. M. hyopneumoniae organisms were detected by IMF in 54% of 13 lungs from transmitter pigs and in 36% of 14 lungs from in-contact pigs ; only lungs with gross lesions were tested. Secondary contaminating bacteria, α-haemolytic Streptococci, were isolated from 1 of 27 control lungs, 4 of 14 lungs with lesions and 3 of 11 grossly normal lungs from challenged pigs.

The growth curves for infected and control groups are shown in Figure 6.5. Major losses occurred between weaning and 14 weeks of age, causing a delay in marketing at 85kgs of 12 days. Table 6.8 shows the

TABLE 6.6

Experiment 2. Details of lung pathology in transmitter and in-contact trial pigs*

Identity	Weeks Post-Exposure When Slaughtered	Percentage of lung Area Affected by EPP	Number of Lobes with Lesions
Transmitter gilts			
1	9	0	0
2		0.1	1
3		0.1	1
4		0.1	1
5		0.1	1
6		6.4	2
7		15.1	6
Transmitter piglets			
1	8	0	0
2		0.2	1
3		2.4	5
4		5.7	5
5		6.6	6
6		11.2	5
7		30	7
8		64.4	7
In-contact pigs			
1	25	0	0
2		0	0
3		0	0
4		0	0
5		0	0
6		0	0
7		0	0
8		0	0
9		0	0
10		0	0
11		0	0
12		0	0
13		0	0
14		0	0
15		0.004	1
16		0.004	1
17		0.04	1
18		0.1	1
19		0.3	1
20		0.3	1
21		0.3	2
22		0.4	3
23		0.6	1
24		2.1	1
25		4.5	5

*Gross lesions of EPP not observed in any control pig.

TABLE 6.7.

Experiment 2. Laboratory confirmation of M. hyopneumoniae infection

Enzootic Pneumonia Status	Gross evaluation of lungs	Number of lungs examined	Histopathology 4	3	2	-	CFT +	-	IMFT +	-
Inoculated sows	Lesions present	6	5	1	0	0	5	1	2	5
	Lesions absent	1	0	1	0	0	1	0	NT	NT
	TOTAL	7	5	2	0	0	6	1	2	5
Inoculated transmitters	Lesions present	7	7	0	0	0	7	0	5	2
	Lesions absent	1	0	0	0	1	1	0	NT	NT
	TOTAL	8	7	0	0	1	8	0	5	2
In-contact (naturally challenged)	Lesions present	17	7	3	-	-	26	3	5	11
	Lesions absent	15	8	7	-	-			NT	NT
	TOTAL	32	15	10	-	-	26	3	5	11

4 - Fourth stage EPP lesions

3 - Third " " "

2 - Second " " "

\- Negative

\+ Positive

*antibody titres at 10 weeks of age

NT = Not Tested

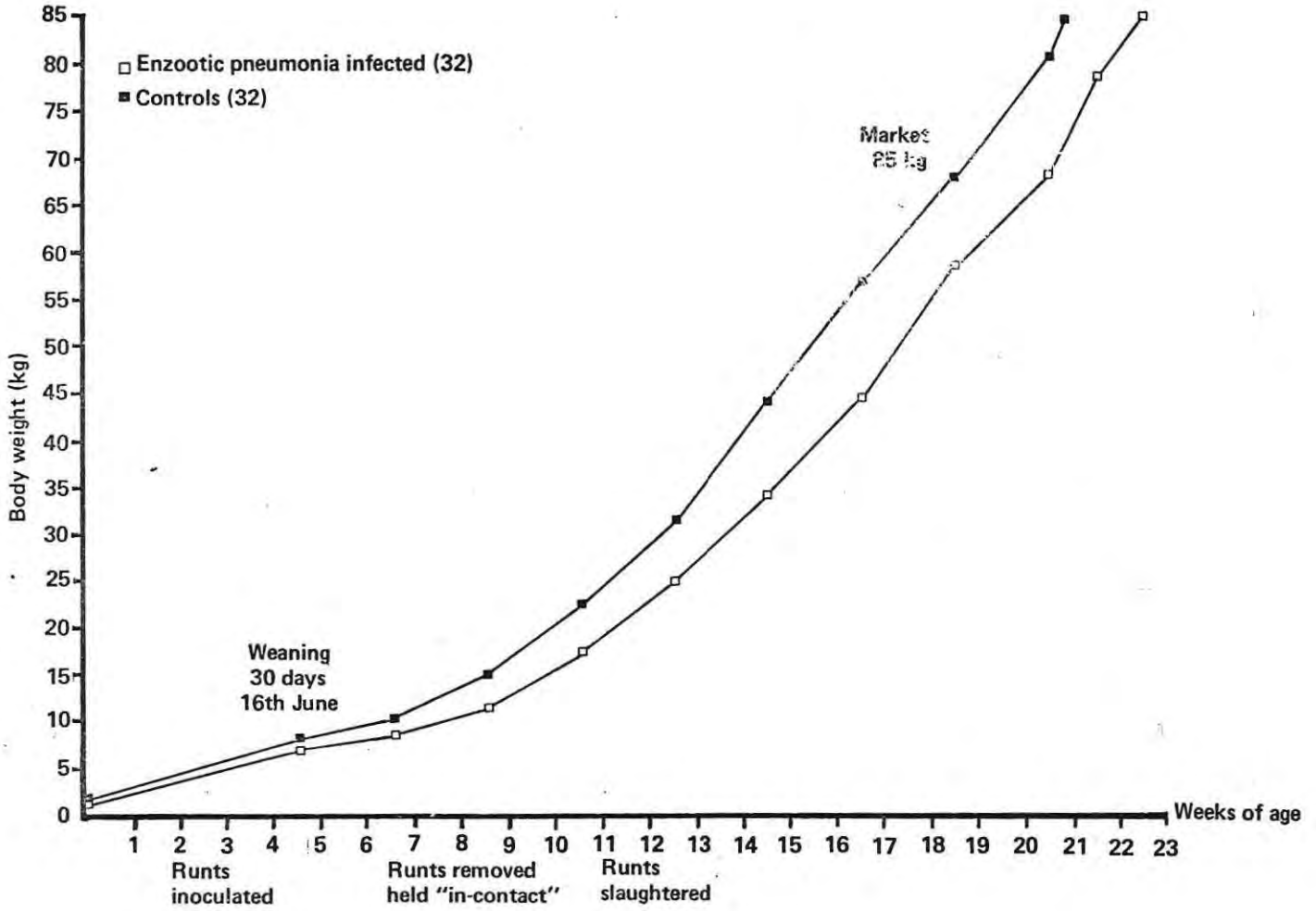


FIGURE 6.5: EXPERIMENT 2. AVERAGE LIVEWEIGHT OF EPP-INFECTED AND CONTROL PIGS DURING ENDEMIC INFECTION.

TABLE 6.8.

Experiment 2. Effect of EPP on growth rate and feed conversion efficiency

	INFECTED GROUP	CONTROL GROUP
Number of animals	32	32
First exposure to EPP	At birth	At birth
Type of housing	Intensive	Intensive
Growth rate gms/day		
8-25kg		
Mean	246***	341
S.D.	±89	±127
Range	71-381	136-696
Decrease in G.R. (%)	27.9	-
25-50kg		
Mean	591***	785
S.D.	±154	±92
Range	171-885	606-928
Decrease in G.R. (%)	24.7	-
50-85kg		
Mean	837	886
S.D.	±135	±145
Range	595-1249	609-1303
Decrease in G.R. (%)	5.5	-
8-85kg		
Mean	545.7***	648.6
S.D.	±58	±62
Range	364-583	455-688
Decrease in G.R. (%)	15.9	-
Feed Conversion ratio†		
10-25kg		
Mean	2.49	2.19
S.D.	NA	NA
Range	NA	NA
Decreased efficiency (%)	13.8	-
25-50kg		
Mean	2.23	2.32
S.D.	NA	NA
Range	NA	NA
Decreased efficiency (%)	-	3.7
50-85kg		
Mean	2.67	2.85
S.D.	NA	NA
Range	NA	NA
Decreased efficiency (%)	-	6.4
8-85kg		
Mean	2.53	2.51
S.D.	NA	NA
Range	NA	NA
Decreased efficiency (%)	0.5	-

*p<0.05 **p<0.01 ***p<0.001

† Daily feed intake/daily LW gain

NA : Not available - individual pig FCE not recorded.

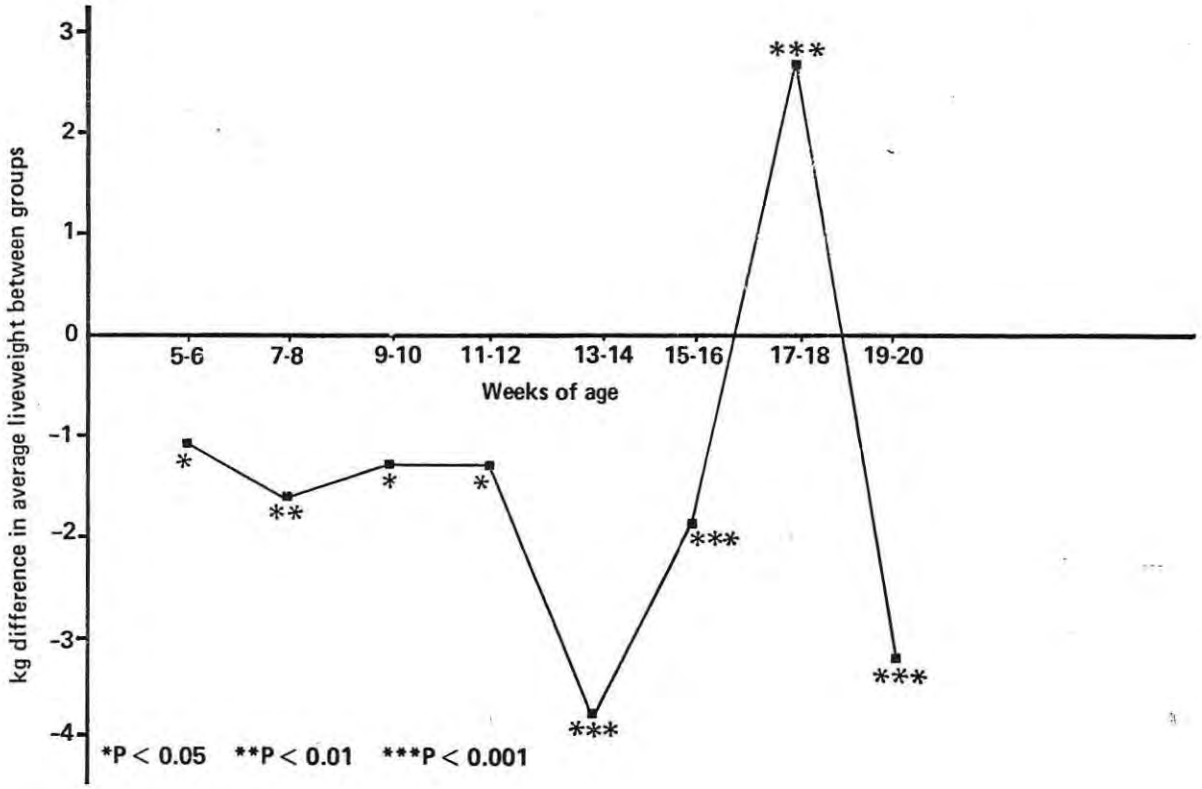


FIGURE 6.6
EXPERIMENT 2. DIFFERENCE BETWEEN AVERAGE FORTNIGHTLY LIVEWEIGHT GAINS OF EPP-INFECTED AND CONTROL GROUPS OF PIGS DURING ENDEMIC INFECTION (INFECTED-CONTROL).

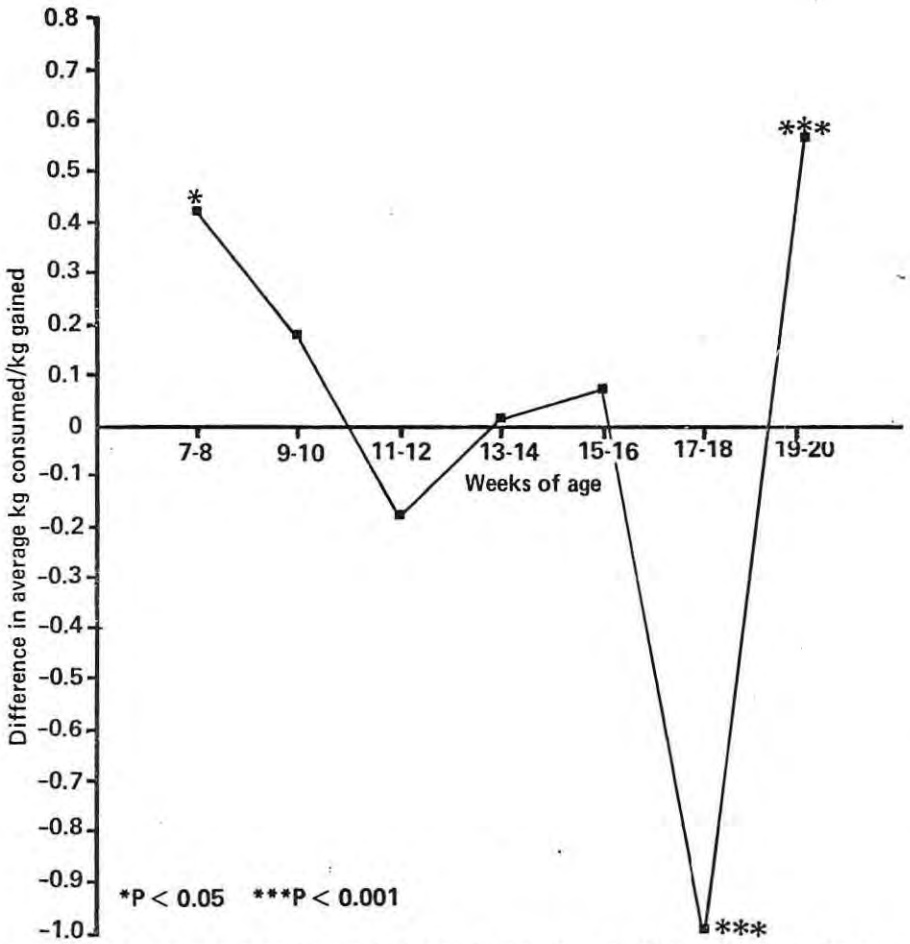


FIGURE 6.7 EXPERIMENT 2. DIFFERENCE BETWEEN AVERAGE FORTNIGHTLY FEED CONVERSION EFFICIENCIES OF EPP-INFECTED AND CONTROL GROUPS OF PIGS DURING ENDEMIC INFECTION (INFECTED-CONTROL).

mean growth rates and feed conversion efficiency for each group with the standard deviation of the mean for the post-weaning, growing and finishing periods as well as the entire trial period. Growth rate was depressed in the post-weaner period by 27.9% ($p<0.001$) and in the grower period by 24.7% ($p<0.001$). From weaning to slaughter growth rate was depressed by 15.9% ($p<0.001$). Feed conversion efficiency was depressed by 13.8% in the post-weaner period, which was marginally short of significance at the 0.05 level.

The difference between weekly average body weight gains of infected and control groups are shown in Figure 6.6. In all but 1 (17th and 18th week) fortnightly analysis period, the growth rate of infected pigs was significantly less than control pigs. The difference between fortnightly FCE of infected and control groups are shown in Figure 6.7. During the second last 2 week recording period the FCE of infected pigs was significantly better than that of control pigs due to the added effect of marginally better FCE among infected pigs and marginally poorer FCE among control pigs. The reason for these transient fluctuations are not apparent.

6.7 Discussion

This study shows that pigs endemically infected with enzootic pneumonia grew 15.9% slower than uninfected pigs between weaning and slaughter at 85kg liveweight

and the feed conversion rate was depressed by 13.8% between weaning and 25kg liveweight. Pigs were reared under environmental and management conditions found commonly on commercial piggeries in South Australia. At the commencement of the trial piglets were challenged naturally by infected dams, which also provided potential for protection from infection via colostral antibody (Durisic et al 1975a, b). Transmitter piglets then maintained a known source of infection during the suckling period and immediately post-weaning. At slaughter 44% of naturally challenged pigs had gross lesions of EPP, a prevalence similar to that recorded for the South Australian pig industry in the abattoir survey (Chapter 4). The results support those obtained in experiment 1, despite the recording of smaller depression in growth rate (12.7%), in association with a higher prevalence of lesions (71%) in that experiment. The probable reason for this observation is that in experiment 1, pigs were challenged from 10 weeks of age when past the major period of susceptibility to the combined effects of infection and suboptimal environmental conditions. In experiment 2 widespread infection with EPP at weaning was also accompanied by a concurrent outbreak of colibacillosis possibly increasing the severity of the pneumonic infection. This would happen frequently in commercial units. That extensive pneumonia did develop in the early post-weaning stages is supported by the wide distribution of lung lesions found in pigs which died during the earlier stages of the experiment (Table 6.5).

The periods when major losses in growth rate occurred were in the post-weaner and grower periods (Table 6.8). Feed conversion efficiency was reduced between 10-25kg liveweight by 13.8%, which was only marginally short of statistical significance at 0.05. This reduction occurred at a time when infection was most severe, supporting previous observations of Betts et al (1955) and Braude and Plonka (1975) which also recorded reductions in FCE due to EPP infection. Reduced growth rate in this period was due to the combined effect of poorer FCE and reduced feed intake (Fig. 6.7 and 6.8). During the second last recording period the performance of the groups was reversed with the infected pigs performing better than controls. This was due to the combined effect of poorer FCE in control pigs and improved FCE among infected pigs. The change was not sustained and no changes in environment or management conditions were noted among either population to account for the observation.

Compensatory growth did not occur among the infected pigs during the latter stages of the trial despite extensive healing of gross lesions. Lesions at slaughter were present in 44% of 25 lungs and were typically chronic, being present in an average of 1.7 lobes per lung and covering an average of 0.78% of the total lung area (Table 6.6). During the finishing period performance of infected pigs did not vary significantly from uninfected pigs (Table 6.8). These results are supported by those of experiment 1, in which resolution of lesions was not accompanied by better

performance than that recorded in uninfected pigs at the same liveweight. In experiment 2, pigs were studied throughout their normal growth period to 85kg, allowing full potential for compensatory growth to occur as is available commercially.

Infection with M. hyopneumoniae was confirmed by CFT and IMFT. A high proportion of both inoculated and in-contact pigs both had detectable CF-antibody after challenge (Table 6.7). All intranasally inoculated gilts were CF negative prior to challenge. The percentage of in-contact pigs which were CF positive declined from 90% at 10 weeks of age to 32% at 22.5 weeks of age. This latter observation contrasts strongly with the findings of Woods et al (1976) who found a higher reactor rate in pigs between 19-24 (69%) weeks than in pigs between 7-12 weeks (42%). The latter results obtained in 8 herds in Illinois indicates a slightly different epidemiology, with a continuing incidence of infection occurring through the growing period . This may be the result of rearing pigs in a colder climate, necessitating the closing of sheds to maintain temperature, which often occurs at the expense of effective ventilation (Muirhead 1979). Despite the waning of CF titres and resolution of lesions in the growing period in this experiment, the CF result at slaughter confirmed the diagnosis of EPP based on gross appearance of lesions (McKean et al 1979). This diagnosis was further confirmed by the demonstration of M. hyopneumoniae organisms by IMF in the lungs of inoculated transmitter and in-contact trial pigs with lesions.

The reduction in performance occurred in the absence of common secondary bacterial invaders known to extend and prolong lung lesions (Smith et al 1973 ; Little 1975 ; Gois et al 1975, 1980 ; Bolske et al 1980). While α-haemolytic Streptococci were more frequently isolated from lungs of challenged pigs, they were isolated equally from affected and normal lungs in this group, suggesting a minor role in the complication of EPP lesions. In this experiment the reduced performance was due primarily to uncomplicated EPP, thus confirming the commercial significance of the "mild" or subclinical form of the disease.

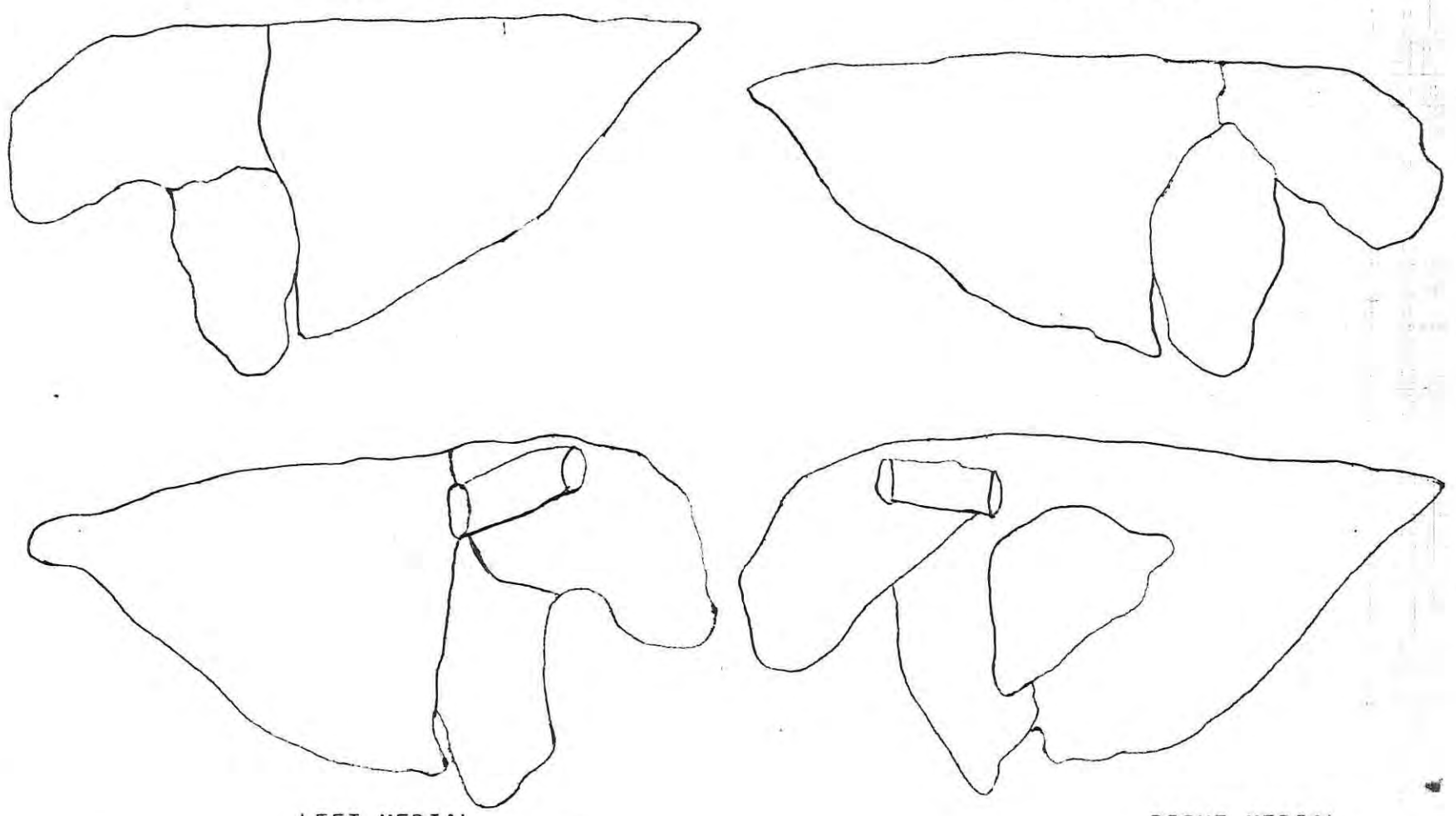
Environmental and management conditions under which the trial was conducted were those commonly found on local commercial piggeries (Chapter 5). The average minimum post-weaning temperature was between the average minimum temperature recorded on small and large herds in winter 1981 (Table 5.5). The diurnal fluctuation was approximately half that recorded commercially due to the limitation of the range of temperatures that could be produced by mechanical ventilation. As in experiment 1 stocking density appears to be marginally less than practised commercially. However, due to allowance of part of the solid floor for dunging, final stocking densities were considered to be effectively greater. Reductions through increasing pen size generally coincided with soiling of the sleeping area. While the facility placed severe limits on the total number of pigs per shed, weaner groups were kept

at a size commonly found commercially. The average ammonia levels recorded in this trial were considered to lie in the mid-range of values recorded in the field study (Table 5.7). While conditions detailed here do not necessarily reflect any particular commercial herd group defined in Chapter 5, the conditions under which the trial was conducted reflect those under which pigs are reared commercially.

The total economic loss caused by EPP infection in this experiment was $7.63/85kg pig produced. It is important to note that this is the average loss per pig to EPP, irrespective of presence or absence of gross lung lesions at slaughter. The major component of this loss was attributable to the cost of extra feed required by pigs to reach the designated slaughterweight. The cost of feeding for an extra 12 days, given a current feed cost of $200/tonne and a daily feed consumption of 2kgs was $4.80 per pig. Further feed costs were caused by the depressed rate of feed conversion in the post weaner (10-25kg) period, which amounted to $1.40/pig. Added to this is the extra cost of labour, housing, electricity, water, etc. which amounts to approximately 10¢/pig/day given current costs and rates of capital depreciation. Another smaller cost was that of medication for pigs which were treated for severe clinical pneumonia, which amounted to 23¢/pig when averaged over all the infected pigs marketed. The opportunity cost of $90 for the 50kg pig which died due to complicated EPP must also be considered. A death rate of 4% due to EPP would be considered high in most herds, being more indicative of a herd with severe complicated EPP.

These experiments demonstrate that substantial economic losses are caused by uncomplicated enzootic pneumonia under environmental and management conditions commonly found on commercial piggeries in South Australia. Furthermore, these losses are similar in nature and magnitude to those recorded in colder northern hemisphere countries where major efforts have been made to control EPP. Now that an estimate of local losses is available, the cost-effectiveness of control procedures developed overseas should be evaluated.

APPENDICES



TOTAL ☐

area x 0.1827 = % of total lung surface consolidated
of lesion(s) (pers. comm. Mercy 1981)

Lung drawn to 1/3 linear scale. Area of affected tissue measured in cm^3 using a planimeter and percentage of lung tissue calculated as above.

APPENDIX II

COMPLEMENT FIXATION TEST - C' CONSUMPTION METHOD

Antigen

The M. hyopneumoniae (strain J) antigen was supplied by CSIRO Melbourne, as raw concentrated culture grown in 6 litres of MH media (Slavik and Switzer 1972 ; Etheridge et al 1979).

The concentrate was prepared for use by thawing and then preparing a smooth suspension using a Griffiths tube. The suspension was then diluted 1/10 with Oxoid CFT diluent and heated in a water bath at 52°C for 30 minutes (Slavik and Switzer 1972).

The treated antigen was then distributed into 200 µl aliquots in glass vials and stored at -80°C.

For use, antigen was thawed and diluted 1/20 with Oxoid CFT diluent. Each batch was titrated to determine the lowest dilution that was antigenic and not anti-complementary for test purposes.

Disposable Micro-titre Plates

The test was performed in micro-titre plates* with U-shaped wells.

(*Micro-titre Plates Type MTP-1, Disposable Products, Adelaide, South Australia).

Diluent

Veronal buffered saline containing Ca++ and Mg++ ions (VBS) was prepared by dissolving CF Test Diluent Tablets (Oxoid Ltd., London, United Kingdom) in glass distilled water.

Complement (C')

Guinea-pig clotted heart blood was kept cool and within 4 hours the serum was separated by centrifugation, distributed into 1 ml aliquots and stored at -80°C or preserved by Richardson's method as detailed by Alton (1977) and stored at 4°C.

Normal Pig Serum

Serum collected monthly from 3, 5 - 10 week old piglets, was pooled and then stored in 1 ml. aliquots at-80°C. This was used as a source of porcine complement to enhance the G.P. complement activity.

Sheep Erythrocytes

Sensitised sheep erythrocytes were prepared by the methods described for the CF test for brucellosis (Alton 1977).

Titration of C'

Using /2 as the multiplication factor, a geometric series of 9 dilutions of guinea-pig serum commencing at 1 in 400 was prepared in cold VBS containing 1% unheated normal pig serum (porcine C'). A unit volume (0.2 ml) of each dilution was added to a unit volume of diluted

(1 in 8), heated (60°C for 30 minutes) normal pig serum and a unit volume of diluted M. hyopneumoniae antigen. The tubes were shaken and placed overnight at 5°C. A unit volume of the suspension of sensitised erythrocytes was then added to each tube; all were shaken and placed in a waterbath at 37°C for 30 minutes, during which they were shaken again at 10 minute intervals. They were then centrifuged and the tube in which 50% haemolysis occurred ($C'H_{50}$ titre) was determined visually. This enhanced titre was usually about 1 in 3 200.

Procedure for CF Test

The presence of CF antibody was demonstrated after a fixation period of 16 to 18 hours at 4°C by comparing the titre of the C' obtained in the presence of the test serum, porcine C' and antigen, with the titre of the C' when antigen was omitted. A simple formula enabled a reduction in C' titre to be expressed as the number of $C'H_{50}$ units fixed.

VBS, guinea-pig serum (C'), normal pig serum (porcine C') and concentrated M. hyopneumoniae antigen were kept at 4°C when not in use.

Test serums were thawed, well mixed and 1 in 8 dilutions prepared in VBS. These were heated at 60°C for 30 minutes; a unit volume (0.2 ml) of each was placed in each of a set of 8 test tubes.

Six doubling dilutions of guinea-pig serum containing 40, 20 etc. $C'H_{50}$ units per unit volume were prepared in cold VBS containing 1% unheated normal pig serum and kept in ice. Unit volumes of each of these dilutions were added to the wells containing the diluted test serums so that in each set, well H contained 40 $C'H_{50}$ units, well G, 20 units, well F, 10 units, well E and C, 5 units, well D and B, 2.5 units and well A, 1.25 units.

The thawed concentrated M. hyopneumoniae antigen was homogenised in a Griffith's tube, diluted in VBS to the level determined above and unit volume was added to wells H, G, F, E and D of each set (C' titration with antigen present).

A unit volume of VBS was added to wells C, B and A of each set in lieu of antigen (C' titration with antigen omitted).

The plates were shaken, covered with a plastic sheet to reduce evaporation and kept at 4°C for 16 to 18 hours (overnight). A unit volume of the suspension of sensitised sheep erythrocytes was then added to each well. The degree of haemolysis was assessed immediately after the microtitre plates had been shaken continuously for 30 minutes on a microtitre shaker at 37°C and centrifuged at 4°C.

Interpretation of CF Test Results

The degree of CF is expressed as the number of $C'H_{50}$ units fixed. Fulton and Dumbell (1949) used the formula:-

$$Z = (x_2 - x_1)/x_1$$

where Z is the number of $C'H_{50}$ units fixed, x_1 is the volume of guinea-pig serum in unit volume of the C' dilution containing one $C'H_{50}$ unit and x_2 is the volume of guinea-pig serum in unit volume of the C' dilution where there is a residue of one $C'H_{50}$ unit in the test proper.

When the guinea-pig serum dilutions used are steps in a geometric dilution series, x_2 may be expressed as $x_1 I^d$ where I is the dilution factor between the increments and d is the number of steps between x_1 and x_2. Substituting this value for x_2 the formula above becomes $Z = I^d - 1$.

The 6 doubling dilutions (I = 2) of guinea-pig serum used in the test were given step numbers 1,2,3, 4,5 and 6 respectively and d was determined for each test serum by subtracting the step number of the C' level where 50% haemolysis occurred in the titration with antigen present, from the step number of the C' level where 50% haemolysis occurred in the titration when antigen was omitted. For example, if 50% haemolysis occurred in well H (step 1) and in well 3 (step 5) then d = 4 and so Z = 15.

Simple interpolation of the step numbers for the C' levels was used when necessary. When d could not be calculated for most of the serums tested, the test was repeated with a more appropriate range of C' dilutions, however, when 50% haemolysis was obtained within the range of C' levels used for the titration with antigen omitted but not in the range used for the titration with antigen present, the serum could be classified as positive if d was greater than 3 or negative if it was less than 1.

Control serum

Control serum was prepared from pigs inoculated with M. hyopneumoniae (Beaufort strain) collected from both pre and post inoculation. Samples were freeze dried and the titre determined by testing against positive control serum supplied by CSIRO. Further sera with low antibody titres were obtained from naturally exposed experimental animals and used to monitor sensitivity of the test.

APPENDIX III

COMPLEMENT FIXATION TEST - DIRECT METHOD

Reagents, their preparations and storage and equipment were the same as described in Appendix II.

Procedure of the CF test

The test procedure was essentially the same as described by Slavik and Switzer (1972). Modifications were made by the addition of normal pig serum (enhancing serum) to restore the CF activity of the heat inactivated test serum. Normal pig serum was added to VBS, producing a 1% solution in which the operating dilution of GPC' was prepared. Test serums were also heated to a higher temperature, 60°C, to reduce the procomplementary activity. The presence of CF antibody was demonstrated after a fixation period of 16 to 18 hours at 4°C by determining the highest serum dilution showing 50% haemolysis of the indicator solution.

VBS, guinea-pig serum (C') and normal pig serum (porcine C') and concentrated M. hyopneumoniae antigen were kept at 4°C when not in use.

Test serums were thawed, mixed well and a unit volume (25 µl) added to an equal volume of VBS in a test tube. These were then incubated in a water bath at 60°C for 30 minutes after which the diluted

serum was added to the first column of a micro-titre plate. Doubling dilutions were prepared from this column in VBS to a dilution of 1/128 for routine use.

The second column containing a serum dilution of 1/4 was treated as an "anti-complementary column" i.e. no antigen was added, allowing for detection of anti-complementary activity of the test serum. A unit volume of VBS was added to this column in place of antigen.

A unit volume of M. hyopneumoniae antigen was added to all wells containing a serum dilution of 1/8 or greater. The working concentration of antigen of 1/8 and GPC' of 1/70 was determined by a checkerboard titration. Finally a unit volume of GPC' diluted 1/70 in VBS containg 1% normal pig serum was added to all test wells. Plates were gently shaken and then stored overnight at 4°C to allow complement fixation.

The method of titrating-back the amount of unfixed complement using a unit volume of sensitized sheep erythrocytes was the same as described in Appendix II. After shaking at 37°C for 30 minutes and centrifugation, rows were examined to determine the serum dilution at which the haemolytic endpoint (50%) occurred.

APPENDIX IV

PARAFFIN SECTIONING FOR IMF STAINING

1. Removed lung and cut section <5mm^2.
2. Placed in 95% ethanol precooled to 4^{o}C.
3. Held at 4^{o}C for 1 hour or longer.
4. Trimmed and placed in capsule.
5. Fixed in 95% ethanol at 4^{o}C for 15-24 hours.
6. Dehydrated in 4 changes of precooled absolute alcohol (1 to 2 hours per change) 4^{o}C.
7. Cleared in 4 changes of precooled (4^{o}C) xylene for 1 -2 hours per change. (Some specimens were stored in xylene at 4^{o}C for 1 to 2 days).
8. Embedding - specimen put through four consecutive baths of filtered paraffin at 56^{o}C for 1 to 2 hours each. Embedded in paraffin at 56^{o}C.
9. Blocks stored at -20^{o}c.
10. Sectioned as usual but flotation on water at 40^{o}C was of short duration. Sections picked up after a few seconds of flotation onto a clean albuminized glass slide.
    Dried for 30 minutes at 37^{o}C.
    Stored in dessicator at 4^{o}C.
11. Removed paraffin by immersing slides in 2 consecutive baths of cold xylene (30 secs. to 1 min. - then 3 consecutive baths of 95% ethanol (30 seconds) - then 3 consecutive baths of cold PBS (1 min. in each)).
12. Stained as described.

APPENDIX V

CULTURE OF PORCINE MYCOPLASMAS

1. Media reagents and stock solutions

(1) Agar (Agarose, Seakem, Rockland ME04841, USA).

(2) Bacitracin (Wellcome, Baconsfield, New South Wales, Australia). Reconstituted by addition of 1 grm of powder to 100 ml d.w.; sterilized by filtration. Stored at -20°C in 1 ml aliquots.

(3) Cycloheximide (Actidione, Upjohn Pty. Ltd., Rydalmere, New South Wales). Reconstituted by addition of 2 grm of powder to 100 ml d.w.; sterilized by filtration. Stored at -20°C in 1 ml aliquots.

(4) Deoxyribonucleic acid (sodium salt) from calf thymus gland (Calf thymus DNA) (B.D.H. Chemicals Ltd., Poole, Dorset U.K.). A stock solution of 0.2% DNA was held for use.

(5) Glucose (D Glucose, Univar, AJAX Chemicals, Sydney).

(6) Heart Infusion Broth-dehydrated (Difco Laboratories, Detroit, Michigan, U.S.A.).

(7) Hanks' balanced salt solution 10 x concentrate (Commonwealth Serum Laboratories (C.S.L.) Melbourne, Victoria).

(8) Hartley's Broth (Oxoid, Basingstoke, Hants., England). Powdered or solution.

(9) Lactalbumin Hydrolysate (ICN Pharmaceuticals, Ohio, United States).

(10) Methicillin (Metin, Commonwealth Serum Laboratories, Melbourne, Victoria).

(11) B nicotinamide adenine dinucleotide (B NAD) (Boehringer Mannheim GmbH, Germany). Reconstituted by addition of 1 grm of B NAD to 100 ml d.w.; sterilized by filtration. Stored at -20°C in 1 ml aliquots.

(12) Phenol Red (Univar, AJAX Chemicals, Sydney). Stock 0.4% solution prepared.

(13) Pig serum was obtained from EPP free pigs (ex. - N.P.R.U.) at slaughter. This was allowed to clot for 16 hours then centrifuged, heated at 56°C for 30 minutes. Sterilized by Seitz filtration (14 cm pads HP/EKS, Carlson-Ford, Sales Ltd., U.K.) and stored in 370 ml volumes at -20°C. Pig serum for the PPLO agar was obtained from C.S.L.

(14) Thallium acetate (B.D.H. Chemicals Ltd.). Prepared as a 1% (w/v) solution in d.w.; sterilized by filtration.

(15) Vancomycin (Vancocin, Lilly, Indianapolis, Indiana, U.S.A.). Reconstituted by addition of 100 ml d.w.; to 2 grms of Vancocin. Stored at -20°C in 1 ml aliquots.

(16) Difco PPLO

(17) Ampicillin (Austrapen, C.S.L.). Reconstituted by addition of 10 ml d.w.; to 500 mg Ampicillin. Stored at 4°C.

(18) Yeast Extract. Prepared by method of Herderschee (1963).

1 kg of fresh bakers yeast was kneaded until mixed thoroughly in 1 L of d.w. The suspension was then heated to 80°C while stirring regularly. The pH is adjusted to 4.5 as the solution approaches 80°C by adding concentrated HCL (AR grade). After holding at 80°C for 20 mins. the suspension is quickly cooled and the pH adjusted to 8.0 by adding 5N Na OH. Centrifuged for 10 mins at 10 000 r.p.m. to remove yeast cell ghosts. Decanted and filtered through Seitz (14 g) filter. Distributed into 20 ml aliquots and stored at -20°C.

Filtration. Unless stated otherwise, sterilization by filtration was performed using membrane filters (Millipore Corp., Bedford, Mass., U.S.A.), of 0.22 µm average pore diameter.

2. Media used in isolation and propagation

The liquid medium used for M. hyopneumoniae (MH medium) and M. hyorhinis consisted of Hanks' balanced salt solution 10 x concentrated, 43 ml; Hartley's broth (Cruikshank et al 1975), 100 ml; dehydrated heart infusion broth, 3.75 g ; lactalbumin hydrolysate, 5 g ; glucose, 10 g ; deoxyribonucleic acid, 0.2% W/V sodium carbonate aqueous solution, 10 ml; yeast extract (Herderschee 1963), 20 ml; serum from EPP free pigs (heated 56°C for 30 minutes, stored at -20°C) 370 ml;

phenol red 0.4% solution, 3 ml; B-nicotinamide adenine dinucleotide (B-NAD), 1% W/V aqueous solution, 14 ml; glass distilled water, 800 ml. After the pH was adjusted to 7.9 the medium was sterilized by Seitz filtration stored at 4°C and used within 4 weeks.

It was made selective prior to use by adding methicillin (150 µg/ml), vancomycin hydrochloride (200 ug/ml), bacitracin (150 µg/ml), thallium acetate (100 ug/ml) and cyc lohexamide (200 µg/ml). The selective pH medium had the same osmotic pressure as serum (7.4 atmospheres at 38°C) as recommended by Friis (1975). The final phenol red concentration was 15 µg/ml.

PPLO agar used for the isolation of M. hyorhinis contained Difco PPLO agar, 80 ml; yeast extract, 10 ml; pig serum, 20 ml; 0.2% deoxyribonucleic acid (B-DNA), 1 ml; 1% thallous acetate, 1 ml; 17% K_2HPO_4, 2 ml; 1% Ampicillin, 1 ml and 1% Cyclohexamide..

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PREVALENCE OF LESIONS OF ENZOOTIC PNEUMONIA OF PIGS IN SOUTH AUSTRALIA: AN ABATTOIR SURVEY

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Prevalences of enzootic pneumonia of pigs (EPP) reported in abattoir surveys in Australia range from 19% - 67% (Pullar 1949a; Edwards et al 1971; Norton 1976). During recent years there has been a world trend toward larger pig herds. As part of this trend it has been demonstrated that prevalence and extent of lesions of EPP increase with increasing herd size (Aalund et al 1976). In the past 7 years in South Australia the mean herd size has increased from 73 to 128 pigs and the number of herds with over 1 000 animals has doubled. Because of this association between prevalence of EPP and herd size, EPP may be an emerging disease in the South Australian pig industry. The prevalence of lesions of enzootic pneumonia at 3 major pig abattoirs in South Australia was recorded over a 12 month period in this survey.

During 1980 lungs were examined from 3 major abattoirs (1, 2 and 3), each slaughtering between 120 000 - 143 000 pigs annually. Approximately 60 lungs, predominantly from bacon-weight pigs, were sampled at one of these 3 abattoirs each fortnight so that each abattoir was sampled twice per season or 8 times in the 12 months. The sample consisted of 30 lungs which were held for later detailed examination (group A), and another 30 which were examined on the viscera table of the abattoirs (group B). To maximise the number of lines of pigs monitored, every second lung was sampled. Each abattoir was visited at least once on each day of the week that pigs were routinely slaughtered. This was done to avoid bias produced by larger units which slaughter on the same day each week. A total of 1 430 lungs were examined; 712 in group A and 718 in group B. Pneumonic

lesions were classified according to the criteria used by Pullar (1949a) and Edwards et al (1971); with typical active lesions being characterized by greyish swelling of lobules in the ventral aspects of the apical lobes and chronic lesions by atelectic plum coloured lobules.

Blood samples were collected concomitantly at slaughter from 412 of the 712 pigs in group A whose EPP lung lesion status was evaluated. Serum samples from these were tested for presence of antibody to Mycoplasma hyopneumoniae using the complement fixation (CF) method of Etheridge and Lloyd (1980).

Lesions typical of enzootic pneumonia in groups A and B were observed in 645 of 1 430 (45.1%) lungs examined during the 12 month survey. When calculated on a proportional basis with regard to the annual number of pigs slaughtered at each abattoir, the overall prevalence of EPP lesions in lungs at these 3 abattoirs was 47%. Prevalence varied on a seasonal basis, the highest prevalence of 51.1% being recorded in summer, and the lowest prevalence of 41.7% in winter, which was marginally short of statistical significance at the $p < 0.05$ level.

The prevalence of lesions at abattoirs 1, 2 and 3 varied significantly ($p < 0.001$) with 22.2%, 41.9% and 71.2% of lungs affected respectively.

The distribution of EPP lesions varied significantly ($p < 0.001$) with the highest prevalence of 83.8% being recorded in the right cardiac lobe followed by the left cardiac, right apical, intermediate left apical, right and left diaphragmatic lobes. Among group A lungs gross EPP lesions most frequently occurred in 1 or 2 lobes of each affected lung, comprising 12.6% and 10.1% of the total prevalence of lesions of 43.3% within this group.

The prevalence of pleurisy for group A lungs was 14.5% which did not vary significantly between seasons. The prevalence of pleurisy varied significantly between abattoirs ($p < 0.001$) with a prevalence at abattoirs 1, 2 and 3 of 6%, 17%, and 20% respectively. The association between pleurisy and pneumonia was highly significant ($p < 0.001$) in that pleurisy was present in 68 of 308 lungs with pneumonia but only in 35 of 404 normal lungs.

The presence of a positive CF titre to M. hyopneumoniae in serum of pigs with gross lesions typical of enzootic pneumonia was highly significant ($p < 0.001$). Where lungs were grossly affected with EPP, 44.9% of 156 pigs had serum samples that were CF positive. In 256 pigs with normal lungs 12.1% had serum samples that were CF positive.

The prevalence of lesions of EPP in South Australia of 47% was higher than that recorded in single abattoir surveys in New South Wales of 19% (Edwards et al 1971) and Queensland of 40% (Norton 1976). This prevalence is considered to be representative of the prevalence within the state as 85% of the annual number of pigs slaughtered occurs at these 3 abattoirs.

In this study the prevalence of lesions of EPP varied significantly between the 3 abattoirs sampled. Consequently if only abattoir 1 or 3 had been sampled, the prevalence recorded would have been a significant misrepresentation of the prevalence of EPP in South Australia. For this reason observations based on single abattoir surveys should not be extrapolated to larger state or country populations. Previously it has been suggested (Whittlestone 1973) that the prevalence of EPP lesions in Australia had declined during the preceding 20 years due to development of improved husbandry standards and disease awareness. Even if this observation, based on single abattoir surveys was correct, the current status of the disease in South Australia indicates a wide distribution with a high morbidity.

The highest seasonal prevalence of EPP in slaughter pigs was recorded in summer, when 51.1% of lungs were affected. This is consistent with previous reports of Pullar (1948) and Edwards et al (1971) who concluded that due to "Australian environmental influence, the disease spreads more rapidly amongst suckers and weaners in the autumn and winter months" causing a peak in prevalence in summer when these pigs are sold.

The prevalence of pleurisy of 14.5% in 712 group A lungs was also higher than the 3.8% reported by Edwards et al (1971). This may be explained by the highly significant association found between the presence of pleurisy together

with lesions of enzootic pneumonia. Predictably the prevalence of pleurisy followed the pattern set by prevalence of enzootic pneumonia at abattoirs 1, 2 and 3.

The distribution of lesions found between lung lobes supports previous observations (Pullar 1949b, Edwards et al 1971) which have attributed this phenomenon to the different anatomical structure of the major airways. The classification of gross lung lesions as EPP was supported by the relationship between the presence or absence of lung lesions and CF titres in 412 corresponding serum samples. The results also indicate that detectable CF antibody frequently dissappears before resolution of lesions. However, the converse also appears to occur, but less frequently.

Results of the survey confirm that EPP and pleurisy are widespread in slaughter pigs in South Australia. At a time when herd size continues to increase this result gives little support to the notion that this trend has been accompanied by better measures to control pneumonia.

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ENZOOTIC PNEUMONIA OF PIGS II: EPIDEMIOLOGY IN 31 HERDS IN SOUTH AUSTRALIA

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Summary: Environmental and managemental conditions of 15 herds with a high-prevalence (> 70%) of enzootic pneumonia of pigs (EPP) at slaughter were compared with 16 herds with a low-prevalence (< 30%) to determine factors commonly predisposing pigs to EPP in South Australia.

Comparisons were made of herds having > 100 sows (fully intensive units) and also small herds having 20-70 sows (sideline units). Half the herds were visited in summer and half in winter to detect seasonal factors.

In small herds factors commonly found associated with a high-prevalence of EPP were a higher gilt replacement rate ($p < 0.1$), purchase of pigs for finishing ($p < 0.07$), larger numbers of pigs per shed section ($p < 0.001$), larger group sizes ($p < 0.01$) and draughty farrowing and weaner accommodation ($p < 0.01$). In large herds factors associated with a high-prevalence were higher pen stocking rate ($p < 0.05$) and airspace stocking rate ($p < 0.05$) and higher atmospheric ammonia levels in summer ($p < 0.1$). EPP was also associated with reduced total

production as substantially more high-prevalence herds marketed pigs at a lighter bodyweight ($p < 0.06$) and older age ($p < 0.06$).

Introduction

The aetiological or predisposing role played by environmental and management factors in complex respiratory disease syndromes of pigs are well documented (Gordon 1963; Backstrom 1973; Lindqvist 1974; Mickwitz et al 1975; Aalund et al 1976; Whittlestone 1976; Flesja et al 1978; Backstrom and Bremer 1978; Muirhead 1979). By using post-mortem observations from many herds these authors showed associations between a variety of factors and enzootic pneumonia of pigs (EPP). Many factors including shed numbers, stocking density, ammonia levels, herd size, continuity of stocking and purchase of stock for finishing have been demonstrated consistently to predispose to infection.

This investigation into the epidemiology of EPP in South Australia was motivated by the demonstration of widespread EPP infection in herds (Pointon and Sloane 1983); the potential for EPP to become an emerging disease in expanding herds (Aalund et al 1976) and the need to identify common predisposing factors to assist in disease control.

The purpose of this study was to determine which factors were commonly involved in predisposing pigs to enzootic pneumonia in South Australia by comparing environmental components between groups of pigs from herds with high and low-prevalences of EPP.

Materials and Methods

Selection of Piggeries

Piggeries were selected on the basis of the prevalence of EPP obtained by monitoring lungs at three abattoirs, during the month prior to the herd visits. Prevalence of lesions typical of EPP were recorded for each line of pigs slaughtered during several days at each abattoir. Monitoring was concluded when sufficient herds were identified which fulfilled the selection criteria. In the case of the smaller herds which generally marketed insufficient pigs at one time to obtain an accurate prevalence of EPP, at least one subsequent line of pigs was monitored.

Two herd categories were established, based on criteria defined by Muirhead (1979). High-prevalence herds were those with a prevalence of lesions in 70% of pigs or greater, while low-prevalence herds were those with 30% or less affected (Table 1). Herds within these pneumonia categories were stratified on the basis of production emphasis, with a division into fully intensive units being herds with > 100 sows and sideline units with between 20-70 sows. These methods were adopted to allow for any influence that increasing herd size could exert on environment or management within the herds. By choosing herd groups with a large difference in the prevalence of EPP lesions, the greatest potential was created for the detection of differences in each factor between high and low-prevalence herd groups. To assess the importance of factors which are influenced by seasonal conditions, a similar number of piggeries were visited in late summer and mid winter of 1981. Herds visited in late summer were excluded from the winter sample due to implementation of management changes aimed at controlling EPP.

Production in all herds was of a continuous nature. All growing pigs in the large herds were intensively housed; in all small piggeries the growing phase included an intensive housing period of at least 8 weeks.

Evaluation of Piggeries

Piggery environment is established by a large number of components whose interactions under field conditions are difficult to estimate and vary between piggeries. Due to the large number of herds required for a study of these interactions, individual factors of biological interest and easy to define were selected for study.

A questionnaire was designed to be answered by the piggery owner or manager in conjunction with one of the researchers during a visit. Criteria used for assessing each factor were standardized by the two researchers together visiting several herds prior to the study. Factors assessed appear in Table 2. About 60 variants in housing environment and management were recorded; however after preliminary study only a limited number of these were selected for detailed analysis.

The number of sows culled during the previous twelve months was recorded and annual sow culling rate calculated.

The criteria for pigs purchased for finishing were all pigs introduced to the property from markets or from other breeders at an age from "slips" (weaners from 5-10 weeks old) to unfinished bacon, for the sole purpose of finishing for slaughter.

Procedures used to reduce the amount of dust generated from the feed included the addition of tallow or oils to the feed rations and

the use of pelleted feed for growing pigs.

In-feed antibiotics recorded were those commonly used to control EPP and its secondary bacterial invaders and included the tetracyclines, tylosin, lincomycin and sulphadimidine.

The average market age and liveweight of pigs were obtained from herd records where possible. In herds in which individual pigs were not identified the age of marketing was estimated from basic herd records. To partially overcome this inaccuracy, the data was stratified (Figure 1).

The total number of pigs in a shed section (ie. undivided shed or separate air space) for any age group included all pigs (including other age groups if present). Group size (Table 4a) was taken as the number of pigs per pen.

Pen area stocking density was calculated by dividing the total weight of pigs of a particular age group (weaners, growers or finishers) by the total area which they occupied at that time. Airspace stocking density was calculated similarly, but used the total mass of pigs in the shed section divided by its volume (ie. if weaners, growers and finishers were present in the same shed, the airspace stocking density was the same for each age group).

Weaner pen design was assessed under four categories, being (1) pens with completely solid floors, (2) partially slatted meshed pens (3) pens above growers or finishers and (4) multi-tiered weaner crates.

Potential for draughts was assessed by presence of obvious faults, eg. holes in walls, incompleted walls, poorly fitted shutters and

absence of flaps at the end of dung channels.

Ventilation methods were classified as either fan forced or natural with side shutters.

The siting of all maximum-minimum thermometers and hygrometers in piggeries prior to the planned recording periods ensured that all piggeries recorded conditions in weaner and grower areas during the same two weeks in summer and winter. Equipment was suspended 0.5 metres above weaner pens and 1 metre above grower pens in all piggeries. All equipment was tested and calibrated prior to each recording period and again upon siting in each new piggery.

Ammonia levels were recorded at normal morning shutter opening time, allowing estimation of overnight accumulation within the finisher unit. Three readings were obtained over the area occupied by finishers. Readings were obtained using a multigas detector* 2 cm above the floor

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* Drager (Aust.) Pty. Ltd., Adelaide, South Australia.

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surface at the junction of the sleeping and dunging area. The temperature and relative humidity at each recording site were also recorded.

Statistical Analysis of Data

The categorical nature of the management data and some of the environmental data was suitable for contingency table analysis (Bishop et al 1975; Plackett 1981). For each management or environmental factor the hypothesis tested was of independence between the factor and the prevalence of EPP.

The Mantel-Haenszel test (Landis et al 1978) simultaneously tests for independence between a t-level treatment factor (eg. pen space stocking density) and an r-level response factor (eg. high or low-prevalence), in each of S-strata (eg weaners, growers, finishers). The independence of the factors in Tables 4a and 4b were also tested with the Mantel-Haenszel test.

Temperatures, relative humidity and ammonia levels were analysed with analysis of variance or linear regression.

Results

Comparison of management data between high and low-prevalence herd groups is presented in Table 3. The small high-prevalence herds had higher sow culling rates ($p < 0.1$) with 7 of 8 herds (85.5%) culling at a rate of > 15% while 4 of 8 low-prevalence affected small herds culled at < 15%. Similarly, more high-prevalence small herds purchased pigs for finishing ($p < 0.07$). Pigs were purchased equally from markets or direct from breeders. Other factors were not significantly different between herd groups.

Analysis of the average market age and bodyweight of pigs from all herds (Figure 1) revealed that more high-prevalence herds had pigs which were marketed at older age ($p < 0.06$) and lower liveweight ($p < 0.06$) than pigs from low-prevalence herds.

Analysis of housing data (Table 4a) for weaners, growers and finishers for the 2 herd groups revealed that significantly more high-prevalence small herds had more pigs per shed section ($p < 0.001$) and

more pigs per group ($p < 0.01$) than low-prevalence herds. Significantly more high-prevalence large herds stocked pens ($p < 0.05$) and airspace ($p < 0.05$) more densely than low-prevalence large herds (Table 4b).

Pen design for weaners did not vary substantially between high-prevalence and low-prevalence herd groups. Weaners were most frequently housed in pens on the floor. Partial or full slats or mesh were used in 14 herds and totally solid floors were used in 7 herds. In 7 herds weaners were housed in partially meshed pens over growers and in 3 herds multi-tiered weaner crates were used.

Mechanical ventilation was used in only one weaner unit with all other age groups in all herds being naturally ventilated.

Average minimum daily temperatures and diurnal temperature fluctuation for each herd group in summer and winter did not vary significantly between herd groups (Table 6). The average minimum temperature in all small and large herds in winter was 10.7°C and 14.4°C respectively. The diurnal fluctuation was significantly greater in the small low-prevalence herds in summer ($p < 0.01$) and in the large high-prevalence herds in winter ($p < 0.1$) for growers only. The average diurnal fluctuations for weaners in winter in large herds was 7.9°C and in small herds was 7.8°C.

Minimum temperatures in weaner houses in winter were highly dependant on the potential for draughts in both small ($p<0.01$) and large herds ($p < 0.05$) (Table 7).

The average relative humidities (3.00 p.m.) for all herd groups in summer and winter are presented in Table 8. In the small herd groups more

high-prevalence herds had higher relative humidities in weaner sections in summer ($p < 0.1$) while in the large herd groups more low-prevalence herds had higher relative humidities in weaner sections in winter ($p < 0.05$).

The average ammonia concentration was higher in more high-prevalence large herds in summer than in low-prevalence herds ($p < 0.1$). The average ammonia concentration in summer of all the large high-prevalence herds was 11.3 ppm compared with 5.9 ppm in large low-prevalence herds (table 9). Ammonia concentration of all individual recordings (93) was found to be dependant on the temperature at the recording site ($r = 0.43$, $p < 0.05$) (Figure 2).

Discussion

The study demonstrated significant differences in environmental and managemental factors between high- and low-prevalence herd groups, within each herd size category. In the small herds a high-prevalence of enzootic pneumonia at slaughter was associated with a high sow culling rate, purchase of pigs for finishing, larger numbers of pigs per shed section, larger group sizes and draughty volume of weaner accommodation. In herds with > 100 sows, factors associated with a high-prevalence of EPP were higher stocking densities of pen space and airspace and high atmospheric ammonia levels in summer. These factors commonly associated with a high-prevalence of EPP in South Australia are the same as those determined to be of importance in similar studies in the northern hemisphere (Lindqvist 1974; Aalund et al 1976; Backstrom and Bremer 1978).

The finding that different factors were important in the different herd size groups clearly justifies the stratification of herds according to size in the analysis. This finding may be explained by changes in emphasis in environmental and managemental conditions which occur as herd size increases. For example, larger herds were found to depend entirely upon the production of their own piglets, while the practice of purchasing pigs for finishing was a common practice among the smaller piggeries. Similarly with the increased financial pressure of a high total cost in construction of larger piggeries, shed population and stocking density is increased (Table 4) in an effort to reduce the cost per unit produced.

It is of particular interest to note that no factor in common was determined to be of importance for both small and large herd groups. While shed section and group numbers were significant factors for the smaller herds this trend was also supported in the larger herds, where they may have assumed significance if more herds had been studied. The emergence of pen and airspace stocking rate as significant factors in the low number of large herds studied, may indicate that stocking rates exert a greater effect than pig populations in large herds. The possibility of factor dominance is supported by results of Lindqvist (1974) who indicated that shed volume may be a more dominant factor than pen lying space. Reasons for the low-prevalence of EPP in large units with shed section populations comparable with or greater than found in small high-prevalence herds, is not clear, but may result from interaction with other factors, eg. efficiency of ventilation and temperature control by the operator.

The study indicates that production in high-prevalence herds was significantly decreased due to depressed growth rate. More high-prevalence herds had pigs which were marketed at an older age and a lighter bodyweight

when compared with low-prevalence herds. Estimates of quantitative losses are unavailable from this study because the effect of EPP varied between herds, probably because of the combined effects of other diseases and environment and management differences.

The aetiological significance of each factor in South Australia is considered in the following discussion.

Annual sow culling rate The importance of this factor in small herds is supported by observations of Goodwin (1965) who demonstrated a reduced prevalence of EPP lesions with age. This implies that infection is most frequently transmitted from gilts and second litter sows to piglets (Muirhead 1979). Therefore by increasing the percentage of gilts (a common practice to speed genetic improvement), the prevalence of EPP in breeding herds is increased along with increased morbidity among suckling piglets. The reasons for this factor being of apparent influence in the small herds but not large herds may be the spasmodic nature of culling in smaller herds leading to differences, as compared with the routine culling programme operating on most large herds.

Purchase of pigs for finishing The purchase of pigs for finishing has long been recognised as a source of infection for herds (Fogedby 1967 - cited by Lindqvist 1974; Aalund et al 1976; Backstrom and Bremer 1978). Under Australian conditions pigs are either purchased from country markets or direct from breeders as weaners, both methods involving stress of transport and sudden changes in environmental conditions. This form of trade ensures introduction of a wide range of bacteria from many sources into susceptible herds.

Pen space and airspace stocking density In large piggeries, stocking rates in high-prevalence herds were higher than those in low-prevalence herds in all age groups of growing pigs. Lindqvist (1974) and Backstrom and Bremer (1978) also found a similar relationship but could not determine if pen space or air volume was the major factor. In this study both factors, when treated individually, influenced the prevalence of EPP. Reduced pen space per pig would increase the potential for contact spread and reduced air space would cause an increase in atmospheric bacterial contamination. These in turn would increase the challenge dose to susceptible pigs continually added to the population. It was observed in all high-prevalence large herds that finisher pen stocking rate was > 100 kg/m^2, whereas 37% of low-prevalence herds stocked at < 100 kg/m^2. Similarly an airspace stocking density of 20 kg/m^3 for finishers in large high-prevalence herds appeared to be a significant threshold level beyond which pigs are more likely to develop severe pneumonia. While these values were chosen arbitrarily for the purpose of statistical analysis, they serve as a guide to establishing maximum stocking densities under current housing standards.

Potential for drafts Potentially draughty farrowing and weaner units were more common among high-prevalence than low-prevalence small herds ($p > 0.01$, Table 5). It was found that the minimum temperatures in weaner units (Table 6) were dependant upon the potential for draughts in both small ($p > 0.01$) and large herds ($p > 0.05$) (Table 7). The combination of low temperatures with draughty conditions would lead to a "chill" situation, causing reduced resistance to infection through cilial damage. This would lead to widespread infection among weaners in winter, which would cause an increased prevalence of lesions to be recorded in summer when these pigs were marketed (Pullar 1948; Edwards et al. 1971).

Atmospheric ammonia More large high-prevalence herds were associated with higher levels of ammonia in summer ($p < 0.1$). The average level for large high-prevalence herds in summer was 11.3 ppm compared with 5.9 ppm in low-prevalence herds. These results are supported by those of Mickwitz et al (1975), Kovacs et al (1967 - cited by Lindqvist 1974) and Curtis (1980 - cited by Backstrom and Curtis 1982) who reported high levels of ammonia in association with pigs with clinical pneumonia and concluded that ammonia levels should be kept below 10 ppm.

Several controlled experimental studies have demonstrated the role of atmospheric ammonia in respiratory infections of poultry (Sato et al 1974; Quarles and Kling 1974), rats (Brodersen et al 1975) and pigs (Drummond et al 1978; Drummond et al 1981). The effect observed has been reduced clearance of inhaled bacteria and the greater multiplication of respiratory pathogens. The prevalence of pneumonia in rats was directly correlated with an increase in environmental ammonia concentrations when exposed to levels commonly encountered in piggeries (20 ppm).

As higher ammonia levels were most frequently recorded in summer it was postulated that levels may be associated with temperature within the pig unit. Further analysis (Figure 2) revealed that this was the case ($r = 0.43$, $p < 0.05$). As house temperatures in winter are often maintained at the expense of ventilation (Muirhead 1979), this latter association between factors assumes greater significance. It therefore appears that ventilation in summer in many herds is inadequate; there is also a high potential for problems to develop in winter as seen in some herds where minimum ventilation is not provided when units are closed to conserve temperature.

Minimum daily and diurnal temperatures The average minimum daily temperatures (Table 6) for weaner accommodation in winter in both small (10.7°C)

and large herds (14.4°C) were well below current recommended levels of 25°C for 3 week old weaners and 21°C for 6 week old weaners (Wright 1981). While they were not significantly different between herd groups, it is likely that low minimum temperatures are important determinants of EPP when combined with drafty conditions.

The diurnal temperature range (Table 6) was also larger than the recommended limit of 5°C (Baxter 1969). Wide diurnal variation predisposes to enteric as well as pneumonic problems (Cargill 1981). The diurnal temperature fluctuation was significantly higher for grower pigs in the large high-prevalence herds in winter. This result may reflect a decreased emphasis placed on temperature control for the older pigs.

Relative humidity (RH) Levels of relative humidity recorded in this study at 3.00 p.m., when temperatures were near the daily maxima, were well below those known to assist in control of EPP. Gordon (1963) reported that RH > 95% in conjunction with temperatures > 27°C were found to be associated with lower levels of EPP. Tonks et al (1972) and Whittlestone (1976) have reported that in conditions of high temperature and humidity atmospheric droplets sediment at a faster rate, decreasing atmospheric bacterial contamination.

In a review of the effect of RH, Whittlestone (1976) stated that midrange RH was more detrimental to the survival of mycoplasma organisms than a RH of either 25% or 95%. In this study the average RH for each herd group in each season was within mid-range values. Clearly, organisms were excreted at a continually high rate in the severely affected herds to maintain the high incidence of infection.

The study highlighted many environmental and managemental factors commonly involved in predisposing pigs to EPP in South Australia. As

these factors are the same as those identified in similar studies in the northern hemisphere, measures developed there should be evaluated in the control of EPP in South Australia.

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Table 1

Abattoir Prevalence (%) of Lesions of EPP for Each Herd Visited

(31 herds)

Season	Herd Size (Sows)	High Prevalence (> 70%)		Low Prevalence (< 30%)	
Summer	20-70	79	(17)	8	(10)
		84	(13)	7	(7)
		72	(18)	6	(8)
	> 100	75	(13)	28	(12)
		93	(8)	25	(13)
		72	(18)	13	(9)
		81	(13)	10	(8)
		72	(18)		
Winter	20-70	75	(18)	28	(16)
		71	(13)	4	(8)
		71	(18)	14	(14)
		71	(16)	6	(11)
				22	(14)
	> 100	85	(5)	19	(10)
		95	(7)	22	(12)
		71	(13)	30	(8)
				27	(13)

Confidence interval ± ( )

Table 2

Information Collected by the On-farm Questionnaire and Measurements

Management Data	• Age of herd (since establishment)	
	• Annual sow culling rate	
	• Purchase of pigs for finishing	
	• Use of anti-dust agents in feed	
	• Presence of antibiotics in feed	
	• Average market age	
	• Average market weight	
Age Group Data - Weaners (W), Growers (G), Finishers (F)		
	• Total pigs in section	- W, G, F
	• Group size	- W, G, F
	• Pen area stocking density	- W, G, F
	• Airspace stocking density	- W, G, F
	• Pen design	- W
	• Potential for draughts	- W, Farrowing Unit
	• Type of ventilation	- W, G, F
Measurements - for 1 week	• Maximum and minimum temperatures	- W, G
	• Relative humidity (at 3.00 p.m.)	- W, G
	• Ammonia - 3 sites/herd	- F

Table 3

Comparison of Management Data between High- and Low-Prevalence Herd Groups

Factors	Herd Size	Significance of Difference Between Herd Groups
Age of herd	All	NS
Annual sow culling rate	20-70	*
	> 100	NS
Purchase of pigs for finishing	20-70	**
	> 100	NS
Use of anti-dust agents in feed	20-70	NS
	> 100	NS
Presence of anti-biotics in feed	20-70	NS
	> 100	NS

NS Not Significant * $P < 0.1$ ** $P < 0.07$

Table 4(a)

Statistical Difference in Housing Data between the Herd Groups

Total Pigs per Section

:d :e	Herd Pneumonia Status	Weaner Unit			Grower Unit				Finisher Unit			Significan of Differenc
		<100	100-199	>200	<100	100-199	200-399	>400	<400	400-799	>800	
70	High-Prevalence	1	2	4	0	2	3	2	0	5	2	***
	Low-Prevalence	3	4	1	3	4	1	0	6	1	0	
		<300	300-499	>500	<400	400-799	>800		<400	400-799	>800	
00	High-Prevalence	2	3	3	1	4	3		2	3	3	NS
	Low-Prevalence	2	4	2	3	3	2		3	3	2	

Average Group Size

		Weaner Unit			Grower Unit			Finisher Unit			
		<15	15-24	>25	<11	11-16	>17	<10	10-11	>12	
70	High-Prevalence	4	0	3	4	0	3	3	1	3	**
	Low-Prevalence	6	2	0	4	4	0	3	4	1	
		<15	15-29	>30	<12	12-17	>18	<11	11-12	>13	
00	High-Prevalence	4	2	2	3	2	3	3	3	2	NS
	Low-Prevalence	2	4	3	3	3	2	3	4	1	

** P < 0.01 *** P < 0.001

Table 4(b)

Statistical Difference in Housing Data between the Herd Groups

Pen Space Stocking Density (kg/m^2)

:d :e	Herd Pneumonia Status	Weaner Unit			Grower Unit			Finisher Unit			Significan of Differenc
		<25	25-39	>40	<40	40-82	>83	<67	67-124	>125	
70	High-Prevalence	3	2	2	3	1	3	2	3	2	NS
	Low-Prevalence	2	2	4	3	4	1	4	2	2	
		<40	40-49	>50	<50	50-82	>83	<100	100-124	>125	
00	High-Prevalence	0	2	6	0	4	4	0	4	4	*
	Low-Prevalence	2	1	4	2	1	5	3	3	2	

Airspace Stocking Density (kg/m^3)

		Weaner Unit		Grower Unit		Finisher Unit		
		<10	>10	<20	>20	<20	>20	
-70	High-Prevalence	2	3	4	1	4	1	NS
	Low-Prevalence	4	2	2	3	1	4	
		<10	10-19	>20	<20	20-29	>30	<20	20-29	>30	
100	High-Prevalence	0	4	4	2	4	2	1	7	0	*
	Low-Prevalence	2	4	1	4	2	1	3	2	2	

* $P < 0.05$

Table 5

Potential for Draughts when Shutters Closed

rd ze	Penumonia Status	Farrowing House		Weaner Unit		Significance of Difference
		Present	Absent	Present	Absent	
-70	High-Prevalence	6	1	6	1	**
	Low-Prevalence	3	5	2	6	
100	High-Prevalence	6	1	0	8	NS
	Low-Prevalence	3	5	2	6	

** $P < 0.01$

Table 6

Temperatures

(Average of 7 Consecutive Days)

Age Group	Season	20-70 Sows		> 100 Sows	
		High-Prevalence	Low-Prevalence	High-Prevalence	Low-Prevalence
Average Minimum Daily Temperature					
Weaners	Summer	18.4	16.2	17.9	18.4
	Winter	9.1 (10.7)	11.8	14.0 (14.4)	14.8
Growers	Summer	19.4	16.7	18.6	18.4
	Winter	8.7	10.5	12.5	14.5
Average Diurnal Temperature Range					
Weaners	Summer	9.9	13.9	10.7	10.1
	Winter	7.9	7.1	8.7	8.1
Growers	Summer	8.6	12.5	9.8	9.3
	Winter	7.6	7.7	10.3	6.9

( ) Average for all small and large herds respectively

Table 7

Association between Minimum Weaner House Temperatures and Potential for Draughts

Season	Weaner House Minimum Temperatures (Average of 7 consecutive days) Herd Size	
	20-70 Sows	> 100 Sows
Summer	NS	NS
Winter	**	*

NS Not Significant * $P < 0.05$ ** $P < 0.01$

Raw data presented in tables 6 and 7

Table 8

Average Relative Humidity (3.00 p.m.)

Age Group	Season	20-70 Sow Herds		> 100 Sow Herds	
		High-Prevalence	Low-Prevalence	High-Prevalence	Low-Prevalence
Weaners	Summer	53	44	53	52
	Winter	78	73	63	70
Growers	Summer	53	43	50	54
	Winter	73	67	72	69

Table 9

Average Overnight Ammonia Accumulation (ppm)
on Each Herd Group in Each Season

Herd Size	Season	High-Prevalence	Low-Prevalence
20-70	Summer	7.7 (3-20)	9.8 (1-22)
	Winter	4.0 (1-10)	7.1 (3-21)
> 100	Summer	11.3 (2-35)	5.9 (3-22)
	Winter	5.8 (1-30)	5.4 (1-18)

( ) range in ppm

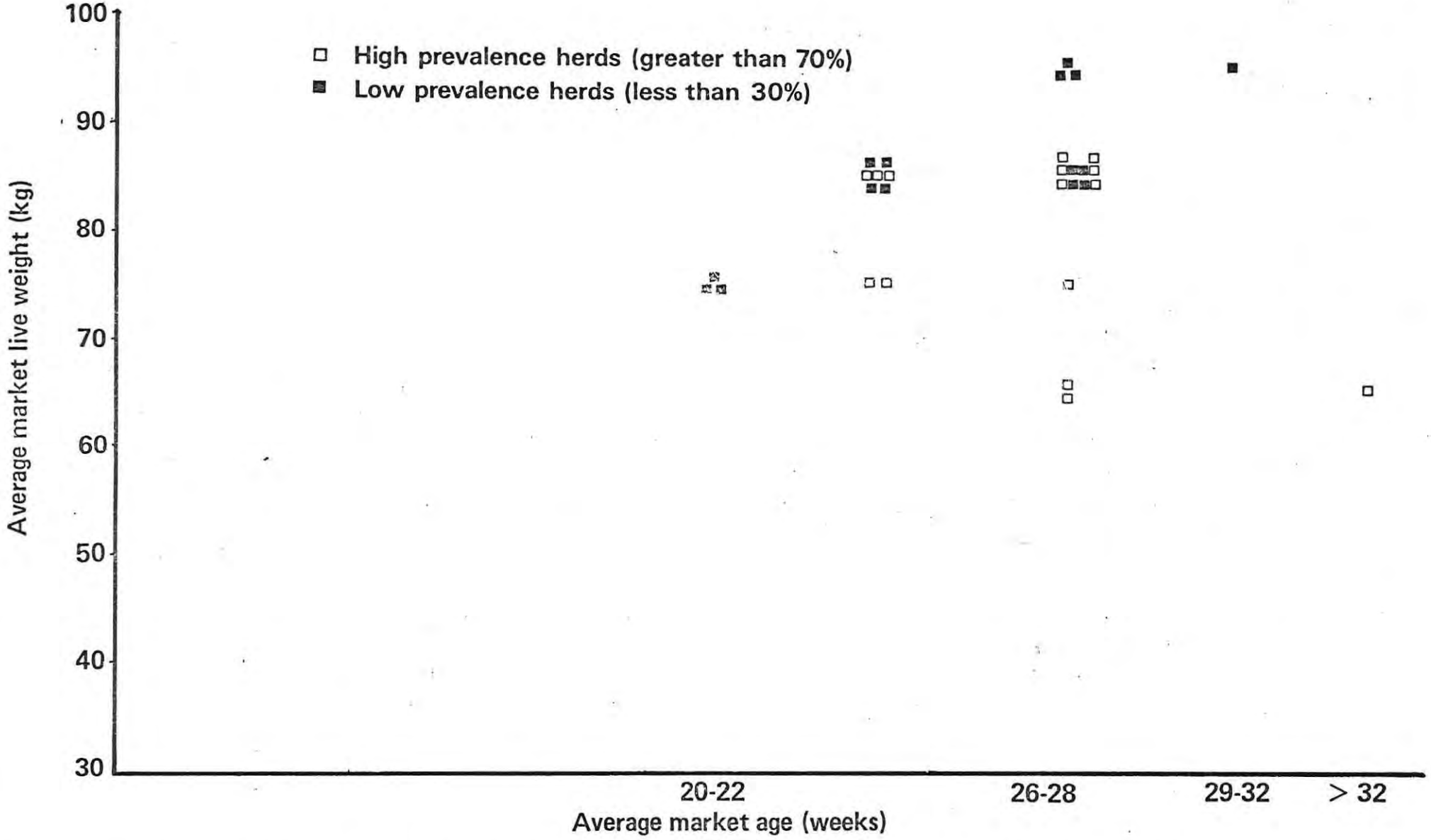

FIGURE 1: AVERAGE MARKET AGE AND LIVEWEIGHT OF 31 PIG HERDS IN SOUTH AUSTRALIA.

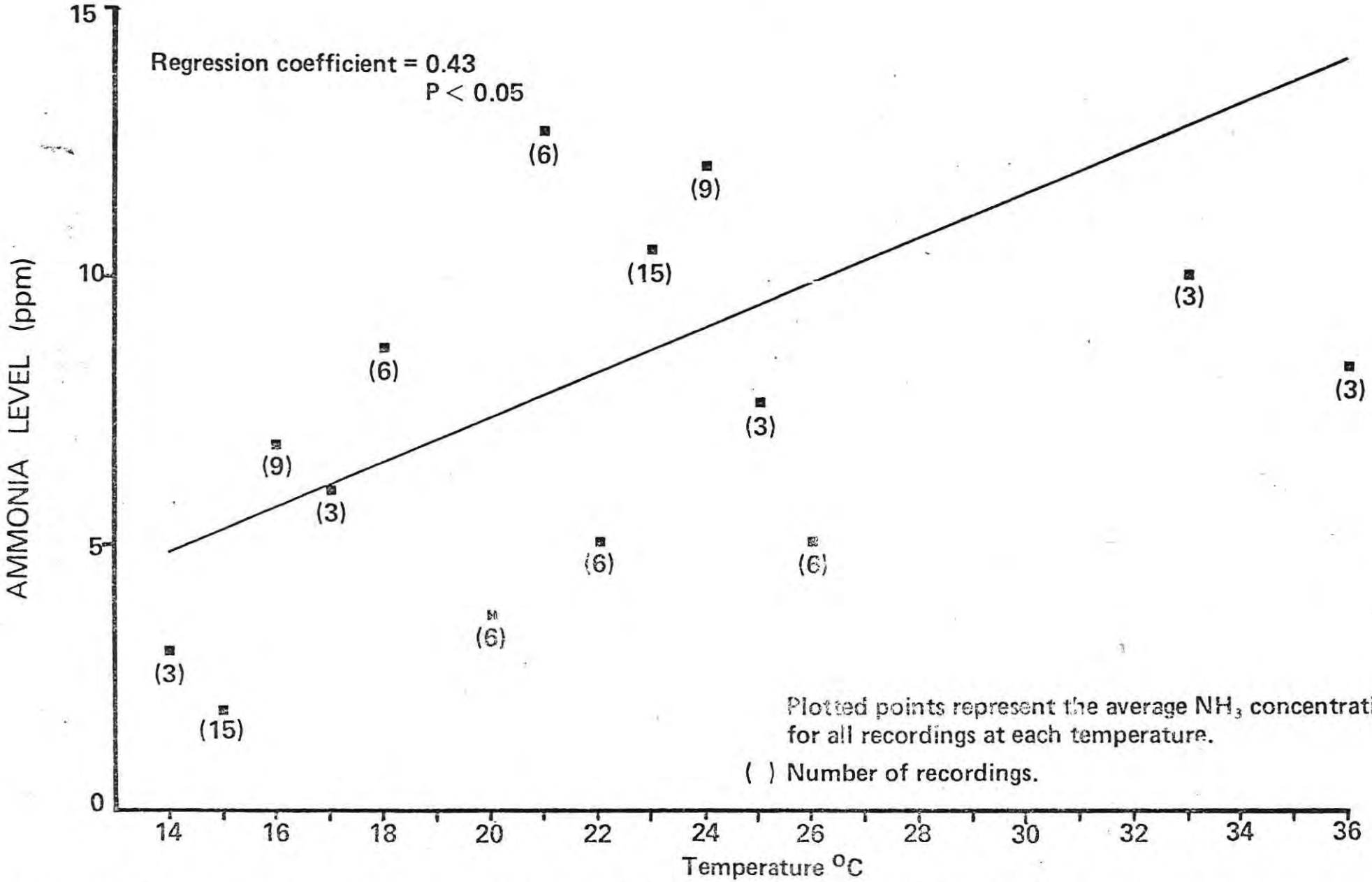

FIGURE 2: ASSOCIATION BETWEEN ATMOSPHERIC AMMONIA LEVELS AND SHED TEMPERATURES.