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AN INVESTIGATION OF THE HETEROGENEITY
OF ISOLATES OF *Mycoplasma ovipneumoniae*
USING RESTRICTION ENDONUCLEASE ANALYSIS.

A THESIS PRESENTED IN PARTIAL FUFILMENT OF
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ABSTRACT

Previous studies of *Mycoplasma ovipneumoniae* by Restriction Endonuclease Analysis (REA) (Mew, 1982) indicated that the species shows exceptional heterogeneity as compared to other species of pathogenic Mycoplasmas. This thesis further investigates this heterogeneity.

To get confirmation of the heterogeneity of *M. ovipneumoniae*, sixty isolates derived from three sheep on each of twenty farms, were examined by REA. All twenty independent isolates (i.e. isolates originating from sheep on different farms) gave REA patterns that were markedly different, with at most, only 5% of bands in common. Isolates from sheep on the same farm were found to be either indistinguishable, similar (i.e. at least 95% of bands in common) or markedly different (i.e. less than 5% of bands in common). Having confirmed the heterogeneity of *M. ovipneumoniae* isolates from sheep on different farms further study was directed at providing an explanation for this heterogeneity.

The stability of the *M. ovipneumoniae* genome was investigated by serial passage of a multiply cloned isolate *in vitro*. Three REA patterns, A, B and C (pattern A was the original pattern) were observed. These pattern changes were non-random in that they were reversible. Thus it appears that an internal rearrangement system is present in *M. ovipneumoniae*. No non-reversed REA pattern changes were seen. It was concluded that the pattern changes seen after serial *in vitro* passage were minimal, and that genomic instability could not explain the heterogeneity seen in *M. ovipneumoniae*.

Changed REA patterns must represent DNA changes which in turn may mean changes in proteins. To attempt to detect protein changes, 3 clones which showed patterns A, B and C respectively were examined by SDS-Polyacrylamide gel electrophoresis of total cellular proteins. No differences were detected. There remains the possibility that antigenic changes occurred

which might not be demonstrable by this method.

A second possible explanation for the heterogeneity seen in *M. ovipneumoniae* is that frequent genetic interchange between initially distinct REA strains might result in the generation of many new REA types that differ markedly from both parental strains. Three approaches were taken to investigate this possibility:

1. "Classical crosses" detected by antibiotic resistance markers.
2. Mixtures of two cultures of *M. ovipneumoniae* with different REA patterns were mixed and propagated together.
 - (a) Clones were selected from a mixed culture after it had been passaged for about 30 generations and examined by REA.
 - (b) "Presumptive recombinants", i.e. clones of *M. ovipneumoniae* which were resistant to two antibiotics, recovered from mixtures of singly resistant clones were examined by REA.
3. *M. ovipneumoniae* was examined for the presence of extra-chromosomal DNA which, if present, could facilitate genetic interchange.

Using these three approaches, we were unable to demonstrate genetic interchange in *M. ovipneumoniae* so it is unlikely that genetic interchange accounts for the considerable heterogeneity seen in the species.

It was concluded that the heterogeneity seen in the species is due to the presence of a large number of strains that are genetically stable with respect to REA, which have evolved over a long time period and which are independantly maintained.

We estimated the minimum number of strains of *M. ovipneumoniae* that must exist in a population so that when 29 independent isolates are examined, all will be different. With 95% certainty, this minimum number is 150.

The possibility that at least 150 *M. ovipneumoniae* strains could be maintained in New Zealand was discussed. By applying general epidemiological principles to *M. ovipneumoniae*, we concluded that many more than 150 could be independently maintained.

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CONTENTS

	<u>Page</u>
TITLE PAGE	i
ABSTRACT	ii
ACKNOWLEDGEMENTS	v
LIST OF CONTENTS	vi
LIST OF FIGURES	xi
LIST OF TABLES	xiv
INTRODUCTION	1
CHAPTER 1: <u>HISTORICAL REVIEW</u>	3
1.1 Chronic Non-Progressive Pneumonia	3
1.11 Occurrence of Chronic Non-Pro- gressive Pneumonia	3
1.12 Economic importance of Chronic Non- Progressive Pneumonia	3
1.13 Aetiology of Chronic Non-Progressive Pneumonia	4
1.14 Transmission studies with Chronic Non-Progressive Pneumonia	5
1.2 Restriction Endonucleases	7
1.21 Discovery of characterisation of restriction endonucleases.	7
1.22 Class 1 Restriction Endonucleases	8
1.23 Class 2 Restriction Endonucleases	8
1.24 Nomenclature of Restriction Endon- ucleases	9
1.25 Specificity of class 2 Restriction Endonucleases	10
1.26 Applications of class 2 Restriction Endonucleases	10
a) Restriction Endonuclease mapping of DNA	10
b) Cloning of genes	11
c) Classification and Identification of Micro-organisms	12

CHAPTER 2: <u>EXAMINATION OF INDEPENDANT ISOLATES</u>	
<u>OF <i>Mycoplasma Ovipneumoniae</i> BY</u>	
<u>RESTRICTION ENDONUCLEASE ANALYSIS</u>	
<u>(REA)</u>	18
2.1 Introduction	18
2.2 Materials and Methods	18
2.21 Origin and Isolation of <i>M.</i>	
<i>ovipneumoniae</i>	18
2.22 Identification of the Mycoplasma	
isolates	19
2.23 Storage of stock culture of <i>M.</i>	
<i>ovipneumoniae</i>	19
2.24 Cloning of <i>M. ovipneumoniae</i>	20
2.25 Restriction Endonuclease Analysis	
(REA) of <i>M. ovipneumoniae</i>	20
a) Preparation of <i>M. ovipneumoniae</i> cells	
for DNA extraction	20
b) DNA Extraction	20
c) Estimation of the concentration of	
DNA in the extract	21
d) Spectrophotometry	22
e) Fluorimetry	22
f) Digestion of the DNA with <i>EcoRI</i>	23
g) Gel Electrophoresis of the DNA samples	25
h) Photography of the gel	26
2.26 Comparison of REA patterns	26
2.27 Gel precipitin tests	26
2.3 Results	28
2.31 Results of Identification tests	28
2.32 Results of REA of Independant isolates	
of <i>M. ovipneumoniae</i>	30
2.4 Discussion	37
CHAPTER 3: <u>STABILITY OF THE <i>M. ovipneumoniae</i> GENOME</u>	39
3.1 Serial passage of <i>M. ovipneumoniae</i> in	
<i>vitro</i>	39

3.11	Introduction	39
3.12	Materials and Methods	40
	a) Cloning of <i>M. ovipneumoniae</i> before serial passage.	40
	b) Analysis of the clone before passage	41
	c) First passage stage	41
	d) Labelling of cultures	41
	e) Second passage stage	42
	f) Third passage stage	42
3.13	Results	42
3.14	Conclusion	53
3.2	Reproducibility of REA pattern differences observed after the serial passage <i>in vitro</i> of <i>M. ovipneumoniae</i>	54
3.21	Introduction	54
3.22	Materials and Methods	54
	a) REA of duplicate cultures	54
	b) Effect of increased enzyme concentration on REA patterns	54
3.23	Results	55
	a) REA of duplicate cultures	55
	b) Effect of increased enzyme concentration on REA patterns	55
3.24	Conclusion	58
3.3	SDS Polyacrylamide gel electrophoresis (SDS-PAGE) of the proteins of <i>M. ovipneumoniae</i> clones which showed different REA patterns after serial passage <i>in vitro</i> .	59
3.31	Introduction	59
3.32	Materials and Methods	60
	a) Preparation of cell lysate	60
	b) Estimation of Protein content of <i>M. ovipneumoniae</i> lysates	60
	c) Preparation of the <i>M. ovipneumoniae</i> protein samples for SDS-PAGE Gel	61
	d) SDS-PAGE	61

3.33 Results	64
3.34 Conclusion	66
3.4 Discussion	67
CHAPTER 4: <u>GENETIC INTERCHANGE</u>	68
4.1 Introduction	68
4.2 "Classical" Antibiotic Resistance crosses	71
4.21 Introduction	71
4.22 Materials and Methods	71
a) Determination of Antibiotic concentrations for use in Recombination experiments	71
b) Selection of Antibiotic Resistant Mutants	71
c) "Classical" Antibiotic Resistance crosses	72
4.23 Results	73
a) Inhibitory concentrations of Naladixic Acid and Rifampicin	73
b) Selection of Antibiotic Resistant mutants	73
c) "Classical" Antibiotic Resistance crosses	73
4.24 Conclusion	75
4.3 REA of clones derived from mixed cultures	76
4.31 Introduction	76
4.32 Materials and Methods	76
a) REA of clones derived from a mixed culture of <i>M. ovipneumoniae</i> that had been passaged <i>in vitro</i> for five days.	76
b) REA of "presumptive recombinant" <i>M. ovipneumoniae</i> derived from "classical" antibiotic resistance crosses	76

4.33	Results	77
a)	REA of clones derived from a mixed culture of <i>M. ovipneumoniae</i> that had been passaged <i>in vitro</i> for five days.	77
b)	REA of "presumptive recombinant" <i>M. ovipneumoniae</i> derived from "classical" antibiotic resistance crosses.	79
4.34	Conclusion	82
4.4	Examination of <i>M. ovipneumoniae</i> for the presence of extrachromosomal DNA	83
4.41	Introduction	83
4.42	Materials and Methods	83
4.43	Results	84
4.44	Conclusion	86
4.5	Discussion	87
CHAPTER 5:	<u>GENERAL DISCUSSION</u>	89
Appendix 1:	<i>M. ovipneumoniae</i> growth and identification media	100
Appendix 2:	REA materials	103
Appendix 3:	SDS-PAGE Materials	106
Appendix 4:	Plasmid identification materials	109
	Bibliography	110

LIST OF FIGURES

FIGURE	<u>Page</u>
1. Standard Fluorimetry curve used to determine the DNA concentration present in <i>M. ovipneumoniae</i> extracts.	24
2. Gel precipitin test used in identification of Mycoplasma species.	29
3. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 1,2 and 3.	30
4. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 4, 5, and 6.	31
5. REA OF <i>M. ovipneumoniae</i> , isolates from CNP lesions of lambs from farms 7, 8 and 9.	32
6. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 10, 11 and 12.	33
7. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 13, 14 and 15.	34
8. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 16, 17 and 18.	35
9. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 19 and 20.	36
10. REA of 8 clones of <i>M. ovipneumoniae</i> strain 5 selected simultaneously after 3 prior serial clonings.	44
11. REA of 8 clones selected after serial passage <i>in vitro</i> of a clone of <i>M. ovipneumoniae</i> strain	45

- 5 for approximately 133 cell generations. 45
12. REA of 8 clones selected simultaneously after serial passage *in vitro* of a clone of *M. ovipneumoniae* strain 5 for approximately 266 cell generations. 46
13. REA of 8 clones selected simultaneously after serial passage *in vitro* of a "variant" clone of *M. ovipneumoniae* strain 5 for approximately 133 cell generations. 47
14. REA of 8 clones selected simultaneously after serial passage *in vitro* of a clone of *M. ovipneumoniae* strain 5 for approximately 399 cell generations. 48
15. REA of 8 clones selected simultaneously after serial passage *in vitro* of a "variant" clone of *M. ovipneumoniae* strain 5 for approximately 133 cell generations. 49
16. REA of 8 clones selected simultaneously after serial passage *in vitro* of a "variant" clone of *M. ovipneumoniae* strain for approximately 266 cell generations. 50
17. REA showing the banding differences of which resulted in *M. ovipneumoniae* clones after serial passage *in vitro*. 51
18. A Flow diagram illustrating the serial *in vitro* passage of a *M. ovipneumoniae* strain 5 clone. 52
19. REA of duplicate cultures of *M. ovipneumoniae* clones that showed different REA patterns after serial passage *in vitro*. 56
20. REA of *M. ovipneumoniae* clones that showed

- different REA patterns after serial passage *in vitro*, digested with increased concentrations of *EcoRI*. 57
21. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of *M. ovipneumoniae* clones that showed different REA patterns after serial passage *in vitro*. 65
22. REA of *M. ovipneumoniae* clones randomly selected from a mixed culture of *M. ovipneumoniae* strains 5 and 10. 78
23. REA of "presumptive recombinant" *M. ovipneumoniae* clones derived from "classical" antibiotic resistance crosses 80
24. REA of "presumptive recombinant" *M. ovipneumoniae* clones derived from "classical" antibiotic resistance crosses. 81
25. Eckhardt plasmid gel of *M. ovipneumoniae* and *Rhizobium trifolii* isolates. 85

LIST OF TABLES

PAGE

Table I:	A Typical spectrophotometry profile for a nucleic acid sample extracted from <i>M. ovipneumoniae</i>	22
Table II:	Number of "presumptive recombinant" <i>M. ovipneumoniae</i> colonies observed on FM4 agar containing 4% of both Naladixic Acid and Rifampicin.	74
Table III:	Background level of Naladixic Acid resistant <i>M. ovipneumoniae</i> also resistant to Rifampicin.	74
Table IV:	Background level of Rifampicin resistant <i>M. ovipneumoniae</i> also resistant to Naladixic Acid.	74

Introduction

Chronic Non-Progressive pneumonia (CNP) is a disease which typically occurs in three to ten month old lambs. It has a complex aetiology, which has still not been unequivocally elucidated. However, it is apparent that *Mycoplasma ovipneumoniae* plays an important role in the pathogenesis of the disease. *M. ovipneumoniae* can be recovered from the nasal tract and lungs, and it is unclear if these represent a different population. Mew (1982) compared nasal and pneumonic strains of *M. ovipneumoniae* by Restriction Endonuclease analysis (REA) and from these studies it became apparent that many strains (as defined by their REA patterns) of *M. ovipneumoniae* could infect both the nasal cavities and lungs of lambs. To further investigate this heterogeneity of strains, Mew (1982) examined nine independent isolates (i.e. isolates which originated from different farms) by REA. All gave REA patterns that were completely different from each other. It therefore appeared that the species *M. ovipneumoniae* was extremely heterogeneous.

This heterogeneity contrasts with almost all other species examined by REA i.e. most microorganisms show either homogeneity e.g. *Brucella ovis* (O'hara et al.,1984) or show limited heterogeneity e.g. *Campylobacter coli* (Kakoyiannis et al., 1984). The present study therefore set out to further investigate the heterogeneity of *M. ovipneumoniae*.

To further examine the conclusion of Mew (1982), i.e. that the species *M. ovipneumoniae* shows considerable heterogeneity, a further sixty isolates of *M. ovipneumoniae*, i.e. three isolates from sheep on each of twenty farms. (thus giving a total of twenty independent isolates), were examined by REA. From this study it became apparent that considerable heterogeneity does exist in the species *M. ovipneumoniae*, so experiments were undertaken to answer the following questions:

1. Does genetic instability (i.e. rapid internal genetic change) in *M. ovipneumoniae* account for the heterogeneity

observed in the species?

This was investigated by analysis of *M. ovipneumoniae* clones before and after serial passage.

2. Does frequent genetic interchange in *M. ovipneumoniae* account for the heterogeneity observed in the species? (i.e. random genetic interchange between what were initially two strains of *M. ovipneumoniae* with markedly different genomes could generate an almost infinite number of strains which varied from their parental strains.

- This was investigated using three experimental approaches:

(a) "Classical" antibiotic resistance crosses, with selection for recombinants.

(b) REA of possible recombinants.

(c) Examination of *M. ovipneumoniae* for plasmids which, if present, might facilitate genetic interchange.

During the course of investigations into the genetic stability of *M. ovipneumoniae*, further questions arose concerning REA pattern changes:

1. Are the REA band changes observed real differences i.e. are they reproducible?

2. Do these changes in the REA patterns affect the antigenicity/protein component of the *M. ovipneumoniae* concerned?

These questions were also investigated.

3. If the strains are stable and independantly maintained, what is the minimum number of strains that is likely to be present and what is the likelihood that this number can be maintained?

Chapter 1. HISTORICAL REVIEW

1.1 Chronic Non-Progressive Pneumonia

1.11 Occurrence of Chronic Non-Progressive Pneumonia

Chronic Non-Progressive pneumonia (CNP) is enzootic in New Zealand sheep and typically occurs in 3 to 10 month old lambs. It differs from progressive pneumonia of sheep in that it normally resolves after a few months. Pathologically, CNP is characterised by varying degrees of dull red to grey consolidation of the anterior lobes. It causes less severe damage to the alveolar epithelium and the lesions are more localised than those in acute bacterial pneumonia.

A similar, probably identical, disease has been described in sheep in other countries including Australia (St George *et al.*, 1971), Great Britain (Jones *et al.*, 1975), The United States (St George and Carmichael, 1975), Iceland (Fris *et al.*, 1976) and Iraq (All-Sultan and Zubaidy, 1978).

A variety of names have been assigned to the disease: Summer pneumonia (St George *et al.*, 1972), Enzootic pneumonia (Kirton *et al.*, 1976), Atypical pneumonia (Stamp and Nisbet, 1963), Proliferative interstitial pneumonia (Carmichael *et al.*, 1972) and Chronic Non-Progressive Pneumonia (Alley, 1975). To avoid confusion, the name Chronic Non-Progressive Pneumonia (CNP) will be used in this thesis synonymously with the names mentioned above, as well as any other names used to refer to an apparently identical disease.

1.12 Economic Importance of Chronic Non-Progressive Pneumonia

It has been suggested that CNP is of great economic importance to New Zealand for two reasons

(1.) The formation of pleural adhesions and subsequent down-grading of such carcasses at the Meat works. (Dyshart, 1976). Pleurisy has been listed as the second most important (31.4%) disease/defect encountered at the Meat works. (Central Districts Farmer (1985) 3 (1))

(2.) Diminished Weight gains of lambs as a consequence of CNP

(St George *et al.*, 1971; Carmichael *et al.*, 1972; Harris and Alley, 1977; Jones *et al.*, 1982).

1.13 Aetiology of Chronic Non-Progressive Pneumonia

Although the disease referred to as CNP was described over two decades ago (Stamp and Nisbet, 1963), its aetiology has still not been unequivocally established.

Many microorganisms (*Mycoplasma ovipneumoniae*, *Mycoplasma arginini*, *Pasteurella haemolytica*, *Neisseria catarrhalis*, *Escherichia coli* and several viruses) have been recovered from the lung lesions, but not all have been consistently associated with the disease.

M. ovipneumoniae can be consistently isolated from the lesions, but may also be isolated from the lungs of apparently healthy sheep, although with decreased frequency and in lower titres (Alley, 1975; Davies *et al.*, 1981). Both *P. haemolytica* and *M. arginini* can be isolated from a smaller proportion of lung lesions than *M. ovipneumoniae*. (Alley, 1975; Jones *et al.*, 1978). *E. coli* and *N. catarrhalis* have only been isolated occasionally from CNP lesions (Alley, 1975; Alley and Clarke, 1979) but these two organisms are probably secondary invaders, as there is no other evidence of association with the disease. Several workers have suggested the Parainfluenza virus type III (PI3) may have a role in the aetiology of CNP eg. Jones *et al* (1979) detected a rise in antibody titre to PI3 in lambs which occurred concurrently with a rise in antibody titre to *M. ovipneumoniae* and the onset of a respiratory disease. However, these lambs were housed, so a rise in antibody titre to PI3, and no doubt a lot of other viruses could be hardly surprising with the stress and close contact imposed on the animals by the housing. Furthermore, PI3 has only been isolated once from lesions resembling those produced by CNP (Davies, 1980), other workers have failed to isolate PI3 from such lesions (St George *et al.*, 1971; Jones *et al.*, 1978, 1979; Brian, 1980).

Isolation alone of a microorganism from the lesions does

not imply causation of the disease. This, as Koch suggested in his postulates, requires transmission studies.

1.14 Transmission studies

Transmission studies have been carried out using different organisms and combination of organisms with CNP to try to elucidate the aetiology of the disease. These studies gave varying results and are summarised below.

Pneumonic Lung homogenate (This was derived from animals with CNP, and contained varying species and titres of the bacteria noted in the previous section). This was inoculated intranasally and produced a disease in over 50% of lambs. This disease resembled CNP (Jones *et al.*, 1978, 1980; Alley and Clarke, 1979, 1980; St George *et al.*, 1971). Transmission studies using lung homogenate as an inoculum allied with administration of antibiotics that inhibit either Mycoplasmas or (cell walled) bacteria significantly reduced the proportion of animals which consequently developed CNP (Alley and Clarke, 1980; Brian, 1980). This indicates that both a Mycoplasma and a (cell walled) bacteria are necessary to produce CNP.

Mycoplasma arginini cultures failed to produce CNP (Foggie and Angus, 1972). Furthermore, CNP has been produced with lung homogenate which did not contain *M. arginini* (Alley and Clarke, 1980).

Pasteurella haemolytica cultures inoculated into Specific Pathogen Free (SPF) lambs failed to produce CNP (Sharp *et al.*, 1978; Davies *et al.*, 1981).

Mycoplasma ovipneumoniae cultures inoculated into lambs have sometimes produced no disease (Davies *et al.*, 1981), but the majority of workers have produced a mild disease which resembled CNP. This disease occurred in a low proportion of the lambs.

Mixed Cultures Jones *et al.*, (1980), using an inoculum which

contained *M. ovipneumoniae*, *M. arginini*, and *P. haemolytica*, have produced a disease similar, if not identical, the CNP in a significant proportion of lambs. CNP has also been produced using a culture that contained *M. ovipneumoniae* and *P. haemolytica* but no *M. arginini* (Jones *et al.*, 1978; Gilmour *et al.*, 1982). Transmission studies so far used cloned cultures of *M. ovipneumoniae*. Thus, even though the isolate was derived from a CNP lesion, there is the possibility that it was one of many strains present in the lesions. These strains might differ in virulence or act in synergy. Jones *et al.* (1982) found that an inoculum which contained *P. haemolytica* and a mixed culture of 6 strains of *M. ovipneumoniae* (all isolated from CNP lesions) produced the disease in a higher proportion of animals than when *P. haemolytica* was used in combination with a single strain of *M. ovipneumoniae*. Thus a method to differentiate between strains of *M. ovipneumoniae* was necessary to further elucidate the aetiology of CNP.

Mew (1982) developed such a method (based on REA) that differentiated between *M. ovipneumoniae* strains. Before the results of this work are discussed, it is first appropriate to discuss the background to Restriction Endonuclease analysis.

1.2 Restriction Endonucleases

1.21 Discovery and characterisation of restriction endonucleases

Luria *et al.*, (1952) were the first to describe the phenomenon that we now know as the Restriction and Modification system. They noticed that bacteriophages which had been propagated on one strain of bacteria displayed variation in their ability to propagate on other bacteria. This phenomenon was seen to be a property of the bacterial cells rather than the bacteriophages and thus led Luria *et al.* (1952) to postulate the existence of some type of cellular mechanism to account for this phenomenon.

Later studies by Arber and Dussoix (1962) on *Escherichia coli* strains B and K, led them to propose a model of restriction and modification to account for these observations. Thus they proposed that the bacterial cell contained two distinct enzymes, with paradoxical functions. One of these is an endonuclease (which recognises a specific sequence of bases within the DNA and consequently makes a double stranded cut of that DNA) and the other is a modification enzyme (which recognises the same sequence and makes a chemical alteration, eg. addition of a methyl group, to a specific site or sites within the recognition site, thus ensuring that it is no longer a substrate for the restriction enzyme). Thus the host (bacterial) DNA would always be modified, and hence protected from destruction by the Restriction enzyme. Any foreign DNA that invaded, provided it was unmodified, would therefore be destroyed.

Some years later the first Restriction endonucleases were isolated by Meselson and Yuan (1968) and Linn and Arber (1968) from *E. coli* K and B respectively. These enzymes (later designated class I restriction enzymes) recognise specific nucleotide sequences in the DNA, but cut at random sites away from the recognition sites. Two years later Smith and Wilcox (1970) and Kelly and Smith (1970) isolated an enzyme from *Haemophilus influenzae*. This enzyme cut the DNA at the

recognition site, and is therefore referred to as a class II enzyme.

1.22 Class I Restriction Enzymes

Class I restriction enzymes are non specific with respect to their cutting sites. They require ATP, Mg^{2+} and S-adenosyl methionine. As proposed by Rosamund *et al* (1979) in a model of the action of *Eco* b, the enzyme binds to both the recognition sequence and some neighbouring bases in the presence of S-adenosyl methionine. While remaining bound to the recognition sequence, the other end of the enzyme utilises the energy from the ATP to form a gradually enlarging loop of DNA, by moving along the DNA to which it is bound. When the loop reaches a size between 1000 and 6000 base pairs, the enzyme produces a gap of about 75 nucleotides in one strand of the duplex. While the first enzyme continues to hold the DNA in the looped configuration, a second attaches and produces a cut in the remaining DNA strand. Thus a double stranded break in the DNA occurs at a site approximately 1000 nucleotides from the original recognition sequence. As Class I restriction enzymes are non-specific and hence produce heterogeneous products, their usefulness in genetic analyses is limited. Further discussion is therefore concerned mainly with class II Restriction Enzymes.

1.23 Class II Restriction Enzymes

Class II enzymes differ from Class I restriction enzymes in that they cut at a particular point in the recognition sequence. Class II enzymes are further divided into subgroups, based on the nature of the base sequence they recognise.

(1) Symmetric i.e. The recognition sequence has 2-fold rotational symmetry and is thus a palindrome.

eg. *Eco*RI 5'GAATTC 3'
 3'CTTAAG 5'

(2) Degenerate symmetric i.e. A portion of the recognition sequence forms a palindrome.

eg. *Hae*II 5'PuGCGPy3' Pu = Any Purine
 3'PyCGCPu5' Py = Any Pyrimidine

(3) Asymmetric i.e. No symmetry is seen in the recognition sequence.

eg. *Eco*PI 5'AGACC 3'
 3'TCTGG 5'

Class II restriction enzymes can produce 3 different types of fragments when they cut the DNA. If they cut in the middle of the recognition site (eg. *Bsu*RI which cuts thus 5'GG/CC3'), all the fragments produced have blunt ends. If they cut to one side of the middle of the recognition sequence, single stranded ends are produced. These ends can have either 5' tails (eg. *Bam* HI which cuts thus 5'G/GATCC 3') or 3' tails (eg. *Pst* I which cuts thus 5'CTGCA/G 3'). The production of these single stranded ends is useful in genetic studies, as will be discussed later (Although double stranded ends can be used in the same studies after modification).

1.24 Nomenclature of Restriction Enzymes

Restriction enzymes are designated by italicized letters representing the genus and the species name of the producing organism. A fourth letter may be added to indicate which specific strain produces the enzyme. When an organism produces more than one enzyme, the enzymes are differentiated by Roman numerals, eg. *Eco*RI is derived from *Escherichia coli* RY13.

About 200 Restriction enzymes have been discovered. (Restriction enzymes appear to be widespread throughout the bacterial kingdom, and each group of bacteria so far examined has produced at least one genus from which a restriction enzyme has been isolated.) As the recognition sequences for these enzymes are 4 to 7 bases long, enzymes which recognise the same sequence have been isolated from a variety of bacteria. These duplicate enzymes are known as isochizomers, and are still named according to the organism they are derived from.

1.25 Specificity of Class II Restriction Enzymes

Although the recognition sequence is necessary and sufficient for cleavage to occur, sequences outside this recognition sequence do influence the rate of cleavage. Furthermore specificity for the recognition sequence can be relaxed by changing the pH and ionic conditions of the reaction. Thus purification of the enzyme to Homogeneity is important. Most enzymes show a broad pH optimum, a broad magnesium optimum and are inhibited by high concentrations of sodium chloride. (Roberts, 1976).

1.26 Applications of Restriction Enzymes

Many applications of Restriction enzymes stem from their ability to produce small DNA fragments which can then be separated. The applications are in three main areas:

- (1) Physical Mapping of DNA.
- (2) Cloning of genes.
- (3) Classification and Identification of microorganisms
(and hence Epidemiological studies.)

Restriction Endonuclease mapping of DNA

Since class II restriction endonucleases are site specific, and thus produce a unique set of fragments from a particular DNA molecule, they can be used in the physical mapping of (viral and plasmid) DNA. These restriction maps are constructed by digestion of the DNA with a restriction endonuclease. The sizes of the fragments thus produced are then deduced by gel electrophoresis (The rate at which fragments move in the gel is a function of their size, with small fragments moving much faster than large fragments) and comparison with DNA fragments of a known size. The order of fragments in the DNA can then be deduced by studying the patterns of fragments produced as the digest proceeds to completion, and by redigestion of the fragments with another restriction endonuclease.

Thus a restriction map shows the positions of an endonucleases

cutting sites on the DNA. Each restriction endonuclease therefore produces a different restriction map for the same DNA molecule. These restriction maps are useful in the determination of regions of biological importance on the DNA.

Many restriction maps have been constructed since Darna and Nathans (1971) constructed the first restriction map (A *Hin* d II map of the circular DNA virus SV40). Thus restriction maps have been constructed for many viruses, including Adenoviruses (Tibbetts, 1977), Herpes Simplex virus type I (Skare and Summers, 1977) etc.

Cloning of genes

The realisation that many restriction endonucleases (eg *Eco* RI) generate specific cohesive ends (i.e. single stranded cohesive ends) that can be later sealed up by DNA ligase was followed by the development of the first practical method for cloning specific DNA fragments, regardless of their origin. The essential factor in the production of these cloned DNA fragments, is the random insertion of the restriction fragments of DNA molecule into a circular replicative plasmid DNA that had also been cut with the same endonuclease. These hybrid plasmids, which contain a section of DNA of interest, can then be used to infect bacteria. Thus each bacterial cell will acquire a recombinant plasmid carrying a specific piece of foreign DNA. Restriction endonucleases which do not produce single stranded cohesive ends can also be used to clone DNA fragments, once the blunt ended DNA fragments they produce have 'sticky ends' of single stranded DNA added to them.

The production of such recombinant DNA has produced almost endless possibilities in genetic manipulations, of which perhaps the most important are the possibilities that recombinant DNA opens up for the analysis of DNA from plants and animals.

Classification and Identification of Micro-organisms

Restriction Endonuclease Analysis (REA) i.e. digestion of the DNA with a restriction endonuclease, followed by gel electrophoresis of the fragments, is a relatively new technique. This technique was used initially with viruses to identify either species or individual strains. Thus species which are members of the Orthopoxviridae family i.e. rabbitpox, vaccinia, monkeypox, variola, cowpox and ectromelia all have REA patterns which are common within each species (Muller *et al.*, 1978; Esposito *et al.*, 1978; Mackett and Archard, 1979). Strains of Herpes simplex virus type 1 can also be distinguished by REA (Skare *et al.*, 1975). This has been used in epidemiological studies. Epidemiological studies using REA "fingerprinting" can in principle be applied to any DNA virus, but have so far been confined mainly to studies of Herpes Simplex virus types 1 and 2 (HSV-1 and HSV-2 respectively). Thus Buchman *et al.* (1978) used REA to investigate a nosocomial outbreak of HSV-1, and showed that the outbreak was the result of two independent introductions of HSV-1. They therefore concluded that REA "has the potential of becoming a powerful tool for tracing the spread of HSV-1 and very likely of other herpesviruses in the human population." Other epidemiological studies using REA have also been carried out on HSV-1 (Linneman *et al.*, 1978; Lonsdale *et al.*, 1979).

REA studies can, in principle, be extended to all DNA viruses, since they have a genome size which limits the number of fragments, so that the resulting banding pattern shows good resolution and allows different banding patterns to be distinguished. The larger genome size of bacteria (giving a correspondingly greater number of fragments) seemed at first to preclude the use of REA in identification of bacteria, but in practice identification of strains, species and even genera of bacteria has proved possible.

Thus Marshall *et al.* (1981) developed an REA technique that distinguished the *Leptospira interrogans* serovars *hardjo* and *balcanica* (which belong to the Hebdomadis serogroup - *Leptospira*

interrogans have at least 130 serovars which are grouped into 18 serogroups). These REA "fingerprints" were characteristic of the serovar, and no difference was seen between isolates derived from different host species or between isolates from the same host species from widely separated geographic areas in New Zealand. The REA "fingerprints" of the serovars *hardjo* and *balcanica* differed from other members of the same (Hebdomadis) serogroup and representatives of other *Leptospira interrogans* serogroups. Marshall *et al.*, (1981) therefore concluded that REA "should be useful for the identification of leptospire and might throw light on problems of their classification" and also suggested that the "the technique may prove useful in identifying bacteria other than leptospire." The latter statement has, indeed, proved to be true.

Thus Marshall *et al.* (1985) in a study of 94 isolates of *Moraxella bovis*, from the U.S.A., the U.K., Australia and New Zealand, were able to put the isolates into one of 26 groups on the basis of their REA patterns. These REA patterns were clearly different from prototype strains of other *Moraxella* species i.e. *Moraxella liquefaciens* and *Moraxella nonliquefaciens*.

Strains of *Campylobacter coli* have also been examined by REA. Thus Kakoyiannis *et al* (1984) examined 99 *C. coli* isolates derived from humans, pigs, gulls and chickens by REA. They found that the *C. coli* isolates from within these species could be grouped into REA groups. Thus the 14 human isolates examined produced 11 distinct REA types, the 43 pig isolates produced 20 REA types, the 10 gull isolates produced 5 different types, and the 32 chicken isolates produced 5 REA types. These REA types appear to be host specific e.g. None of the REA types which infected pigs were found in humans. (Note: one REA appeared to be non-host-specific, i.e. one human isolate was indistinguishable from a poultry isolate, however no direct epidemiological link between these isolates could be inferred however, as the isolates came from different areas). Some epidemiological observations can however be made from the results of Kakoyiannis *et al* (1984), apart from

the apparent host specificity e.g. Suckling piglets generally had identical *C.coli* REA types as their dams, and if they did vary, the *C.coli* REA type was similar to a type carried by a nearby sow to which the piglet had access. Thus Kakoyiannis *et al* (1984) concluded that REA "shows great promise for use in epidemiological studies of *C.coli*."

In some instances it is possible to use REA to distinguish a bacterial species and strains within the species. Thus Mielenz *et al* (1979) were able to distinguish the species *Rhizobium trifolii*, *Rhizobium meliloti*, and *Rhizobium japonicum* by their REA patterns. Strains within these species could also be detected by minor banding differences, on the species-specific background banding pattern, The value of REA in identification of *Rhizobium* species was highlighted by the identification of an isolate originally thought to be a *R.trifolii* mutant strain as a *R.japonicum* derivative. Mielenz *et al* (1979) summarised by suggesting that REA "will provide a useful tool for bacterial classification." The ability of REA to identify some genera supports this suggestion.

O'hara *et al* (1984) examined prototype species of *Brucella abortus*, *Brucella canis*, *Brucella melitensis* and *Brucella ovis* and found that they had between 80 and 90% of bands in common. So it appears that the genus *Brucella* can be identified by REA.

From the above review, it is apparent that REA can be used to identify bacterial strains, species, or even genera. Since this thesis is concerned with *M. ovipneumoniae*, we now review studies of the class Mollicutes by REA.

The class Mollicutes, with their genome of intermediate size between bacteria and viruses, have been extensively examined by REA, Thus species from the families Spiroplasmataceae, Acholeplasmataceae, and Mycoplasmataceae have all been examined.

The family Spiroplasmataceae consists of one genus, genus *Spiroplasma*, which in turn has two species within it. (Bergey's Manual of Determinative Bacteriology, 1974). The type species, *Spiroplasma citri* has been examined by Bove and Saillard (1979), who found that isolates of *S. citri* could be put into one of two groups. The first group consists of *S. citri* that have no extrachromosomal DNA and have identical REA patterns. The second group consists of *S. citri* that have varying complements of extrachromosomal DNA and hence has many REA patterns (which probably result from different complements of extrachromosomal DNA).

The family Acholeplasmataceae also consists of one genus, genus *Acholeplasma* within which there are 8 species. (Bergey's Manual of Determinative Bacteriology, 1974). Several of these have been examined by REA. For example Bove and Saillard (1979) showed that several different strains of *Acholeplasma laidlawii* were identical by REA. Similarly, Razin *et al* (1983a) found that the REA patterns of five *Acholeplasma oculi* strains showed only a few minor band difference, against a common background of bands. However, Razin *et al* (1983a) also found that eight *Acholeplasma axanthum* isolates (from widely diverse habitats) showed seven different REA patterns. Thus it seems that the species within the family may vary from homogeneity to great heterogeneity.

The family Mycoplasmataceae consists of two genera, genus *Mycoplasma* and genus *Ureaplasma* (Bergey's Manual of Determinative Bacteriology, 1974).

The solitary species of the genus *Ureaplasma*, *Ureaplasma urealyticum*, can be grouped into 2 distinct clusters by REA (Razin *et al.*, 1983b). Strains within a cluster differ by a few bands only. This led Razin *et al* (1983b) to suggest that the species *U. urealyticum* might represent two distinct species, as represented by the 2 distinct REA patterns.

The genus *Mycoplasma* consists of over sixty species. Of these, eight species (*Mycoplasma pneumoniae*, *Mycoplasma*

hypneumoniae, *Mycoplasma flocculare*, *Mycoplasma gallisepticum*, *Mycoplasma genitalium*, *Mycoplasma pulmonis*, *Mycoplasma hyorhinis* and *Mycoplasma ovipneumoniae*) have been examined by REA. Razin *et al* (1983a) examined three isolates of *M. genitalium*, two isolated from the urethra of patients with non-gonococcal urethritis and one from the urethra of an experimentally infected chimpanzee, and found that they had identical REA patterns. Similarly, Chandler *et al* (1982) and Razin *et al* (1983b) found that even though strains of *M. pneumoniae* may differ in their virulence and their adherence capabilities, they all have almost identical REA patterns. *M. gallisepticum* isolates have similarly been shown by Bove and Saillard (1979) and Razin *et al* (1983a) to have REA patterns very similar to each other, with at most only a few band differences. Two isolates of *M. pulmonis*, one a pathogen and the other not, were found by Bove and Saillard (1979) to be indistinguishable by REA. Chan and Ross (1984) have found that the species *M. flocculare* and *M. hyopneumoniae* are readily distinguishable by their REA patterns. Darai *et al* (1982) were even able to identify a *M. hyorhinis* cell culture contaminant by REA. The identity of the contaminant was later confirmed by classical means. This led Darai *et al* (1982) to suggest that "a more extensive use of restriction endonuclease analyses of different mycoplasma genomes ... will lead to a new basis for a more meaningful classification of mycoplasmas." Thus one might conclude from the above review that species of the genus *Mycoplasma* appear to be either homogeneous or show limited heterogeneity. However, when Mew (1982) examined *M. ovipneumoniae*, the results were remarkably different. Thus she (Mew, 1982) examined nine independent isolates of *M. ovipneumoniae* (i.e. isolates from sheep on different farms) by REA and all showed REA patterns that were completely different. Indeed, when *M. ovipneumoniae* isolates were taken from lambs on a single farm over a six month period, six more different strains were detected. Thus, this species shows considerable heterogeneity. This is in contrast to all other reports of species of the genus *Mycoplasma* examined by REA (which show either homogeneity or limited heterogeneity) and indeed almost all species of the class Mollicutes.

This thesis is thus concerned with the heterogeneity seen in *M. ovipneumoniae*.

Chapter 2 EXAMINATION OF INDEPENDENT ISOLATES OF
M. ovipneumoniae BY RESTRICTION
ENDONUCLEASE ANALYSIS (REA)

2.1 Introduction

In a previous study (Mew, 1982) of independent isolates of *M. ovipneumoniae* (i.e. isolates from sheep on different farms) by REA, the species showed considerable heterogeneity. Thus each isolate showed a REA pattern that differed completely from all other isolates. This is in contrast to almost all other studies of bacteria by REA, as most species are either homogenous, e.g. *Brucella ovis* (O'Hara *et al.*, 1984), *Mycoplasma hyopneumoniae* (Chan and Ross, 1984), *Mycoplasma genitalium* (Razin *et al.*, 1983) etc., or show limited heterogeneity e.g. *Ureaplasma urealyticum* (Razin *et al.*, 1983), *Moraxella bovis* (Marshall *et al.*, 1985), *Campylobacter coli* (Kakoyiannis *et al.*, 1984), etc. Only *Acholeplasma axanthum* (Razin *et al.*, 1982), shows heterogeneity approaching the magnitude of that seen in *M. ovipneumoniae*.

However, Mew (1982) examined only nine independent isolates, so it is possible that the number of different REA patterns that occur for the species *M. ovipneumoniae* might not greatly exceed nine.

This chapter therefore attempts to obtain a better estimate of how many strains of *M. ovipneumoniae* exist, by examination of more isolates.

2.2 Materials and Methods

2.21 Origin and isolation of *M. ovipneumoniae*

The lungs of lambs from 20 different farms were examined at the abattoir for the presence of CNP lesions after slaughter. Thus 3 lungs that showed CNP lesions were collected from each of the 20 farms. (This gave a total of 60 lung specimens). The lungs were placed in individual plastic bags to prevent cross contamination. The three lungs from

each farm were labelled with a number to indicate the farm they came from, and were also labelled A, B or C to distinguish them.

A sample (approximately 3ml) of CNP lesion was aseptically removed from each of the lungs. Approximately 2ml was frozen at -70°C for future reference. The remaining portion was cut into small pieces, using sterile scissors, and placed in 3.0ml of FM4 broth (see Appendix 1.1). The resulting suspension was shaken, and the material allowed to settle. A 0.3ml aliquot of the supernatant was transferred to 3.0ml of FM4 broth and incubated at 37°C on a shaker until a pH change occurred, at which time a 0.1ml sample was spotted onto FM4 agar. The plates were incubated at 37°C for 4 or 5 days and examined for Mycoplasma colonies using a plate microscope. If no contaminants were present, the culture was serially passaged a further 3 times in FM4 broth, and the final culture was stored at -70°C ready to be cloned.

2.22 Identification of the Mycoplasma isolates

Presumptive identification of mycoplasmas isolated from the CNP lesions as *M. ovipneumoniae* was based on several observations:

- (a) all were glycolytic - i.e. utilised glucose as the major energy source;
- (b) all gave "vacuolated", centreless colonies on agar;
- (c) all were isolated from CNP lesions of lambs.

The isolates were further identified by gel precipitin tests performed during the REA process (see Section 2.27). If they gave lines of identity in the gel precipitin test (using antiserum raised against *M. ovipneumoniae* strain 5 as test antiserum), they were considered to be *M. ovipneumoniae*.

2.23 Storage of Stock Cultures of *M. ovipneumoniae*

Cultures of *M. ovipneumoniae* in the exponential phase (containing approximately 10^8 CFU/ml) were stored at -70°C in 1.5ml aliquots.

2.24 Cloning of *M. ovipneumoniae*

An aliquot of *M. ovipneumoniae*, stored at -70°C was thawed and 1ml inoculated into 10ml of FM4 broth and incubated at 37°C over night on a rotary shaker. When the colour of the medium showed that it had reached pH 7.0 approximately (thus indicating the culture to be in the exponential phase. Major *et al.*, 1979), an aliquot was removed using a syringe, and passed through a sterile 0.45µm Millipore filter. 0.1ml aliquots of the filtrate were spotted on to several FM4 agar plate and incubated at 37°C. When colonies of *M. ovipneumoniae* were clearly visible (usually after 3 to 4 days), at least 5 were individually removed, along with a small plug of agar, using a pasteur pipette, and each was inoculated into 3ml of FM4 broth. These were incubated on a rotary shaker at 37°C until a colour change was observed. Aliquots of 1.5ml of the cloned culture were stored at -70°C.

2.25 RESTRICTION ENDONUCLEASE ANALYSIS OF *M. ovipneumoniae*.

Preparation of *M. ovipneumoniae* cells for DNA extraction

A 1.5ml aliquot of *M. ovipneumoniae* stored at -70°C was thawed, inoculated into 10ml of FM4 broth and incubated at 37°C overnight on a rotary shaker. When a colour change was seen, indicating the culture had reached approximately pH7.0, the culture was added to 100ml of FM4 broth and again incubated over night at 37°C on a rotary shaker. Cells were harvested when the colour change indicated that the culture had reached approximately pH7.0, and thus was in the exponential phase.

The *M. ovipneumoniae* cells were collected by centrifugation at 14500g for 20 minutes at room temperature. The supernatant was decanted and the cells washed twice with 20ml of sterile Phosphate Buffered Saline (PBS) (see Appendix 2.1), and resuspended in 1.5ml of Tris-EDTA solution (see Appendix 2.2). 1.0ml of this suspension was frozen at -70°C for DNA extraction, while the remaining 0.5ml was stored at 4°C for Gel precipitin tests (see Section 2.27).

DNA Extraction

1.0ml of the cell suspensions were thawed and mixed with

0.1ml of 10% SDS and 0.1ml of 10mg/ml Pronase (see Appendix 2.3). The mixtures were incubated overnight at 50°C, then 0.3ml of 0.48g/ml Sodium Perchlorate was added to each (i.e. to approximately 1M concentration) and the lysates were then incubated at 50°C for 1 hour. At this time, a Phenol/Chloroform/Iso-Amyl alcohol (see Appendix 2.4) solution was made up in the proportions 25:24:1 respectively. 1/10 volume of working strength STE Buffer (see Appendix 2.5) was added and the mixture was cooled to room temperature in the dark. The lower layer represented a buffer-equilibrated mixture of Phenol, Chloroform, and Iso-Amyl alcohol.

4.0ml of working strength STE buffer was added to each 1.55ml sample of celllysate after incubation, and the solutions poured into screw capped glass centrifuge tubes. 5.0ml of the (buffer equilibrated) Phenol/Chloroform/Iso-Amyl alcohol mixture was added and the two resulting layers were mixed by gently rocking the tube. After 5 minutes the solutions were centrifuged at 1000-2000rpm for 5 minutes at room temperature.

5.0ml serological pipettes, with the tips cut off (to prevent shearing the DNA) were used to remove the upper aqueous layer from the tubes into more screw capped centrifuge tubes, Care was taken to ensure that the interface of precipitated cellular material was not disturbed. The removed aqueous portion was re-extracted twice more by centrifugation with the (buffer-equilibrated) Phenol/Chloroform/Iso-Amyl alcohol mixture.

After the final extraction, the upper aqueous layer was again removed, and gently added to dialysis tubing (see Appendix 2.6), which were sealed and labelled. Extracted DNA was dialysed at least four times with 1l of Tris-EDTA Buffer (see Appendix 2.7).

Estimation of the concentration of DNA in the extract

The nucleic acid extract was removed from the dialysis tubing

by holding one end with forceps, while the lower end was placed in a sterile bijou bottle. First the lower end, and then the upper end of the dialysis tubing were cut, and the nucleic acid extract was allowed to drain into the bijou. These extracts were labelled, and stored at 4°C.

Spectrophotometry

0.4ml of each nucleic acid sample was loaded into quartz cuvettes and the optical density of each was measured using a Unicam Spectrophotometer SP500, against a dialysis buffer blank, at the following wave lengths 230nm; 260nm; and 280nm. The 260:280 ratio was checked to be within the range 1.8-2.5, thus showing minimal contamination with protein. The 270nm reading was also checked (since phenol absorbs strongly at 270nm, peak readings at 270nm indicate phenol contamination and the need to re-dialyse the sample).

The Nucleic acid concentration was estimated from the 260nm reading, assuming that an optical density reading of 1.0 was equivalent to 50µg/ml of nucleic acid. A typical spectrophotometry profile is shown below in Table I.

Table I: A typical spectrophotometry profile for a nucleic acid sample extracted from *M. ovipneumoniae*.

Sample	Wavelength				$\frac{260}{280}$	Nucleic Acid (µg/ml)
	230nm	260nm	270nm	280nm		
637	0.44	0.81	0.63	0.39	2.08	40.5

Fluorimetry

Measurements were taken using a Fluorimeter with a 365nm, filter, as described by Le Pecq and Paoletti (1966).

The volume of Nucleic acid extract to be used in the fluorimetry assay was calculated as follows:

$$\text{Volume for fluorimetry} = \frac{0.5 \times 2000}{\text{Nucleic acid concentration}} \mu\text{l}$$

Thus for the sample described in Table I,

$$\text{Volume for fluorimetry} = \frac{0.5 \times 2000}{40.5} = 25\mu\text{l}$$

A Fluorimetry assay mix was made up for each sample containing: 200 μl of 10 x STE Buffer, 800 μl of 5 $\mu\text{g/ml}$ Ethidium Bromide solution (see Appendix 2.8), the volume of Nucleic acid extract calculated as above, and distilled water to give a final volume of 2000 μl . A standard fluorimetry curve was prepared by using Calf Thymus DNA (See Appendix 2.9) instead of the nucleic acid extract in the Fluorimetry assay mix above, to give the final concentrations: 0; 0.2; 0.4; 0.8 and 1.0 $\mu\text{g/ml}$ of Calf Thymus DNA.

The DNA concentration of the extract was estimated by comparison with the standard fluorimetry curve (see Figure 1) prepared above.

Digestion of the DNA with *EcoRI*

The volume of nucleic acid extract that contained 2.0 μg of DNA was calculated using the following formula:

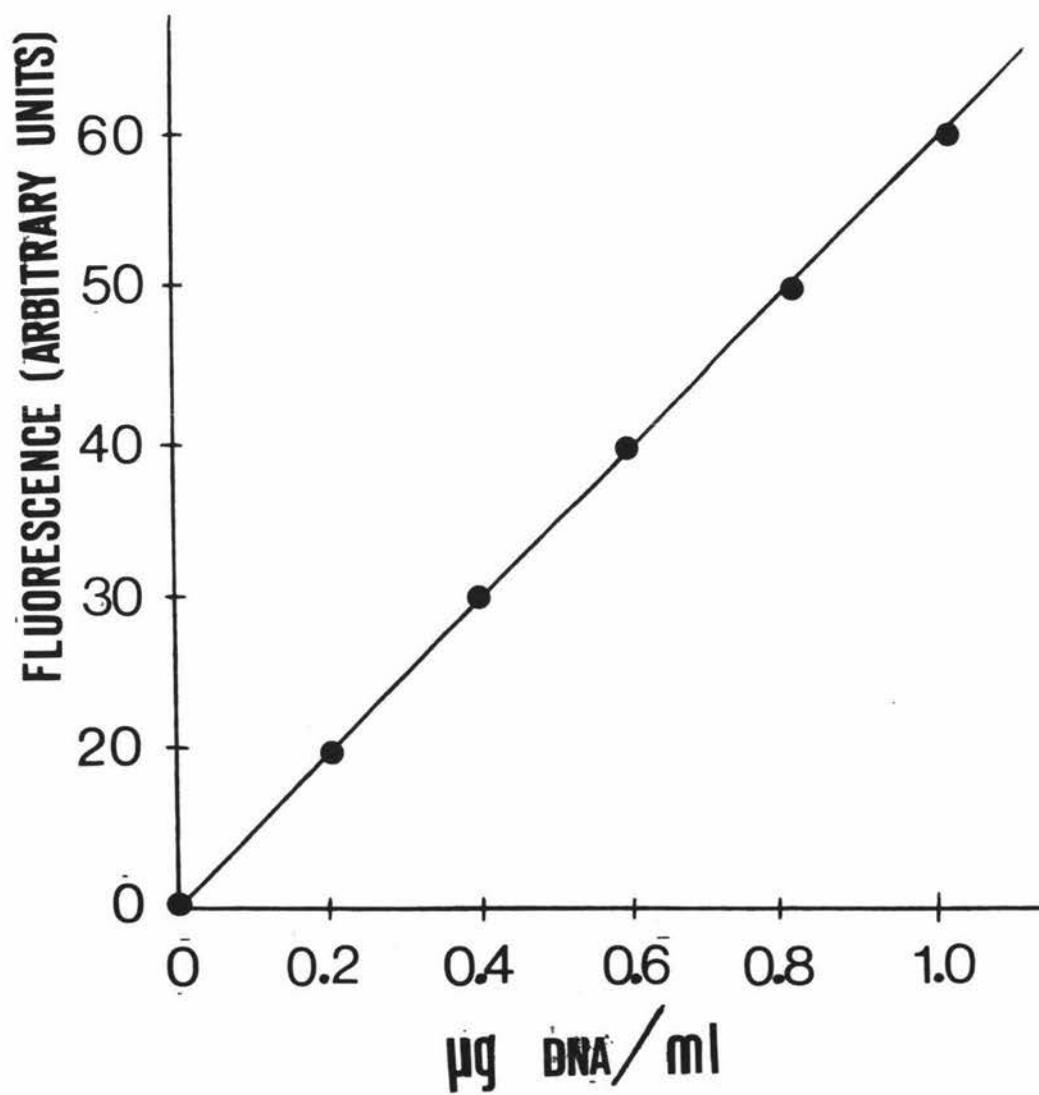
$$\begin{array}{l} \text{Volume of nucleic acid} \\ \text{extract containing 2.0}\mu\text{g} \\ \text{of DNA} \end{array} = \frac{2000}{\text{DNA concentration } (\mu\text{g/ml})}$$

This volume of nucleic acid was added to a 1500 μl Microfuge Tube (Eppendorf) containing 20 μl of 5 x *EcoRI* Buffer (see Appendix 2.10). Distilled water was used to make up the final volume to 100 μl . 2 μl (8 units) of *EcoRI* (see Appendix 2.11) was then added, and the contents mixed by gently tapping the microfuge tube.

As a control to ensure that the digest was working, and to act as a molecular market, 0.325 μg of lambda DNA (see Appendix 2.12) was also digested with 8 units of *EcoRI* in the same way as the Mycoplasma DNA for each REA.

Figure 1. Standard Fluorimetry curve prepared using calf Thymus DNA.

Note: A standard Fluorimetry curve was prepared for each batch of *M. ovipneumoniae* DNA samples.



The digestion mix was incubated at 37°C for 45 minutes then heated at 65°C for 10 minutes to inactivate the enzyme. The digested DNA was precipitated by adding 5 μ l (i.e. $\frac{1}{20}$ volume) of 5M NaCl and 200 μ l (i.e. 2 volumes) of absolute ethanol, and the contents mixed by inverting the microfuge tubes several times. The tubes were then placed at -20°C for at least 30 minutes, after which they were spun at 8500g for 5 minutes.

The supernatant was discarded and the pellet washed gently with 70% ethanol, and dried at 37°C for 15 minutes. 45 μ l of Running Buffer (see Appendix 2.13) was added to each tube which was tapped gently and left at room temperature overnight to resuspend the pellet.

Gel Electrophoresis of the DNA samples

100 μ l of 2.5 μ g/ml Ethidium Bromide solution was added to 1.5l of working strength E-Buffer (see Appendix 2.14). This was used to prepare 100ml of 0.7% Agarose (Biorad. Electrophoresis grade). This agarose was dissolved by boiling in a flask fitted with a reflux condenser, and cooled to approximately 40°C before pouring on to a level clear glass plate (20cm x 15cm) around the edge of which sellotape had been stuck, partially projecting vertically to contain the agarose. A perspex "comb" was placed near one end to form wells in the agarose, and the agarose allowed to solidify. This agarose gel was either allowed to set for 20 minutes before use, or stored at 4°C overnight for use the next day.

Before the gel was used, the comb and sellotape were removed, and the glass plate containing the gel was placed in the electrophoresis tank. The tank compartments were filled with the E-Buffer (containing Ethidium Bromide) that the gel had been made from. Each well in the gel was washed at least twice with the E-Buffer prior to loading the samples. Before the samples were added, the microfuge tubes were gently agitated to ensure that the DNA was resuspended in the running

buffer, and the 2.5 μ l of Bromophenol Blue in 50% glycerol was added to the lambda sample to act as an electrophoresis marker. The samples were loaded into the wells, and topped up with E-Buffer. Electrophoresis was performed at 8V/cm for 3.5 to 4 hours (i.e. until the Bromophenol Blue marker had travelled 12cm).

Photography of the gel

After the current was switched off, the glass plate containing the gel was removed and rinsed with the remaining E-Buffer to remove any dust that had settled on the gel. The gel was removed from the glass plate and placed on to a filter plate which was illuminated from beneath by 4 15W germicidal ultraviolet lamps.

The gel was photographed on Kodak Tri-X film using a 120 format plate camera and a Wratten 23A gelatin filter.

The film was exposed for 1 minute, developed for 3.5 minutes, fixed for 5 minutes, washed for 15 minutes and hung to drip dry.

2.26 Comparison of REA patterns

Photographs of the REA patterns shown by all the 60 lung isolates of *M. ovipneumoniae* from the 20 farms were obtained and prints taken from each. These prints were cut into strips - each of which contained the restriction pattern of one isolate. These were labelled on the reverse side, and each strip was aligned against every other strip in order to ascertain whether they showed the same or a different pattern. Thus independent isolates were compared with one another.

2.7 Gel precipitin tests

Each isolate (propagated in FM4 medium, centrifuged and resuspended in 0.5ml of Tris EDTA solution - see Section 2.25), was diluted 1:2 with Tris EDTA solution; sonicated for 2 x 10 seconds, and had Triton X-100 added to a final concentration of approximately 1%.

Wells were cut in the agar (see Appendix 1.3) using a template. The central well was filled with 50 μ l of anti-serum prepared to *M. ovipneumoniae* strain 5 (see Appendix 1.4) and the peripheral wells were filled with 50 μ l of the different antigen suspensions prepared from the farm isolates. Samples of *M. ovipneumoniae* strain 5 antigen suspension and FM4 medium were used as positive and negative controls.

The plate was left over night at room temperature and examined through a plate microscope for lines of identity.

2.3 Results

The results of the gel precipitin test for isolates from farm 1 are shown in figure 2. Isolates from all the other farms gave similar results i.e. all farm isolates were identified as *M. ovipneumoniae*.

The results of REA of independant isolates of *M. ovipneumoniae* are shown in figures 3 through 9.

Figure 2. Gel precipitin test of *M. ovipneumoniae* isolates from CNP lesions of lambs on farm 1.

Content of wells.

Centrewell: Antiserum to *M. ovipneumoniae*, strain 5.

Well 1: Strain 5 antigen; well 2: isolate 1a antigen; well 3: isolate 1b antigen; well 4: isolate 1c antigen; well 5: strain 5 antigen; well 6: FM4 medium.

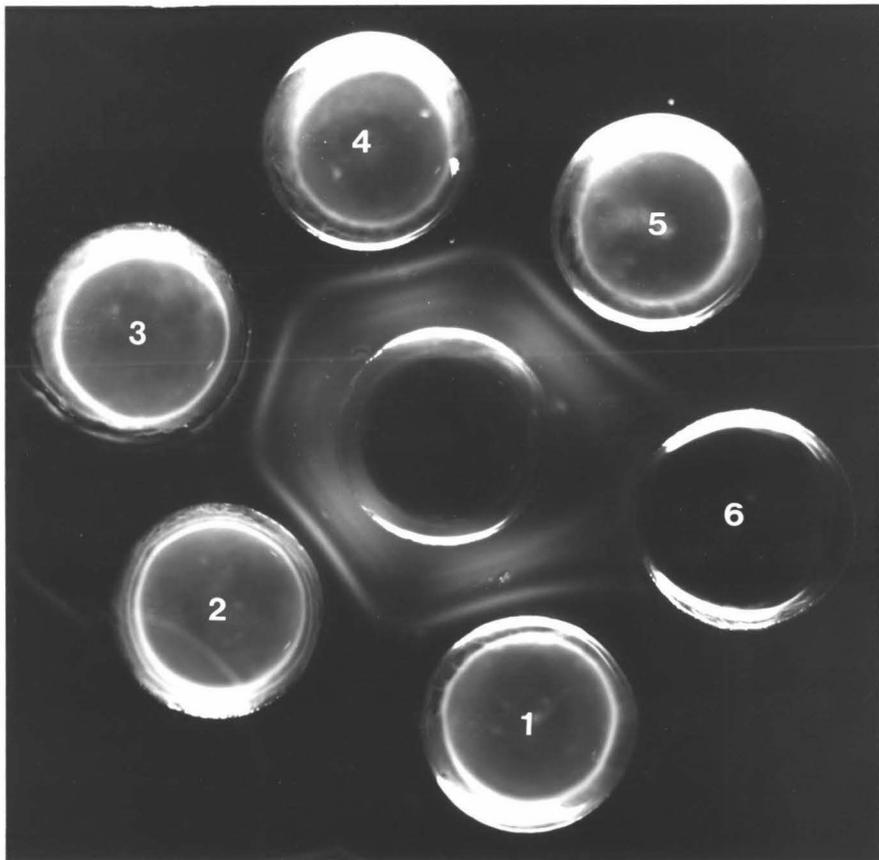


Figure 3 REA of 9 *M. ovipneumoniae* isolates from CNP lesions of lambs. The isolates came from 3 lambs from each of 3 farms. (Farms 1, 2 and 3).

Key: Lane 1: Lambda
Lanes 2, 3, 4: Farm 1, isolates a, b and c respectively.
Lanes 5, 6, 7: Farm 2, isolates a, b and c respectively.
Lanes 8, 9, 10: Farm 3, isolates a, b and c respectively.

The 3 isolates from farm 1 are almost indistinguishable i.e. 1a and 1b (lanes 2 and 3) are indistinguishable but isolate 1c (lane 4) differs in a few bands in the high molecular weight region.

Isolates 2b and 2c are almost indistinguishable but vary in the intensity of one of the high molecular weight bands. Isolate 2a is markedly different from isolates 2b and 2c.

The 3 isolates from farm 3 differ markedly from each other.

Isolates from different farms differ markedly from each other.

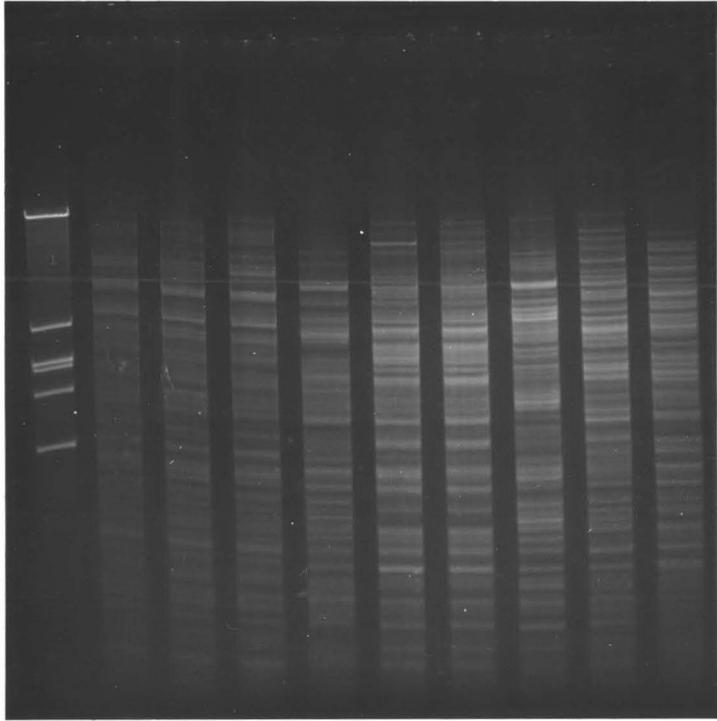


Figure 4. REA of 9 *M. ovipneumoniae* isolates from CNP lesions of lambs. The isolates came from 3 lambs from each of 3 farms (Farms 4,5 and 6).

Key: Lane 1: Lambda
Lanes 2, 3, 4: Farm 4, isolates a, b and c respectively.
Lanes 5, 6, 7: Farm 5, isolates a, b and c respectively.
Lanes 8, 9, 10: Farm 6, isolates a, b and c respectively.

The 3 isolates from farm 4 differ markedly from each other. The 3 isolates from farm 5 differ markedly from each other. Isolates 6b and 6c are indistinguishable. Isolate 6a is markedly different from isolates 6b and 6c.

Isolates from different farms differ markedly from each other.

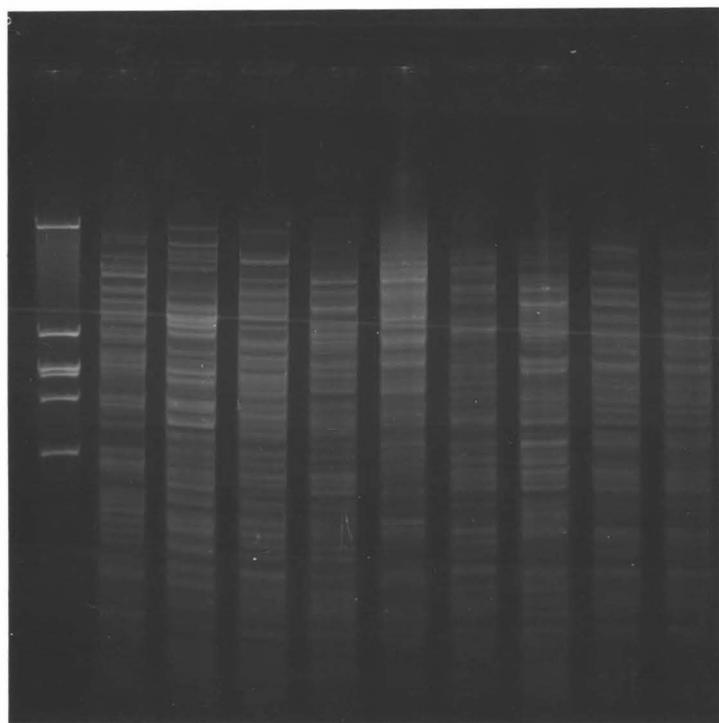


Figure 5. REA of 9 *M. ovipneumoniae* isolates from CNP lesions of lambs. The isolates came from 3 lambs from each of 3 farms (farms 7, 8 and 9.)

Key: Lane 1: Lambda

Lanes 2, 3, 4: Farm 7, isolates a, b and c respectively

Lanes 5, 6, 7: Farm 8, isolates a, b and c respectively

Lanes 8, 9, 10: Farm 9, isolates a, b and c respectively.

Isolates 7a and 7b are indistinguishable. Isolate 7c appears to differ slightly from isolates 7a and 7b in the higher molecular weight region.

The 3 isolates from farm 8 differ markedly from each other.

Isolates 9a and 9b show many similarities, but differ in a number of bands in the medium molecular weight region. Isolate 9c is markedly different from isolates 9a and 9b.

Isolates from different farms differ markedly from each other.

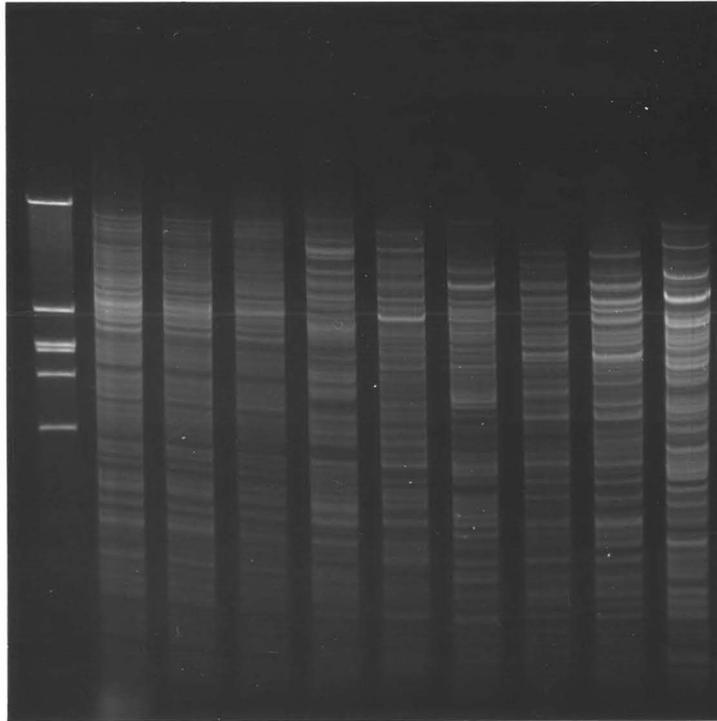


Figure 6. REA of 9 *M. ovipneumoniae* isolates from CNP lesions of lambs. The isolates came from 3 lambs from each of 3 farms (farms 10, 11 and 12).

Key: Lane 1: Lambda
 Lanes 2, 3, 4: Farm 10, isolates a, b and c respectively.
 Lanes 5, 6, 7: Farm 11, isolates a, b and c respectively.
 Lanes 8, 9, 10: Farm 12, isolates a, b and c respectively.

The 3 isolates from farm 10 differ markedly from each other.

Isolates 11a and 11b appear to be similar, if not indistinguishable. Isolate 11c differs markedly from isolates 11a and 11b.

The 3 isolates from farm 12 differ markedly from each other.

Isolates from different farms differ markedly from each other.

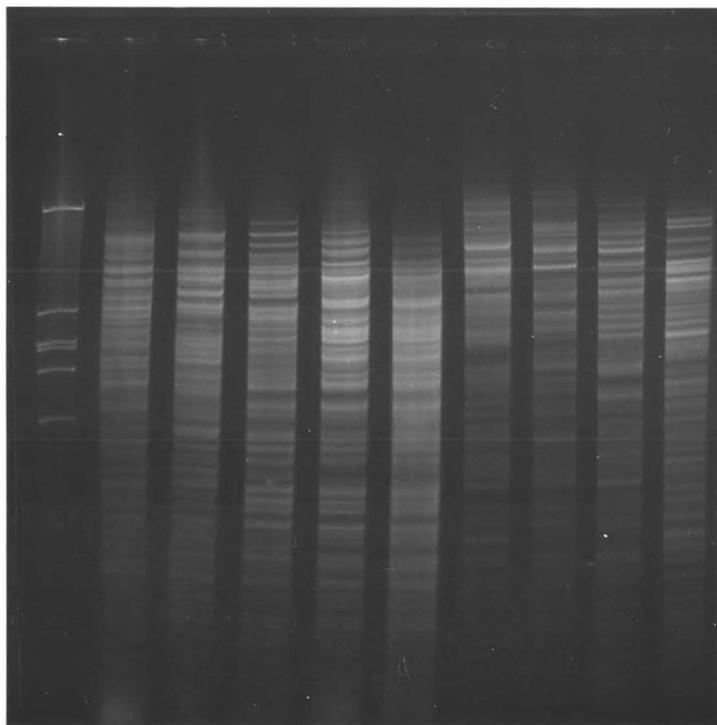


Figure 7 REA of 9 *M. ovipneumoniae* isolates from CNP lesions of lambs. The isolates came from 3 lambs from each of 3 farms (farms 13, 14 and 15).

Key: Lane 1: Lambda
Lanes 2, 3, 4: Farm 13; isolates a, b and c respectively.
Lanes 5, 6, 7: Farm 14, isolates a, b and c respectively.
Lanes 8, 9, 10: Farm 15, isolates a, b and c respectively.

Isolates 13b and 13c are almost indistinguishable, and vary only in the appearance of a band in the high molecular weight region of isolate 13b. Isolate 13a is markedly different from isolates 13b and 13c.

The 3 isolates from farm 14 differ markedly from each other.

Isolates 15a and 15c are indistinguishable, but 15b differs markedly from these.

Isolates from different farms differ markedly from each other.

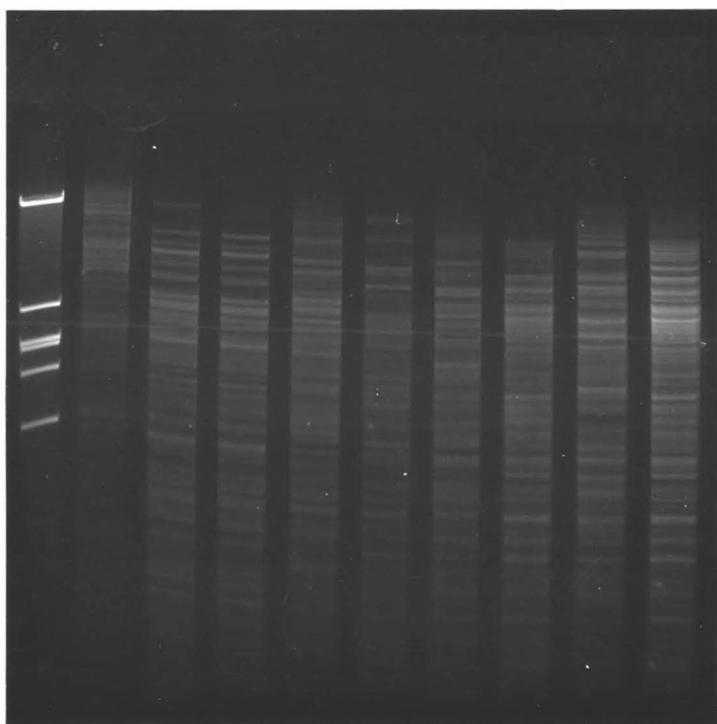


Figure 8. REA of 9 *M. ovipneumoniae* isolates from CNP lesions of lambs. The isolates came from 3 lambs from each of 3 farms. (farms 16, 17, and 18).

Key: Lane 1: Lambda
 Lanes 2, 3, 4: Farm 16, isolates a, b and c respectively.
 Lanes 5, 6, 7: Farm 17, isolates a, b and c respectively.
 Lanes 8, 9, 10: Farm 18, isolates a, b and c respectively.

Isolates 16b and 16c appear indistinguishable apart from minor differences in the high molecular weight region. Isolate 16a differs markedly from isolates 16b and 16c.

The 3 isolates from farm 17 differ markedly from each other.

The 3 isolates from farm 18 differ markedly from each other.

Isolates from different farms differ markedly from each other.

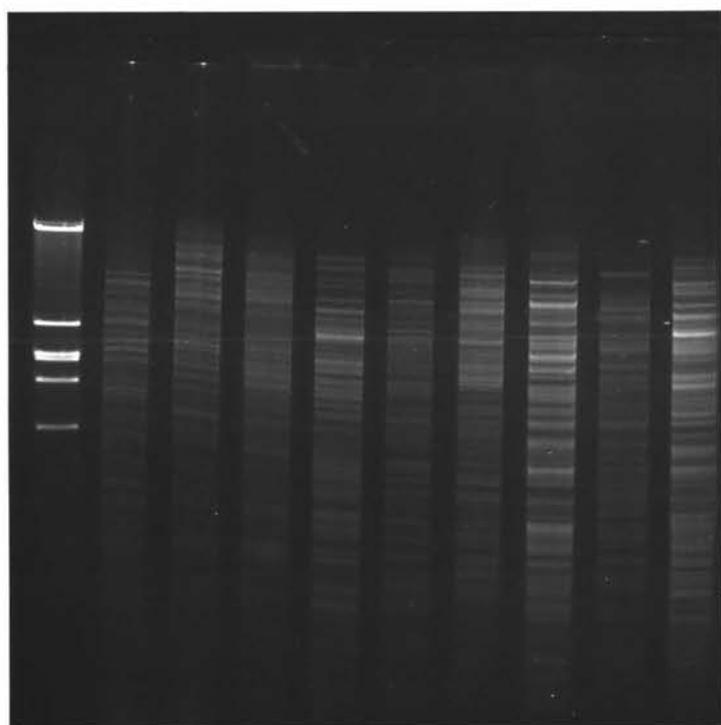


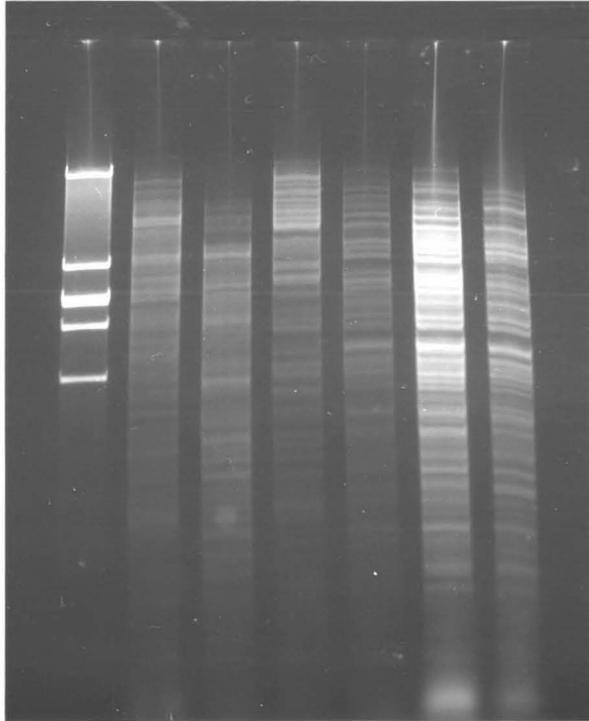
Figure 9. REA of 6 *M.ovipneumoniae* isolates from CNP lesions of lambs. The isolates came from 3 lambs from each of 2 farms (farms 19 and 20).

Key: Lane 1: Lambda
 Lanes 2, 3, 4: Farm 19, isolates a, b and c respectively.
 Lanes 5, 6, 7: Farm 20, isolates a, b and c respectively.

The 3 isolates from farm 19 differ markedly from each other.

Isolates 20a and 20b are indistinguishable from each other. Isolate 20c varies from isolates 20a and 20b in a few bands in the high molecular weight region.

Isolates from different farms differ markedly from each other.



2.4 Discussion

From the examination of these sixty isolates (i.e. three isolates from CNP lesions of three sheep on each of twenty farms), we observed that

(1) Isolates from sheep on different farms gave REA patterns which are markedly different from each other. However, a few bands common to many isolates were seen in the low molecular weight region. The common bands represent, at most, 5% of the total bands.

(2) Isolates from sheep on the same farm varied in their relationship to each other. Thus they could be:

(a) Indistinguishable from each other.

(b) Closely related to each other, with at least 95% of bands in common. The differences were normally confined to the high molecular weight region. (possibly due to the consequently greater number of regions on these large pieces of DNA that need only minor alteration by e.g. deletion or insertion of bases to become recognisable to *EcoRI* as a cutting site) or

(c) Markedly different from each other, with little or no similarity i.e. less than 5% of bands in common.

Furthermore all sixty of the isolates gave REA patterns that were markedly different from those observed in the study by Mew (1982). Thus the species *M. ovipneumoniae* shows considerable heterogeneity.

There are three general hypotheses which might explain why *M. ovipneumoniae* is heterogeneous with respect to REA.

1. A large number of different strains of *M. ovipneumoniae* which have evolved over a long time period, may be independantly maintained in the sheep population of New Zealand.

2. The genome of *M. ovipneumoniae* is "unstable" and

undergoes rapid internal genetic change due to "classical" type mutations (e.g. deletion/insertion mutants, point mutations, etc). or an internal rearrangement system (e.g. Transposons, Insertion sequences or Inversion sequences).

3. Frequent genetic interchange may occur between strains of *M. ovipneumoniae*. Thus random genetic interchange of what were initially two strains of *M. ovipneumoniae* with markedly different genomes, could generate an almost infinite number of strains which varied from their parental strains.

The first possibility i.e. that a large number of different strains of *M. ovipneumoniae* are independantly maintained, is almost impossible to prove directly, but gains plausibility by excluding other explanations, so most of the remainder of this thesis is concerned with the last two possibilities.

Chapter 3: Stability of the *M. ovipneumoniae* genome

3.1 SERIAL PASSAGE OF *M. ovipneumoniae* in vitro

3.11 Introduction

The greater heterogeneity of REA patterns of *M. ovipneumoniae* isolates (as seen in the previous chapter) when compared to other microorganisms (including other mycoplasmas), raises the question - "Why is the species *M. ovipneumoniae* so heterogeneous?".

One explanation is that the species undergoes rapid genetic change (without genetic interchange). This could be due to a variety of reasons including a high "classical" mutation rate (e.g. point mutations, deletion and/or insertion mutations) or the presence of insertion sequences, inversion sequences or transposons which facilitate internal genomic rearrangement.

For REA to be useful in epidemiological studies, the REA patterns must be stable. Zweerink *et al.*, (1981) examined the DNA of Varicella-Zoster virus before and after passage in cell culture. Only minor changes were seen, so they concluded that 'Varicella-Zoster virus DNA was sufficiently stable after multiple passages ... to make restriction endonuclease analysis a valuable epidemiological tool for strain differentiation'.

In a study of Herpes Simplex virus, type 2 (HSV-2) Smith *et al.*, (1981) suggested that HSV-2 REA patterns may vary after serial passage *in vivo*, especially if restriction enzymes that produced large numbers of fragments were used, and thus urged caution in the epidemiological application of restriction enzyme analysis. However, in reply, Roizman and Tognon (1982) suggested that the variation which occurred could be expected due to a normal rate of spontaneous mutation; and concluded "Interpretation of restriction endonuclease patterns, like that of all epidemiological tools, requires judgement and knowledge"(!).

Although the above results apply to viruses, the homogeneity of REA patterns of independent isolates of bacteria suggests that their patterns too are stable. For example Kakoyiannis *et al.*, (1984) found that REA patterns were conserved when 4 *Campylobacter coli* isolates, with different REA patterns, were subcultured on agar 23 times over a period of 45 days. Similarly O'Hara (personal communication) found that *Brucella ovis* reisolated from the same sheep several months after the initial isolation showed an identical REA pattern, and thus concluded that *B. ovis* were stable after passage *in vivo*.

Hintermann *et al.*, (1981) have further found that different growth conditions - e.g. varying the media, temperature and time of harvest - do not affect the REA patterns of the species *Streptomyces glaucescens*.

There is evidence that the REA patterns of some strains of *Moraxella bovis* may change after prolonged periods of *in vitro* passage (Marshall *et al.*, 1985), but these changes are minor and were not observed in all strains of the species. Field isolates collected from cattle in New Zealand over a four year period still show the same pattern (Marshall *et al.*, 1985).

The organisms discussed above appear to be genetically stable - i.e. few, if any, band changes occur after prolonged serial passage, but they are all relatively homogenous within a species. In contrast *M. ovipneumoniae* shows great heterogeneity when different isolates are compared, and it is thus possible that *M. ovipneumoniae* may undergo greater genetic change than the "average" microorganism. This section therefore investigates the stability of the *M. ovipneumoniae* genome by comparing REA patterns before and after passage.

3.12 Materials and Methods

Cloning of *M. ovipneumoniae* before serial passage

To maximise the probability that the selected *M. ovipneumoniae*

culture was derived from a single cell, the cloning procedure (see Chapter 2.24) was repeated three times. Aliquots of each clone were stored at -70°C .

Analysis of the Clone before passage

An aliquot of a clone, that had been cloned previously three times, was removed from storage and used to initiate a fresh culture. This culture was cloned once more by filtration through a $0.45\mu\text{m}$ filter and $100\mu\text{l}$ of the filtrate spotted on FM4 agar. 8 clones were selected and examined by REA (see Chapter 2.25 for a description of REA).

First passage stage

A clone (clone 4, see Figure 10) was selected at random from the 8 clones isolated before passage. This clone was passaged *in vitro* as follows: The (3.0ml) culture was diluted 1:100 each day into FM4 medium and incubated at 37°C on a rotary shaker. (1:100 was the maximum dilution which reproducibly allowed a colour change to develop within each 24 hour period and represents about 6.6 generations). Each day for the 20 days (representing about 133 generations), a 1.5ml aliquot was taken from the culture, and frozen at -70°C for future reference. Note: After the 20th day of passage, 8 clones were selected (see Chapter 2.24) and examined by REA (see Chapter 2.25).

Labelling of Cultures

Clones derived from the experiments which examined the REA patterns of *M. ovipneumoniae* before and after serial passage *in vitro* were labelled to indicate the number of days elapsed since the first day of passage and also indicate the sequence of clones they were derived from. Thus, for example, clone P60N4636 represents the fourth (of 8 clones) selected from the clone P40N463 after 20 days serial passage *in vitro*. Similarly clone P40N463 was the third (of 8 clones) selected from the clone P20N46 after 20 days serial passage *in vitro*. Thus clone P20N46 was

the sixth clone (of 8 clones) selected from the clone PON4 after 20 days serial passage *in vitro*, and clone PON4 was the fourth (of 8 clones) selected from the original triply cloned isolate of *M. ovipneumoniae*.

Second passage stage

Two clones (P2ON45 and P2ON46) derived from the same initial clone (PON4), but which differed slightly in their REA patterns after the first stage of passage (see results: Figure 11) were selected and passaged for a further 20 days as described in the first passage stage. 8 clones were selected from each culture (see Chapter 2.24) and examined by REA (see Chapter 2.25).

Third passage stage

Three clones (P4ON461, P4ON463 and P4ON464) derived from the same initial clone (P2ON46) but which differed slightly in their REA patterns after the second stage of passage (see results: Figures 13 and 14) were selected and passaged for a further 20 days as described in the first passage stage.

8 clones were selected from each culture (see Chapter 2.24) and examined by REA (see Chapter 2.25).

3.13 Results

The REA patterns of the DNA of *M. ovipneumoniae* before and after several passages can be seen in Figures 10 through to 16. Each Figure is accompanied by a flow diagram on its facing page which indicates the relationship of the Figure to the serial passage of *M. ovipneumoniae*. (The Figure represents the clones within the box in the flow diagram).

Figure 10 shows the REA patterns of the 8 clones selected before passage.

Figure 11 shows the REA patterns of the 8 clones selected after the first passage stage, which represents approximately

133 cell generations.

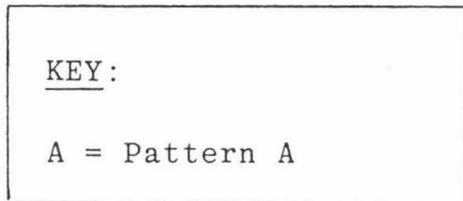
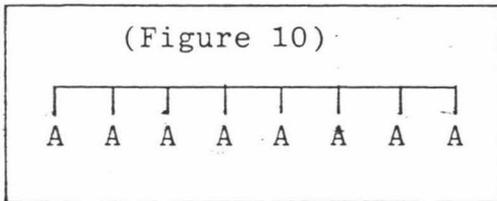
Figures 12 and 13 show the REA patterns of the clones selected after the second passage stage, which represents a further 133 cell generations approximately.

Figures 14, 15 and 16 show the REA patterns of the clones selected after the third passage stage, which represents a further 133 cell generations approximately.

Figure 17 shows the differences detected in REA patterns, and Figure 18 shows a flow diagram of the above experiments.

Figure 10. *M. ovipneumoniae*, strain 5, was re-cloned three times and 8 clones were selected from the resulting culture. Lane 1 contains lambda DNA, the other lanes correspond to the letters in the box in the flow diagram below. All clones show an identical pattern (pattern A).

M. ovipneumoniae cloned 3 times



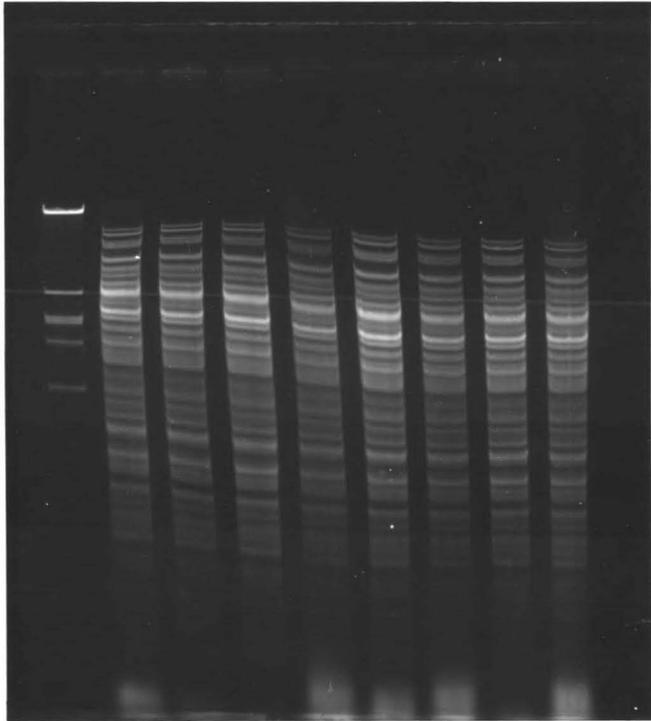
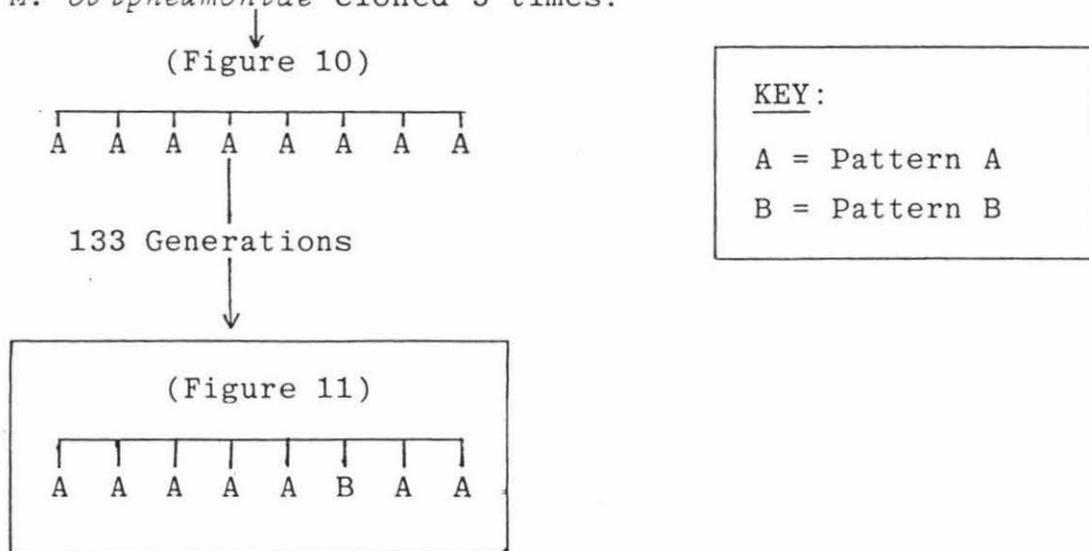


Figure 11. A clone of *M. ovipneumoniae*, strain 5, showing pattern A was serially passaged *in vitro* for approximately 133 generations and 8 clones were selected from the final culture. The lanes correspond to the letters in the box in the flow diagram below. Two patterns can be seen: Pattern A (lanes 1,2,3,4,5,7 & 8), and a new pattern, pattern B (lane 6). These patterns differ in several regions, the most clearly defined difference being the appearance of two closely opposed bands in the high molecular weight region.

M. ovipneumoniae cloned 3 times.



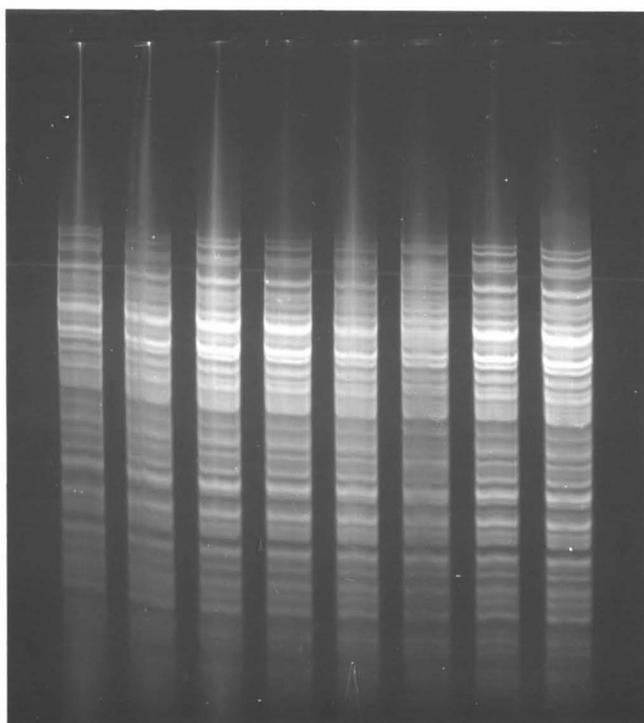


Figure 12. A clone of *M. ovipneumoniae*, strain 5, showing pattern A was serially passaged *in vitro* for approximately 133 generations and 8 clones were selected from the final culture. Lane 1 contains lambda DNA, the other lanes correspond to the letters in the box in the flow diagram below. Two patterns can be seen: Pattern A (lanes 3,4,6,8 & 9) and pattern B (lanes 2,5 & 7). See figure 17 for a description of the differences between patterns.

M. ovipneumoniae cloned 3 times

(Figure 10)

A A A A A A A A

133 Generations

(Figure 11)

A A A A A B A A

133 Generations

(Figure 12)

B A A B A B A A

KEY:

A = Pattern A

B = Pattern B

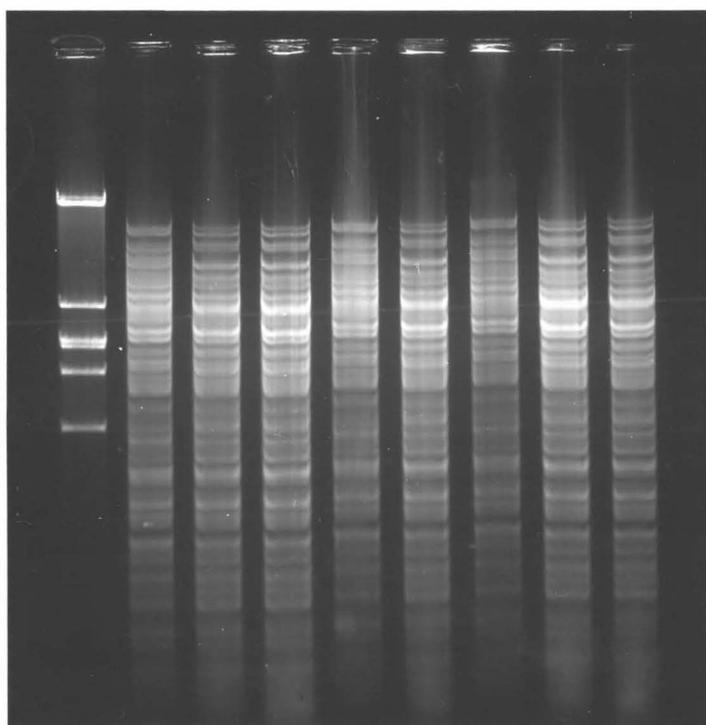
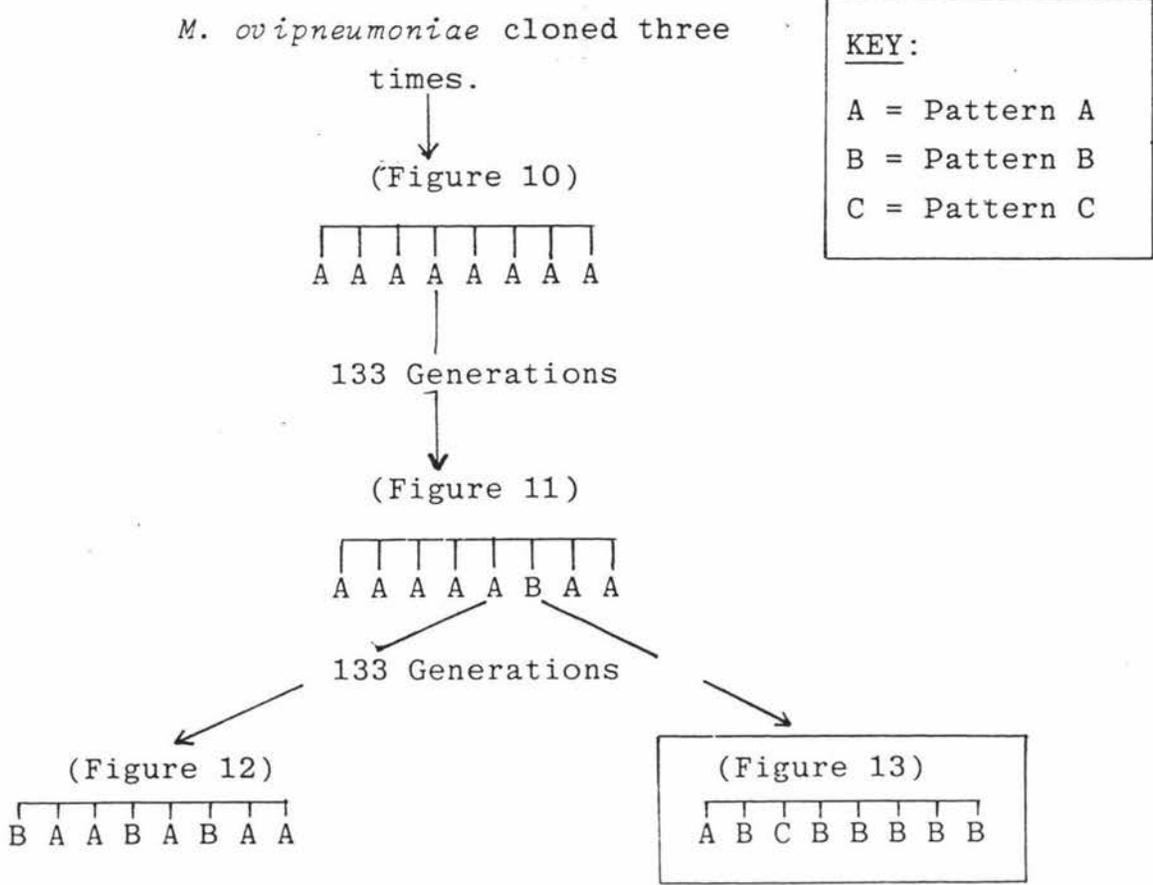


Figure 13. A clone of *M. ovipneumoniae*, strain 5, showing pattern B was serially passaged *in vitro* for approximately 133 generations and 8 clones were selected from the final culture. Lane 1 contains lambda DNA, the other lanes correspond to the letters in the box in the flow diagram below. Three patterns can be seen: Pattern A (lane 2), pattern B (lanes 3,5,6,7,8 & 9), and a new pattern - pattern C (lane 4), which closely resembles pattern B, only differing in an additional 2 bands in the high molecular weight region (arrows). - See figure 17 for a description of the differences between patterns.



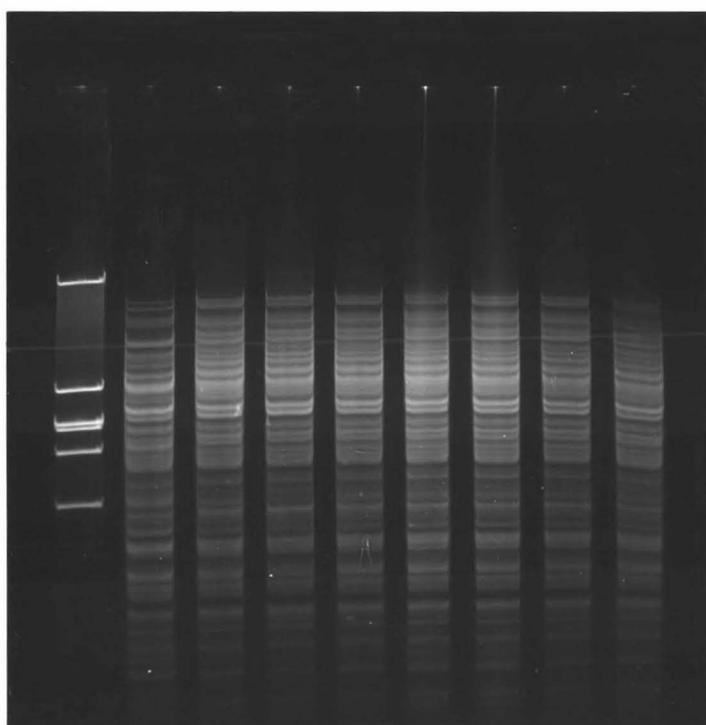
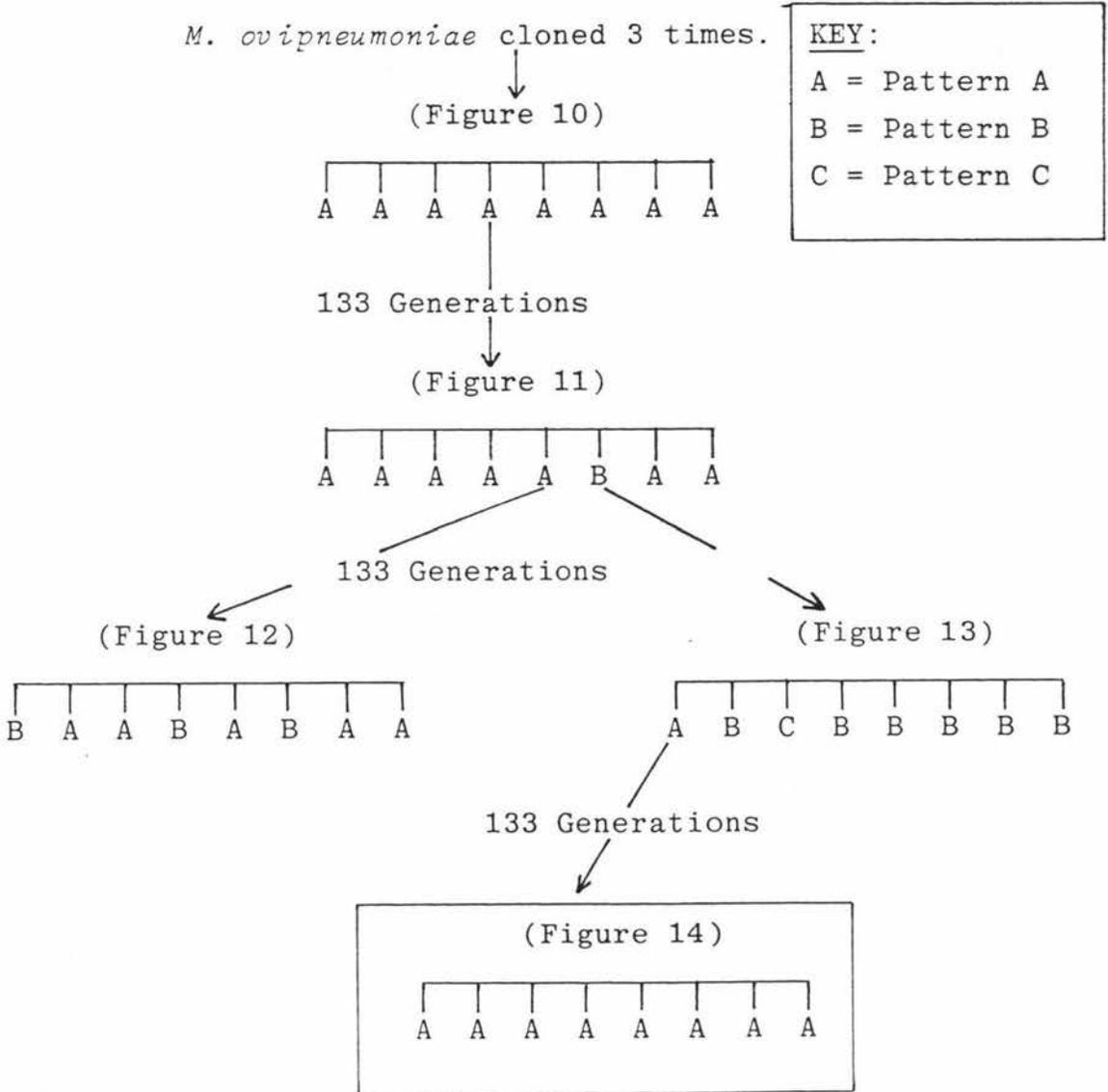


Figure 14. A clone of *M. ovipneumoniae*, strain 5, showing pattern A was serially passaged *in vitro* for approximately 133 generations and 8 clones were selected from the final culture. Lane 1 contains lambda DNA, the other lanes correspond to the letters in the box in the flow diagram below. All clones show an identical pattern (pattern A).



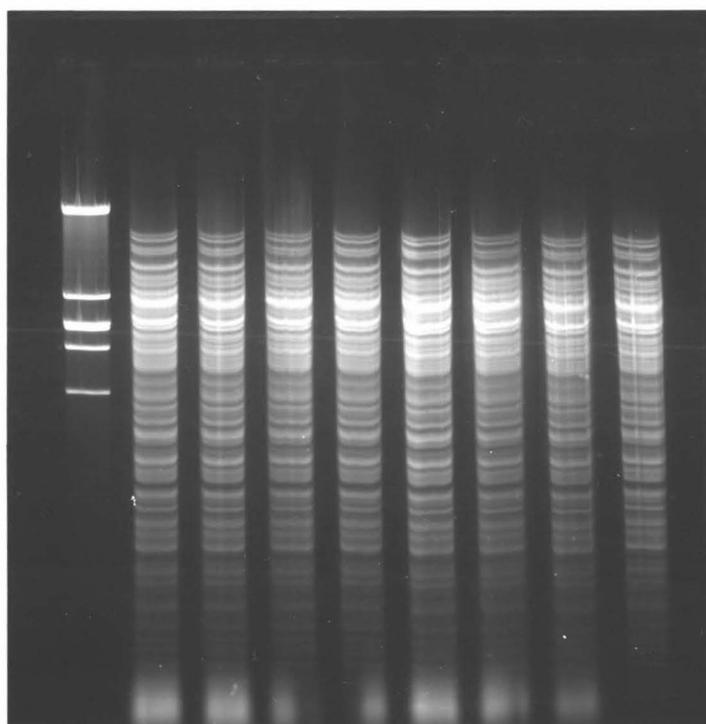
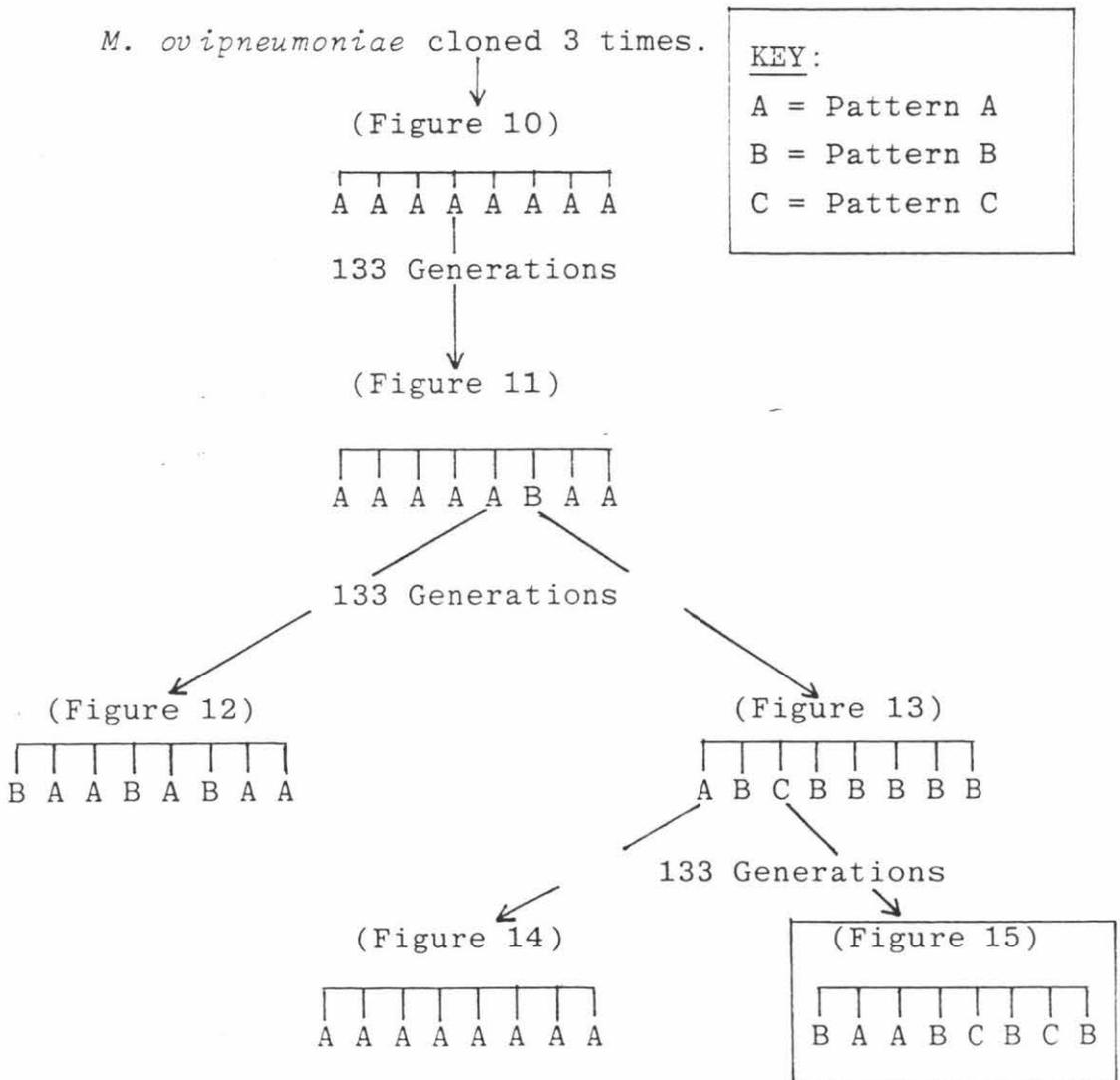


Figure 15. A clone of *M. ovipneumoniae*, strain 5, showing pattern C was serially passaged *in vitro* for approximately 133 generations and 8 clones were selected from the final culture. Lane 1 contains lambda DNA, the other lanes correspond to the letters in the box in the flow diagram below. Three patterns can be seen: Pattern A (lanes 3 & 4), pattern B (lanes 2,5,7 & 9), and pattern C (lanes 6 & 8). - See figure 17 for a description of the differences between patterns.



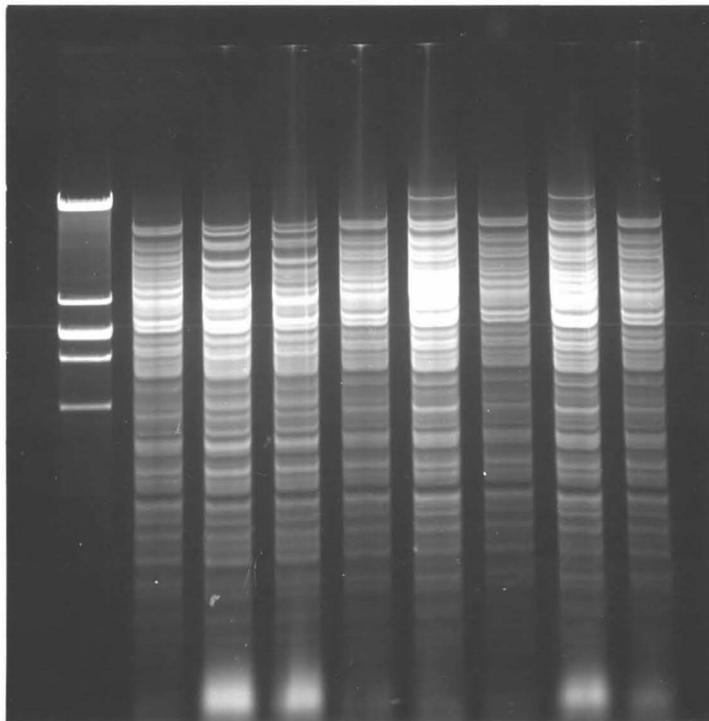
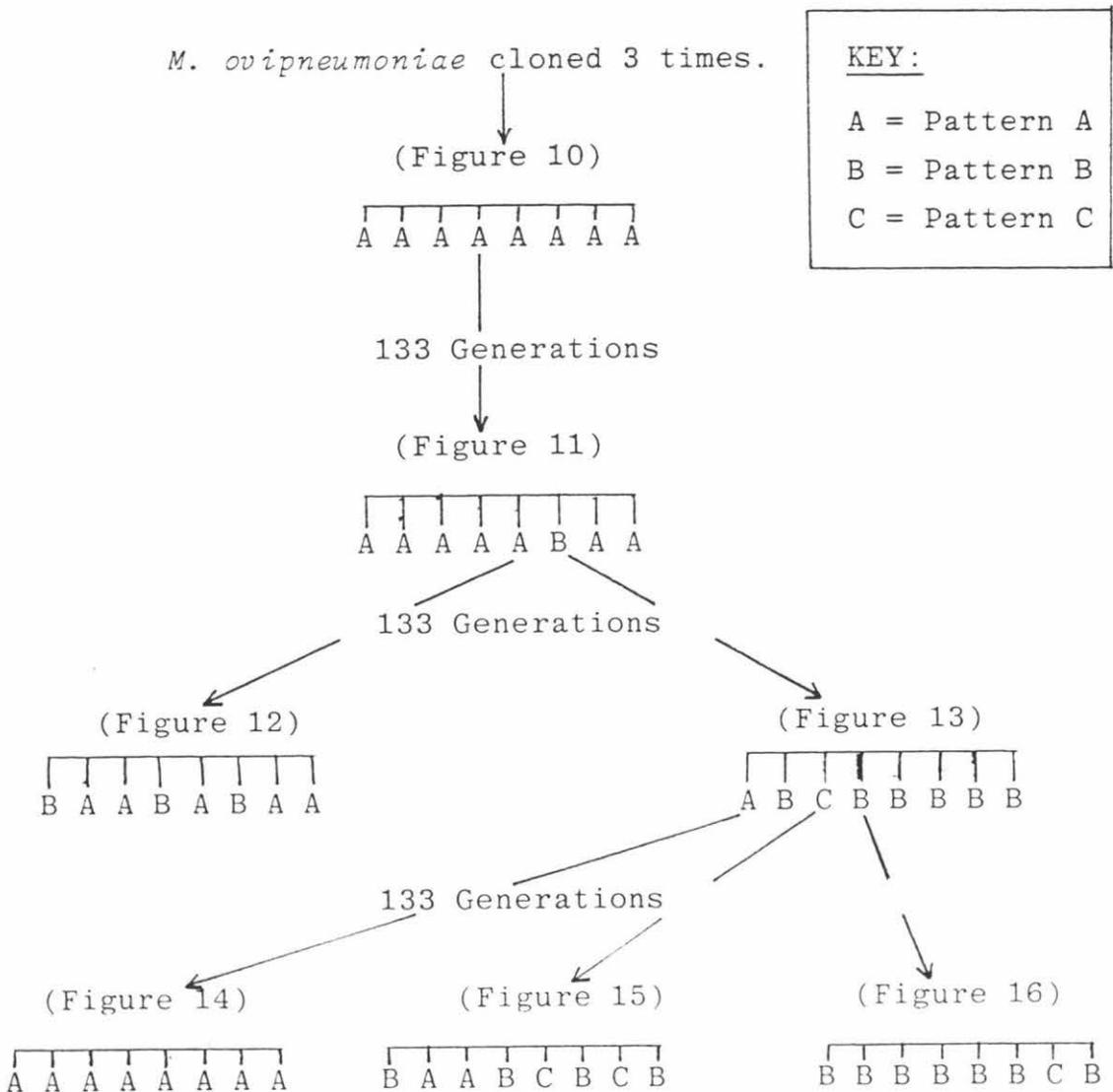


Figure 16. A clone of *M. ovipneumoniae*, strain 5, showing pattern B was serially passaged *in vitro* for approximately 133 generations and 8 clones were selected from the final culture. Lane 1 contains lambda DNA, the other lanes correspond to the letters in the box in the flow diagram below. Two patterns can be seen: Pattern B (lanes 2,3,4,5,6, 7 & 9) and pattern C (lane 8). - See figure 17 for a description of the differences between patterns.



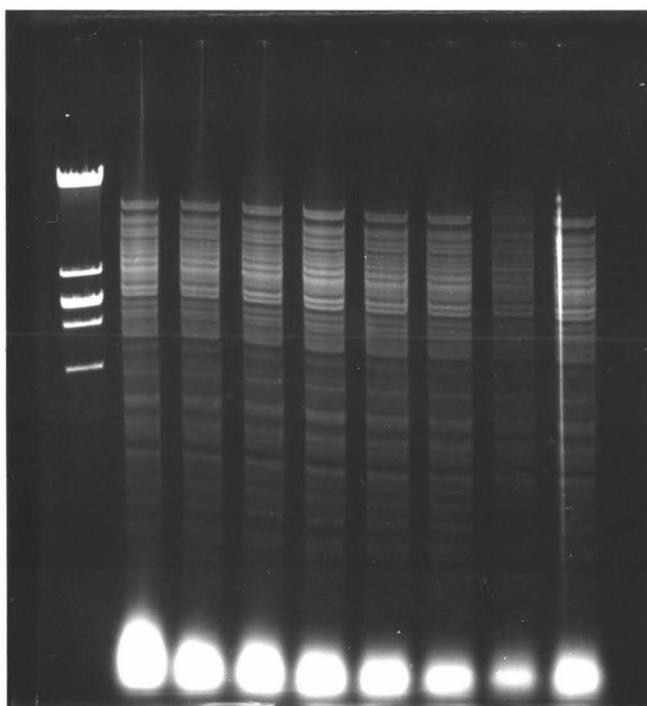


Figure 17: REA showing the differences between patterns A, B and C (lanes 1, 2 and 3 respectively). Pattern A represents the original clone before passage. Patterns B and C appeared after this clone has been passaged *in vitro*. Pattern B differs from pattern A in at least 4 regions. The most clearly defined difference is the appearance of two closely opposed bands in the higher molecular weight region (). Pattern C is similar to pattern B, but has 2 additional bands in the higher molecular weight region ().

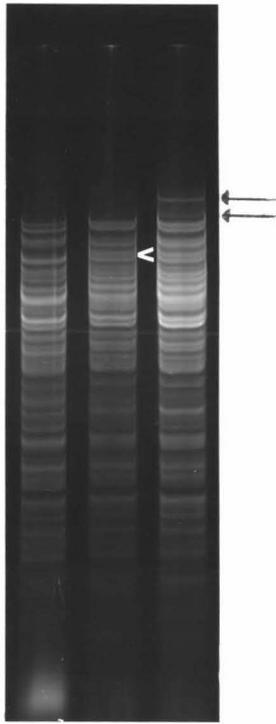


FIGURE 18. Flow diagram illustrating the serial *in vitro* passage of a *M. ovipneumoniae* strain 5 clone - See figure 17 for a description of the differences between patterns.

M. ovipneumoniae cloned 3 times.

(Figure 10)

A A A A A A A A

133 Generations

(Figure 11)

A A A A A B A A

133 Generations

(Figure 12)

B A A B A B A A

(Figure 13)

A B C B B B B B

133 Generations

(Figure 14)

A A A A A A A A

(Figure 15)

B A A B C B C B

(Figure 16)

B B B B B B C B

KEY:

A = Pattern A
 B = Pattern B
 C = Pattern C

3.14 Conclusion

Several changes were detected in the REA patterns of a clone of *M. ovipneumoniae* after serial passage *in vitro*. Thus three different patterns (patterns A, B & C) were detected. Before the implications of these pattern changes are considered, it is first necessary to determine if these differences are in fact real.

3.2 REPRODUCIBILITY OF REA PATTERN DIFFERENCES OBSERVED AFTER THE SERIAL PASSAGE *in vitro* OF *M. ovipneumoniae*

3.21 Introduction

It was found in the previous section that some changes occur in the REA patterns of strain of *M. ovipneumoniae* after serial passage *in vitro*. Thus after serial passage, three patterns (patterns A, B, and C) were observed (see Figure 17).

The same patterns reoccurred later in passage stages, and were repeatable under the same digest conditions. This indicates that the differences are real. Nevertheless, it is possible that the different patterns observed may merely be due to unidentified technical differences in processing.

To show that the differences are real, duplicate cultures of clones that showed the A, B, or C. patterns were recultured, independently processed and re-examined.

In further experiments the enzyme concentration was varied to find if this affected the REA patterns.

3.22 Materials and Methods REA of duplicate cultures

Frozen stock cultures of *M. ovipneumoniae* clones that showed REA patterns A, B, and C after serial passage *in vitro* (see Figure 17) were thawed and duplicate cultures of each prepared. DNA was extracted from each of the 6 cultures individually, and each was independently examined by REA (see Chapter 2.25).

Effect of increased enzyme concentration on REA patterns

Nucleic extracts from *M. ovipneumoniae* clones that showed patterns A, B, and C after serial passage *in vitro* (see Figure 17) were examined by REA using the following concentrations of *Eco*RI: 8 units, 20 units and 40 units.

The volumes of digest mixture were equalised for

centrifugation by addition of appropriate volumes of distilled water to give a total digest volume of 102 μ l

3.23 Results

REA of duplicate cultures

The results of REA of duplicate cultures of clones that showed REA patterns A, B, and C after serial passage *in vitro* (see Figure 17) can be seen in Figure 19. The REA patterns were reproduced by the duplicate cultures, and were the same as the REA patterns originally seen in these clones.

Effect of increased enzyme concentration on REA patterns

The results of increased enzyme concentration on the REA patterns of clones that showed REA patterns A, B and C after serial passage *in vitro* (see Figure 17) can be seen in Figure 20. No changes were observed within the clones that showed patterns A, B or C when an increased concentration of enzyme was used.

Figure 19 REA of duplicate cultures of *M. ovipneumoniae* clones that showed REA patterns A, B and C (See Figure 17) after serial passage *in vitro*.

Key:	Lane 1:	Lambda
	Lanes 2 and 3:	Pattern A
	Lanes 4 and 5:	Pattern B
	Lanes 6 and 7:	Pattern C

No differences were seen between REA patterns of duplicate cultures (i.e. Lanes 2 and 3, Lanes 4 and 5, Lanes 6 and 7).

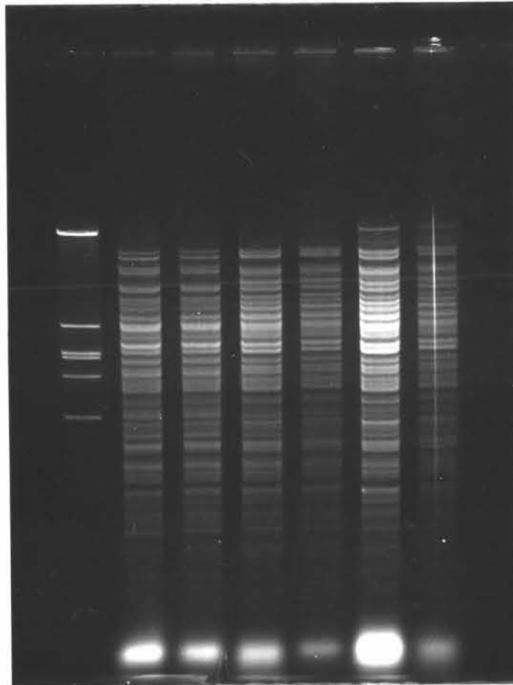


Figure 20 REA of *M. ovipneumoniae* clones that showed patterns A, B and C (see Figure 17) after serial passage *in vitro*, digested with increased concentrations of *EcoRI*.

Key: Lane 1: Lambda

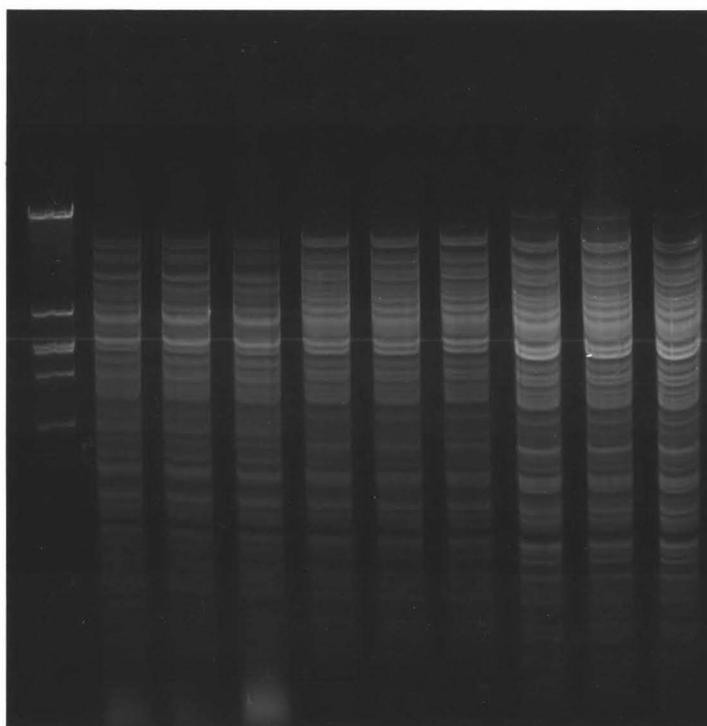
Lanes 2, 3 and 4: Pattern A digested with 8, 20 and 40 units of *EcoRI* respectively.

Lanes 5, 6 and 7: Pattern B digested with 8, 20 and 40 units of *EcoRI* respectively.

Lanes 8, 9 and 10: Pattern C digested with 8, 20 and 40 units of *EcoRI* respectively.

Note: The normal amount of *EcoRI* isolates of *M. ovipneumoniae* were digested with was 8 units.

No changes in the REA patterns A, B and C are seen (i.e. Lanes 2, 3 and 4; Lanes 5, 6 and 7; Lanes 8, 9 and 10).



3.24 Conclusion

Duplicate cultures prepared independantly gave identical REA patterns. Similarly, when the *EcoRI* concentration was varied for a particular isolate, the resultant REA patterns were identical for that isolate.

We therefore concluded that the changes in REA patterns of *M. ovipneumoniae*, strain 5, which occurred after serial passage *in vitro* are reproducible and therefore represent real changes in the DNA.

3.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE PROTEINS OF *M. ovipneumoniae* CLONES WHICH SHOWED DIFFERENT REA PATTERNS AFTER SERIAL PASSAGE *in vitro*

3.31 Introduction

The appearance of reversible genome rearrangements in *M. ovipneumoniae* seen after serial passage *in vitro* (see Chapter 3.1) prompted the question: "Do these reversible changes in the genome have any antigenic significance?"

CNP lesions have a long duration and may persist for up to 7 months (Gilmour *et al.*, 1982). Antigenic changes in the species *M. ovipneumoniae* could account for this long duration. Thus each strain of *M. ovipneumoniae* might have a number of antigenic conformations and elimination of the organism may require the presence of antibody to all of these forms.

Borelia recurrentis, the causative organism of relapsing fever in humans, causes relapses of the disease by such a mechanism. That is, in each successive attack of the disease the organism is antigenically different. It has been suggested that this antigenic variation occurs as a limited, reproducible sequence of change (Garling and Basman., 1980).

Salmonella species may also undergo antigenic changes. These reversible "phase" changes occur in the flagellar (H) antigens and are due to a reversible inversion of a segment of DNA (Davis., 1980).

In the limited time available, serological tests to determine any variability between the clones of *M. ovipneumoniae* showing different REA patterns after serial passage *in vitro* were not feasible. However, proteins are ultimately responsible (either directly or indirectly) for the antigenic identity of all organisms. Protein changes may be detectable by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), so in this section the proteins of each of the three variants

are compared by this method.

3.32 Materials and Methods

Preparation of cell lysate

A 1.5ml stock culture of *M. ovipneumoniae* at -70°C was thawed and inoculated into 15ml FM4 medium. This culture was incubated over night at 37°C on a rotary shaker until a colour change was observed. This was then inoculated into 300ml of FM4 medium, and incubated again over night at 37°C on a rotary shaker until a colour change indicated that the medium had reached pH 7.0 approximately. The cells were collected by centrifugation at 14500g for 15 minutes at room temperature, washed twice in 20ml PBS and finally PBS was added to give an approximately 10% suspension.

The cells were lysed by sonication at 20kc/s with 8 x 15 second bursts, using an MSE 1-0 watt ultrasonic disintegrator. The lysed suspension was frozen at -70°C until required.

Estimation of Protein content of *M. ovipneumoniae* lysates

Preparation of standard curve

A standard curve was prepared by using a range of Bovine serum albumin (BSA) solutions (see Appendix 3.1) in 0.2M NaOH and heated at 100°C for 3 minutes (to simulate the preparation of *M. ovipneumoniae* proteins). The solution was cooled to room temperature, 5 ml of Comassie Blue reagent (see Appendix 3.2) was added to each sample, and mixed by inversion.

The absorbance of the solutions was measured at 595nm using a Spectronic 20 and a standard curve prepared.

Protein Assay of *M. ovipneumoniae*

A 0.1ml aliquot of the lysed suspension was thawed and

diluted $\frac{1}{3}$, $\frac{1}{10}$, $\frac{1}{30}$, and $\frac{1}{100}$ in 0.2M NaOH to give a final volume of 0.1ml. Proteins were solubilised by heating at 100°C for 3 minutes. The solutions were cooled to room temperature, 5ml of Coomassie-Blue reagent was added to each, and the contents mixed by inversion. The absorbance of each was measured at 595nm, using a Spectronic 20, and the protein content determined from the standard curve (see previous section).

The volume of lysate which contained 80 μ g of protein (this being the optimum load for good resolution of the gels) was calculated for each sample.

Preparation of the *M. ovipneumoniae* Protein Samples for SDS-PAGE

To a 0.1ml aliquot of the original lysed call suspension, 24 μ l of SDS-sample buffer (4 x concentrated - see appendix 3.3) and 12.5 μ l of Bromophenol Blue (10 x concentrated - see Appendix 3.4) were added. This was thoroughly mixed and heated to 100°C to solubilise the protein. The solution was cooled to room temperature, for SDS-PAGE.

Preparation of SDS-PAGE Gel

General Description

A vertical slab gel system was used for SDS-PAGE. The gel solution was polymerised between two glass plates, and electrophoresed vertically between the upper cathode reservoir buffer, and the lower anode reservoir buffer.

Preparation and Assembly of the Gel Apparatus

Two rectangular plates (5.5 x 170 x 130mm) were used, one of which had a rectangular portion removed from the upper end, to allow contact between the upper reservoir buffer, and the gel.

Immediately before assembly, the glass plates and spacers (135 x 12 x 1.5mm perspex) were washed in 5% Alconox

and dried. The assembly procedure consisted of running a small thread of petroleum jelly near the edge of the two sides and the base of the plate with the rectangular portion removed. The 3 spacers were placed on the jelly, ensuring that the base spacer could be easily removed at a later stage, and another thread of petroleum jelly was placed on top of the spacers. The other glass plate was placed on top of the spacers, secured by two 'bull-dog' clips, and clamped in a stand ready for the gel solution to be added.

Preparation of Running gel

Initially, 100mg of Ammonium Persulphate was dissolved in 1ml of distilled water. While the Ammonium Persulphate was dissolving, 6.7ml of Acrylamide solution (see Appendix 3.5) was added to 5.0ml lower Tris Buffer (see Appendix 3.6), then 8.3ml of distilled water, 0.05ml of the Ammonium Persulphate solution and finally 0.01ml of TEMED (see Appendix 3.7) were added and the solution mixed between each addition.

The running gel was poured into the gel mould, to a level approximately 110mm from the base spacer. (Note: The plates were held at about a 45° angle to the horizontal to ensure that no air bubbles were trapped within the gel). Approximately 1.0ml of distilled water was layered on top of the gel. This provided the anaerobic environment necessary for gel polymerisation, which took approximately one hour at room temperature (although on some occasions, the gel was left overnight at 4°C to polymerise).

Preparation of the Stacking Gel

100mg of Ammonium Persulphate was dissolved in 1.0ml of distilled water. While it was dissolving, 1.5ml of Acrylamide solution was added to 2.5ml of Upper Tris Buffer (see Appendix 3.8). Then 6.0ml of distilled water, 0.01ml of TEMED and 0.03ml of the Ammonium Persulphate solution was added to the solution (Note: The solution was thoroughly mixed after each addition).

The layer of water was decanted from the polymerised running gel. Approximately 1.5ml of the unpolymerised stacking gel was added and then decanted. (This washing procedure allowed the stacking gel to adhere to the running gel).

The stacking gel was added slowly (avoiding air bubbles) to the top of the running gel and a perspex "comb" was pushed between the glass plates to a distance of 10mm above the running gel/stacking gel interface.

Polymerisation took approximately 20 minutes at room temperature. After polymerisation, the perspex "comb" was removed, and each well was rinsed with Tris-glycine buffer, pH8.3 (see Appendix 3.9) immediately prior to use. The lower spacer was also removed.

SDS PAGE

Application of the Protein samples to the Gel

The apparatus containing the gel was clamped to the stand and Tris-glycine buffer, pH8.3, was added to the upper and lower reservoirs. This filled the wells of the gel. *M. ovipneumoniae* cell lysate protein solutions containing 80ug of protein (see Chapter 3.22, 'Protein Assay of *M. pneumoniae*') were added to the wells. (The glycerol in the Bromphenol Blue dye ensured that the samples settled to the bottom of the wells).

Electrophoresis

Samples were electrophoresed through the stacking gel at an initial current of 10mA. This was increased to 15mA when the Bromophenol Blue tracking dye reached the running gel. Electrophoresis continued until the tracking dye had moved almost 110mm through the running gel.

Processing of the Gel

Once Electrophoresis was completed, the gel was removed from between the glass plates, a small piece cut from the

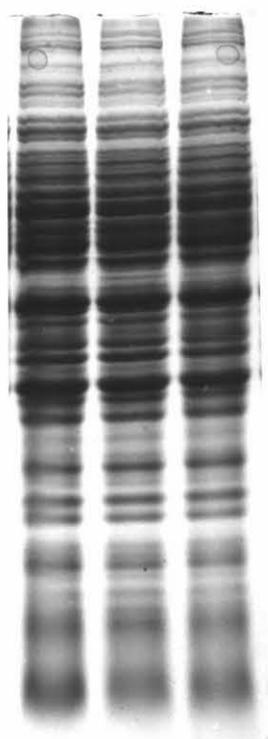
lower left hand corner (to aid orientation of the gel at a later stage), and the gel was stained over night in Coomassie Blue solution. (see Appendix 3.10).

The gel was destained by washing in several changes of 10% (w/v) acetic acid until the background was clear. After the final wash, the acetic acid was replaced by distilled water, The gel was washed in this distilled water until no more acetic acid could be detected by smell. The gel was then photographed.

3.33 Results

The results of SDS-PAGE of the cellular proteins of *M. ovipneumoniae* clones which showed REA patterns A, B and C (see Figure 17) after serial passage *in vitro* (see chapter 3.1) can be seen in Figure 21.

Figure 21. SDS-PAGE of the cellular proteins of *M. ovipneumoniae* clones which showed REA patterns A, B and C (see Figure 17) after serial passage *in vitro* (see Chapter 3.1). No differences can be detected in the SDS-PAGE patterns.



3.34 Conclusion

No protein differences were detected between clones of *M. ovipneumoniae* (that showed different REA patterns after serial passage *in vitro*) by SDS-PAGE.

3.4 Discussion

Changes occurred in the REA patterns of a clone of *M. ovipneumoniae* after serial passage *in vitro*. Thus 3 slightly different patterns, *viz.* A, B and C, occurred. The sequences of pattern changes seen were as follows: A changed to B, B changed to A or C, and C changed to patterns A or B. The pattern changes were therefore reversible. This reversibility would be unlikely to occur if the changes were due to "Classical" type mutations (i.e. Mutations which occur during DNA synthesis or are due to the effect of a mutagen e.g. point mutations, deletion, and insertion mutations, etc.). It is therefore concluded that the changes which occurred after serial passage of *M. ovipneumoniae in vitro* are due to a controlled internal rearrangement system e.g. Insertion sequences, Inversion sequences, Transposons etc.

The reversible changes seen in the REA patterns of *M. ovipneumoniae* after serial passage *in vitro* were not paralleled by changes in the protein component of the organism, as seen by SDS-PAGE of the cellular proteins.

While this gives no support to the hypothesis that antigenic change may occur in serially passaged *M. ovipneumoniae* it does not disprove it either i.e. if a protein was no longer produced but was replaced by another of the same molecular weight. This would not be detectable by SDS-PAGE.

These observations will be further considered in the general discussion.

Chapter 4: GENETIC INTERCHANGE

Introduction

The extreme heterogeneity seen in *M. ovipneumoniae*, when examined by REA, can be explained in several ways, one of which is the possible presence of a system of genetic interchange. Thus, for example, if two *M. ovipneumoniae* strains which showed major differences in REA patterns frequently exchanged portions of DNA, it could lead to the existence of a large number of "ever-changing" REA patterns.

There are three mechanisms by which exchange of DNA could occur: Conjugation, Transduction and Transformation. We consider here the limited evidence of such transfers in the class Mollicutes.

Conjugation

Conjugation relies on the presence of transmissible plasmids, so the presence of extrachromosomal DNA might indicate an ability to undergo conjugation.

Extrachromosomal DNA (in the form of closed circular DNA) has been reported in four species representing three genera belonging to the class Mollicutes i.e. *Mycoplasma arthritidis* (Haller Lynn, 1968), *Mycoplasma hominis* (Zouzias *et al.*, 1973), *Acholeplasma laidlawii* (Dugle and Dugle, 1971), *Spiroplasma citri* (Bove and Saillard, 1979; Randhard *et al.*, 1980). These species have been shown to contain plasmids of a molecular weight between 1×10^6 and 28×10^6 daltons). This of course does not mean that plasmids are ubiquitous in Mycoplasmas or if they do occur that they are transmissible. To date, there has been no reports of evidence to indicate the presence of a transmissible plasmid in the class Mollicutes.

Transduction

Since Transduction requires a bacteriophage vector, the presence of a bacteriophage within the class Mollicutes may

indicate the possibility that species within the class may undergo transduction. Most viruses detected that infect the class Mollicutes infect the family Acholeplasmataceae, however a few have been detected in the family Mycoplasmataceae (Gourlay *et al.*, 1982; Maniloff *et al.*, 1982). None of these viruses have so far been shown to be associated with Transduction.

Transformation

In bacteria which can undergo transformation, DNA appears to be first bound and then taken up at zones of cell wall synthesis (Davis, 1980). Transfection (a form of transformation) can be induced in bacteria by first removing the cell wall (i.e. to form spheroplasts) and then treating these spheroplasts with (e.g.) polyethylene glycol or CaCl_2 to make the membrane more permeable. The absence of a cell wall in the class Mollicutes, suggests that they may be amenable to transformation/transfection.

Folsome (1967) showed that *Achdeplasma laidlawii* was capable of binding DNA, as in transformation, but was unable to conclusively prove that any were in fact transformed. Sladek and Maniloff (1983) have since transfected *A.laidlawii* (treated with polyethelene glycol) at low efficiency (10^{-4} transfectants per CFU) with DNA from a Mycoplasma virus. Furness and Cerone (1979), using DNA extracted from a tetracycline resistant strain of *Mycoplasma hominis*, reported that *M. hominis* and *Mycoplasma salivarium* were transformed to tetracycline resistance. However, the evidence presented did not rigorously prove their conclusion, and the alleged increase in resistance was minimal.

There are no reports in the literature of genetic interchange within *M. ovipneumoniae* strains, but the preceding reports suggest that it is at least a possibility and could include any of the three classical methods.

This section attempts to demonstrate genetic interchange in *M. ovipneumoniae* using three experimental approaches.

1. "Classical" antibiotic resistance crosses

Two cultures, each resistant to a different antibiotic, were mixed, incubated overnight, and the resulting culture examined for the presence of cells resistant to both antibiotics.

2. REA of clones derived from mixed cultures

Two cloned strains of *M. ovipneumoniae* which showed completely different REA patterns were mixed, and this mixed culture serially passaged for five days. Clones selected from the final culture were examined by REA for any changes in the REA patterns.

In further work, this approach was combined with 1 above. i.e. we examined clones derived from the antibiotic resistance experiments. These "presumptive recombinant" clones were either true recombinants or had spontaneously mutated to resistance to the second antibiotic.

3. Examination of *M. ovipneumoniae* for the presence of extrachromosomal DNA.

M. ovipneumoniae isolates were examined for the presence of extrachromosomal DNA by the method of Eckhardt (1978).

NOTE: This last approach did not address itself to the question of the actual occurrence of recombination, but looked instead for plasmids which, if present, might facilitate the process.

4.2 "Classical" antibiotic resistance crosses.

4.21 Introduction

Genetic markers (e.g. Antibiotic resistance, defective operons, etc) have long been used in "classical" genetic analyses of genetic interchange between bacteria. This section investigates the possibility that genetic interchange may occur in *M. ovipneumoniae* by testing for the possible transfer of resistance to either Naladixic acid or Rifampicin i.e. two cultures, each resistant to one antibiotic were mixed and propagated. The resultant culture was then examined for clones which were resistant to both antibiotics.

4.22 Materials and Methods

Determination of Antibiotic concentrations for use in recombination experiments

In order to determine the concentration of Naladixic Acid and Rifampicin which inhibit the growth of *M. ovipneumoniae* colonies on agar (strains 1, 5, 10 and L3C3 - see appendix 1.5 for the source of each strain), a series of doubling dilutions of Naladixic acid (100 $\mu\text{g}/\text{ml}$ to 1.5 $\mu\text{g}/\text{ml}$ and Rifampicin (50 $\mu\text{g}/\text{ml}$ to 1.5 $\mu\text{g}/\text{ml}$) were prepared in FM4 agar. 100 μl aliquots of a fresh culture of each cloned strain was spotted onto each antibiotic containing plate, which were then incubated at 37°C until colonies were clearly visible on those plates with a low antibiotic concentration. (This usually took four to five days). The titre of *M. ovipneumoniae* in each 100 μl aliquot was concurrently assayed on FM4 agar.

The number of colonies on each antibiotic plate were counted and compared with the controls. The concentration which reduced confluent growth or near confluent growth to not more than 100 colonies per plate was noted, and this antibiotic concentration was taken arbitrarily as 1 inhibitory unit (IU) of antibiotic.

Selection of Antibiotic Resistant mutants

A 100 μ l aliquot (containing approximately 10^6 to 10^7 CFU) of a fresh culture of a cloned strain of *M. ovipneumoniae* was spotted on FM4 agar plates which contained either Naladixic Acid, or Rifampicin, at 1IU, 2IU, 4IU and 8IU. These plates were incubated at 37°C until colonies were clearly visible (Usually 4 to 5 days.) Four colonies of each strain were then individually removed along with a plug of agar, using pasteur pipettes, from the highest concentrations of antibiotic that allowed growth of that strain, and added to FM4 medium containing the same antibiotic concentration. (see results). They were then incubated at 37°C on a shaker until a colour change was observed.

0.3 ml of the resulting antibiotic resistant culture was inoculated into another 3ml of FM4 containing the same antibiotic concentration, and incubated overnight at 37°C on a shaker. After a further subculture, in the absence of antibiotic, two 1.5ml aliquots were stored at -70°C .

"Classical" antibiotic resistance crosses

Cloned strains of *M. ovipneumoniae*, stored at -70°C , that were resistant to either Naladixic Acid or Rifampicin, were thawed, and 0.3ml aliquots independantly subcultured into 3ml aliquots of FM4 medium. These cultures were incubated overnight at 37°C on a shaker. A 0.2ml aliquot of a Naladixic acid resistant culture, and a 0.2ml aliquot of a Rifampicin resistant culture were then added to a 3.0ml aliquot of FM4. The mixed culture was incubated at 37°C on a shaker, until a colour change indicating a reaction of approximately pH7.0 was reached. (Cultures of either Naladixic acid or Rifampicin resistant strains were sub-cultured concurrently into 3.0ml aliquots of FM4 medium to act as controls). A series of ten-fold dilutions were made from each antibiotic cross, and control culture. 100 μ l of each dilution were spotted on separate FM4 agar plates containing 4IU of both antibiotics, and the plate incubated at 37°C . When colonies were clearly visible (normally after 4 to 5 days) they were counted and compared

to the background level of controls that grew on plates containing 4IU of both antibiotics.

4.23 Results

Inhibitory Concentrations of Naladixic Acid and Rifampicin

The concentrations of either Naladixic Acid or Rifampicin that inhibited the growth of the *M. ovipneumoniae* (strains 1, 5, 10 and L3C3) on agar, was approximately 6.25 μ g/ml. This concentration was taken arbitrarily as 1 inhibitory unit (1 IU) of antibiotic.

Selection of Antibiotic resistant mutants

The growth of *M. ovipneumoniae* was completely inhibited at concentrations of greater than 4IU (25 μ g/ml). Antibiotic Resistant colonies for the genetic interchange experiments were thus selected from plates containing 4IU of antibiotic.

"Classical" Antibiotic Resistance crosses

The results of the "classical" antibiotic resistance crosses are shown in Tables II, III and IV.

Note: An 0.1ml aliquot of the *M. ovipneumoniae* culture which contained approximately 10^4 CFU was added to each FM4 agar plate which contained 4IU of both Naladixic Acid and Rifampicin.

Table II. Number of "presumptive recombinant" *M. ovipneumoniae* colonies observed on FM4 agar containing 4IU of both Naladixic acid and Rifampicin.

Average number of colonies/plate		Naladixic Acid Resistant Strains			
		1	5	10	L3C3
Rifampicin resistant strains	1	0	12	0	1
	5	2	4	2	7
	10	1	4	4	0
	L3C3	0	6	1	0

Table III: Background level of Naladixic Acid resistant *M. ovipneumoniae* also resistant to Rifampicin.

Naladixic Acid Resistant Strain	1	5	10	L3C3
Average number of colonies/plate	0	14	0	0

Table IV: Background level of Rifampicin resistant *M. ovipneumoniae* also resistant of Naladixic Acid.

Rifampicin resistant strain	1	5	10	L3C3
Average number of colonies/plate	0	3	0	1

4.24 Conclusion

The number of "presumptive recombinant" *M. ovipneumoniae* (i.e. *M. ovipneumoniae* resistant to both antibiotics resulting from the mixing of two strains, one resistant to each antibiotic) was not above the background level of mutants resistant to both antibiotics. Thus genetic interchange was not detected by this approach.

4.3 REA of clones derived from mixed cultures

4.31 Introduction

If genetic interchange is to account for the heterogeneity seen in *M. ovipneumoniae* the interchange must be frequent and significantly affect the REA patterns of the organism. This section therefore examines the REA patterns of clones derived from cultures in which such genetic interchange may have occurred.

4.32 Materials and Methods

REA of clones derived from a mixed culture of *M. ovipneumoniae* had been passaged *in vitro* for five days

0.2ml aliquots (fresh culture) of *M. ovipneumoniae* strains 5 and 10 (which have completely different REA patterns), were mixed in 3.0ml of FM4 broth. The resulting mixed culture was incubated overnight at 37°C on a rotary shaker. This culture was serially passaged *in vitro* for a further 5 days (by dilution of 0.3ml of exponential phase culture into 3.0ml of FM4 broth each day see chapter 3.12). After 5 days serial passage, 6 clones were randomly selected as described in chapter 2.24 and examined by REA (see chapter 2.25).

REA of "presumptive recombinant" *M. ovipneumoniae* derived from "classical" antibiotic resistance crosses

Colonies resistant to both Naladixic acid and Rifampicin were randomly selected from the "classical" antibiotic resistance experiments discussed in chapter 4.1, and individually placed in 3.0ml of FM4 broth, and incubated at 37°C on a rotary shaker. When a colour change indicated that a reaction of approximately pH 7.0 had been reached, the clone was further propagated, and examined by REA as described in chapter 3.25.

4.33 Results

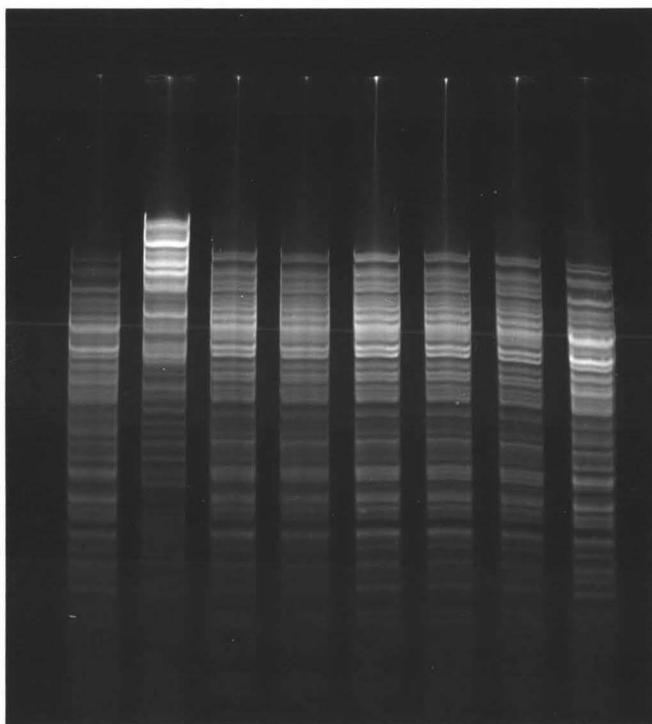
REA of clones derived from a mixed culture of *M. ovipneumoniae* that had been passaged *in vitro* for five days.

The results of REA of six clones randomly selected from a mixed culture (of *M. ovipneumoniae* strains 5 and 10), that had been further serially passaged for five days can be seen in figure 22.

Figure 22. Examination of *M. ovipneumoniae* clones derived from mixed cultures in an attempt to detect evidence of genetic interchange. Lanes 1 and 2: Pure cultures of strains 5 and 10, lanes 3 through 8 represent 6 clones randomly reselected from a mixed culture of *M. ovipneumoniae* strains 5 and 10 which was serially passaged *in vitro* for about 30 generations.

The REA patterns of all the clones derived from the mixed culture are indistinguishable from that of strain 5. There is no evidence that genetic interchange has occurred.

Note: Strain 5 can have several REA patterns (patterns A, B and C - see figure 17) which vary slightly from each other in the high molecular weight region. The two outside patterns (lanes 1 and 8) are pattern A. Lanes 3 through 7 are pattern B.



REA of "presumptive recombinant" *M. ovipneumoniae* derived from "Classical" antibiotic resistance crosses

The results of REA of "presumptive recombinant" *M. ovipneumoniae* resistant to both Naladixic acid and Rifampicin can be seen in figures 23 and 24.

Figure 23. Examination of "presumptive recombinant" *M. ovipneumoniae* clones in an attempt to detect evidence of genetic interchange. Lanes 1 to 5 represent lambda and pure cultures of strains 1, 5, 10 and L3C3 respectively. Lanes 6 through 12 represent "presumptive recombinant" clones derived from the "classical" Antibiotic resistance crosses, Lane 13 represents bacteriophage lambda.

The REA patterns of the "presumptive recombinant" *M. ovipneumoniae* (lanes 6 through 12) are indistinguishable from that of one of their parent strains i.e. strains 1, 5, 10 or L3C3 (lanes 2 through 6). There is no evidence that genetic interchange has occurred.

Note: Strain 5 can have several REA patterns (patterns A, B and C - see figure 17) which vary slightly from each other in the high molecular weight region. Lanes 3 and 12 are pattern A, lanes 7 and 9 are pattern B.

Key to actual content of lanes containing clones resulting from mixed cultures:

Nal^{R} = Naladixic Acid resistant, Rif^{R} = Rifampicin resistant.

Lane 6: Strain 10 (Nal^{R}) x strain 10 (Rif^{R}) - resultant pattern = strain 10.

Lane 7: Strain 5 (Nal^{R}) x strain L3C3 (Rif^{R}) - resultant pattern = strain 5.

Lane 8: Strain 1 (Nal^{R}) x strain 10 (Rif^{R}) - resultant pattern = strain 1.

Lane 9: Strain 5 (Nal^{R}) x strain 10 (Rif^{R}) - resultant pattern = strain 5.

Lane 10: Strain 10 (Nal^{R}) x strain 10 (Rif^{R}) - resultant pattern = strain 10.

Lane 11: Strain 10 (Nal^{R}) x strain L3C3 (Rif^{R}) - resultant pattern = strain L3C3.

Lane 12: Strain L3C3 (Nal^{R}) x strain 5 (Rif^{R}) - resultant pattern = strain 5.

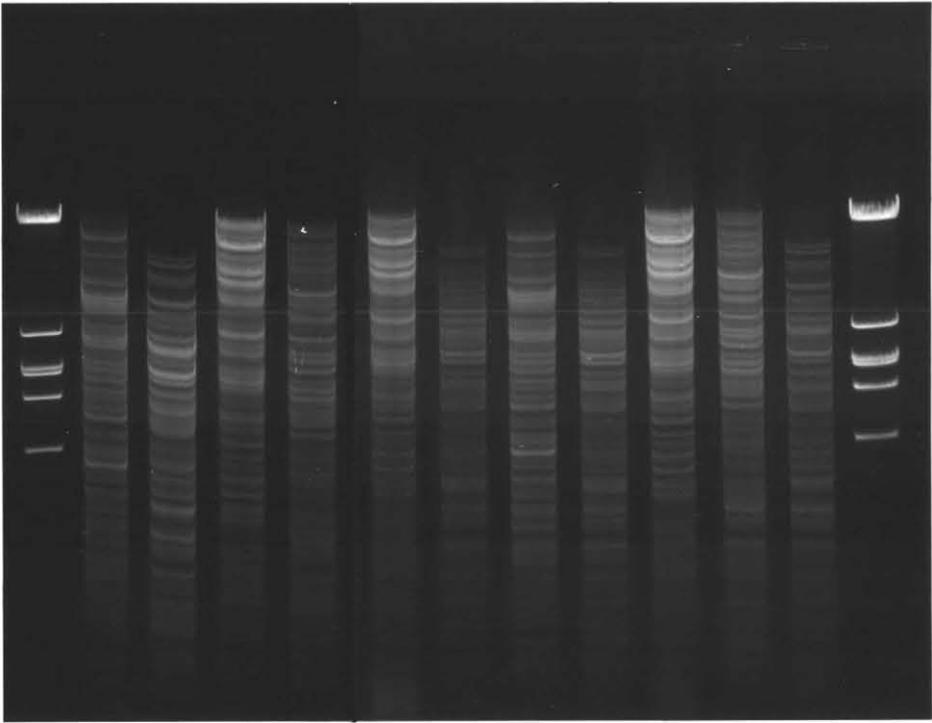


Figure 24. Examination of "presumptive recombinant" *M. ovipneumoniae* clones in an attempt to detect evidence of genetic interchange. Lanes 1 to 5 represent lambda and pure cultures of strains 1, 5, 10 and L3C3 respectively. Lanes 6 through 12 represent "presumptive recombinant" clones derived from the "classical" Antibiotic resistance crosses.

The REA patterns of the "presumptive recombinant" *M. ovipneumoniae* (lanes 6 through 12) are indistinguishable from that of strain 5, i.e. a parent strain. There is no evidence that genetic interchange has occurred.

Note: Strain 5 can have several REA patterns (patterns A, B and C - see figure 17) which vary slightly from each other in the high molecular weight region. Lanes 3, 6, 7 and 8 are pattern A, lanes 9, 10, 11 and 12 are pattern B.

Key to actual content of lanes containing clones resulting from mixed cultures:

Nal^{R} = Naladixic Acid Resistant, Rif^{R} = Rifampicin resistant.

Lane 6: Strain 1 (Nal^{R}) x strain 5 (Rif^{R}) - resultant pattern = strain 5.

Lane 7: Strain 10 (Nal^{R}) x strain 5 (Rif^{R}) - resultant pattern = strain 5.

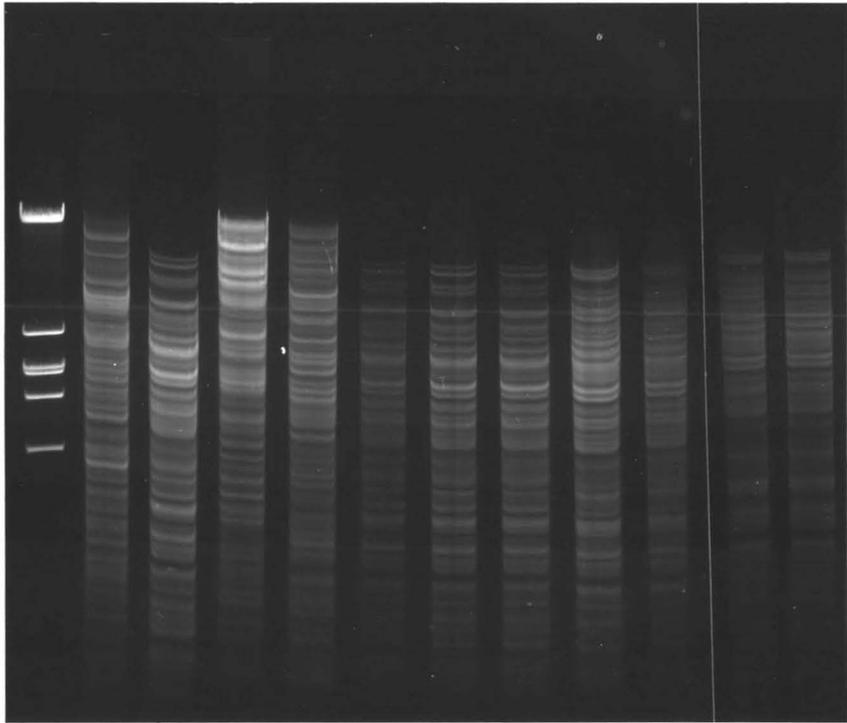
Lane 8: Strain 5 (Nal^{R}) x strain L3C3 (Rif^{R}) - resultant pattern = strain 5.

Lane 9: Strain L3C3 (Nal^{R}) x strain 5 (Rif^{R}) - resultant pattern = strain 5.

Lane 10: Strain 5 (Nal^{R}) x strain 10 (Rif^{R}) - resultant pattern = strain 5.

Lane 11: Strain 10 (Nal^{R}) x strain 5 (Rif^{R}) - resultant pattern = strain 5.

Lane 12: Strain 5 (Nal^{R}) x strain 5 (Rif^{R}) - resultant pattern = strain 5.



4.34 Conclusion

The REA patterns of isolates derived from mixed cultures of *M. ovipneumoniae* were invariable from one or other of the parent strains. When strain 5 was one of the parent strains, it outgrew the other parent strain initially present in the mixed culture.

Genetic interchange, as evidenced by the formation of new composite REA patterns, was not observed. These observations are further discussed at the end of this chapter.

4.4 Examination of *M. ovipneumoniae* for the presence of extrachromosomal DNA

4.41 Introduction

Although the presence of extrachromosomal DNA does not necessarily imply the ability of an organism to undergo genetic interchange, its presence may facilitate the process. This section therefore examines *M. ovipneumoniae* for the presence of extrachromosomal DNA.

4.42 Materials and Methods

A modification of the Eckhardt procedure for the identification of plasmid DNA in bacteria (Eckhardt, 1978) was used to examine strains of *M. ovipneumoniae* for the presence of extrachromosomal DNA.

Preparation of cultures

Four strains of *M. ovipneumoniae* (strain 5 and the farm isolates 9A, 10C and 15C examined in chapter 2 of this thesis) were propagated by inoculating 0.3ml of a frozen aliquot of the strain into 3mls of FM4 broth. These cultures were incubated overnight at 37°C on a rotary shaker. When the colour of the media indicated that the cells were in the exponential phase, a 1.0ml aliquot of each culture was centrifuged at 14500g for 2 minutes. The supernatant was decanted and discarded, and the pellet resuspended in 20µl of Ficoll solution I (see appendix 4.1). At this stage a horizontal agarose gel was prepared.

Preparation of agarose gel

Working strength Tris borate buffer (see appendix 4.2) was used to prepare 50ml of 0.7% Agarose (Biorad Electrophoresis grade). The agarose was dissolved by boiling in a flask, which was cooled to approximately 40°C before pouring. A template for the gel was constructed in the gel box by

placing a perspex strip at each end of the electrophoresis plate. (i.e. the gel was not prepared on a separate glass plate as in REA). The template was sealed by running a small portion of agarose around the template which was allowed to set, thus sealing the joins. The 50ml of agarose was then poured into the template and a perspex "comb" was placed at one end to form wells in the gel. The gel was allowed to set for 20 minutes, whereupon the "comb" and perspex strips were removed. The reservoir compartments were filled with working strength Tris Borate buffer to just below the gel top, and the wells were washed out with the buffer prior to loading of the *M. ovipneumoniae* samples.

Gel electrophoresis

The *M. ovipneumoniae* samples (resuspended in Ficoll solution I) were loaded onto the gel, and left for 15 minutes at room temperature, after which 20 μ l of Ficoll solution II (see appendix 4.3) was added. 20 μ l of Ficoll solution III (see appendix 4.4) was then overlaid. The gel was electrophoresed for 1 hour at 20V, this was then increased to 120V for a further 10 hours. After electrophoresis, an X-ray film was pushed under the gel, thus lifting it off the perspex. The gel was stained for 2 hours in 500ml of working strength Tris-Borate buffer which contained 0.025mg Ethidium Bromide, and photographed as described in chapter 2.25.

4.43 Results

The results of the examination for the presence of extra-chromosomal DNA can be seen in Figure 25.

Note: 8 *Rhizobium trifolii* samples known to contain plasmids of various sizes, were used as controls. These *R. trifolii* samples were prepared in a similar manner to the *M. ovipneumoniae* samples.

Figure 25. An Eckhardt plasmid gel of 4 randomly selected *M. ovipneumoniae* isolates, and 8 *Rhizobium trifolii* isolates. Lanes 1 through 4 contain *M. ovipneumoniae*, and lanes 5 through 12 contain *R. trifolii*.

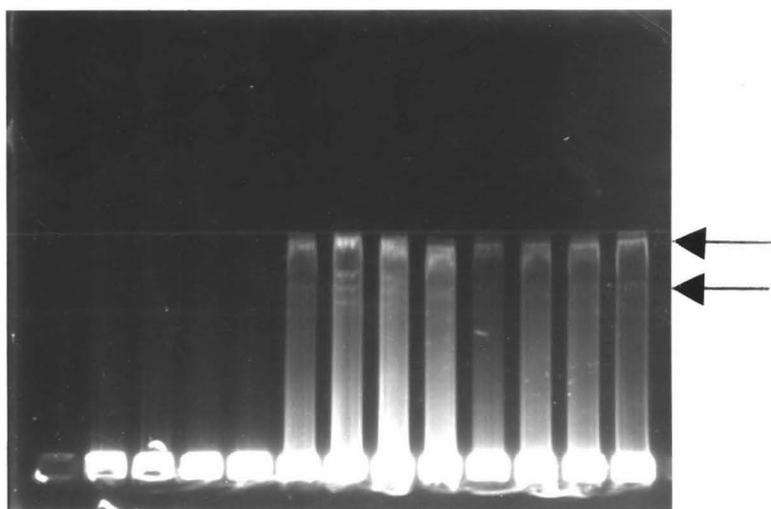
M. ovipneumoniae - Lanes 1 to 4 (left to right)

Lane 1: Farm isolate 9A; Lane 2: Farm isolate 10C;
Lane 3: Strain 5; Lane 4: Farm isolate 15C.

No plasmids were detected.

R. trifolii - Lanes 6 to 13

Various strains of *R. trifolii* that show one or more plasmids (arrows).



4.44 Conclusion

No plasmids were detected in the four *M. ovipneumoniae* isolates examined by this technique. This observation will be further considered in the following discussion.

4.5 Discussion

The number of "presumptive recombinant" *M. ovipneumoniae* resistant to both Naladixic Acid and Rifampicin recovered from a mixed culture was not significantly different from the naturally occurring background level of such double mutants. Furthermore, the REA patterns of these "presumptive recombinants" were indistinguishable from one or other of the two parental strains. Similarly, the REA patterns of clones randomly selected from a serially passaged mixed culture of two strains (with markedly different REA patterns) were indistinguishable from one or other of the parental strains. Thus no genetic interchange was detected. Apart from the obvious reason that a system of genetic interchange might not be present in *M. ovipneumoniae*, there are several other reasons that might account for the apparent lack of genetic interchange. For example, conditions might not have been suitable for genetic interchange to take place, the antibiotic resistance markers might not be on transmissible genetic material, or low level genetic interchange might not be detected by these methods (although for genetic interchange to account for the considerable heterogeneity of REA patterns seen in *M. ovipneumoniae*, one might expect some change in the REA patterns to occur). These possibilities were not further investigated in the present work.

The last section of this chapter examined for extrachromosomal DNA which, if present, might facilitate genetic interchange. Four *M. ovipneumoniae* strains were investigated for extrachromosomal DNA by the method of Eckhardt (1978). No extrachromosomal DNA was detected. Apart from the obvious reason that no extrachromosomal DNA exists in *M. ovipneumoniae*, there remains the possibility that extrachromosomal DNA outside the range size detected by this technique (i.e. less than 2 megadaltons or greater than 150 megadaltons) may exist within *M. ovipneumoniae*. There also remains the possibility that (normally) extrachromosomal DNA was integrated within the *M. ovipneumoniae* genome when the isolates were examined.

We conclude that the experiments detailed in this chapter give no support to (but do not uniformly disprove) the possibility that the heterogeneity seen in *M. ovipneumoniae* is due to extensive genetic interchange.

Chapter 5: General Discussion

Examination of independant isolates of *M. ovipneumoniae*

In a previous study of independant isolates (i.e. isolates from sheep on different farms) of *M. ovipneumoniae* by REA (Mew, 1982), each independant isolate gave an REA pattern that was completely different from all other independant isolates. However, only nine such isolates were examined, so it is possible that the number of distinct strains of *M. ovipneumoniae* might not greatly exceed this number.

The present work examined a further sixty isolates. Of these, twenty were "independant isolates". That is we examined isolates from three sheep on each of twenty farms. We were able to show that:

(1) Isolates from sheep on different farms gave REA patterns which were markedly different from each other. However, a few bands common to many isolates were seen in the low molecular weight region. The common bands represent, at most, 5% of the total bands.

(2) Isolates from sheep on the same farm varied in their relationship to each other. Thus they could be:

- (a) Indistinguishable from each other, or
- (b) Closely related to each other, with at least 95% of bands in common, or
- (c) Markedly different from each other, with little or no similarity i.e. less than 5% of bands in common.

When small differences were seen in similar isolates, the differences were normally confined to the high molecular weight region. All of the isolates gave REA patterns that were markedly different from those observed in the study by Mew (1982). By combining the results of Mew (1982)

with the present study, 29 different *M. ovipneumoniae* REA types have been observed from 29 independent isolations.

This confirms and extends the earlier conclusion that *M. ovipneumoniae* is extremely heterogeneous with respect to REA. Having confirmed the heterogeneity (which is in marked contrast to almost all other species examined by REA) this thesis attempted to explain it.

We first excluded the possibility that the observed heterogeneity was due to technical factors, e.g. preparation, incomplete DNA digestion, electrophoresis etc., by demonstrating that each unique pattern was reproducible even when replicate cultures were prepared independently. This was considered in chapter 3 and is not further discussed here.

Possible explanations for the Heterogeneity seen in *M. ovipneumoniae*

There are three general explanations as to why *M. ovipneumoniae* is heterogeneous with respect to REA.

1. A large number of different strains of *M. ovipneumoniae*, which evolved over a long time period, are independently maintained in the sheep population of New Zealand.
2. "Genetic instability" i.e. the genome of *M. ovipneumoniae* may undergo rapid genetic change. This could be due to either "classical" type mutations (e.g. deletion and insertion mutants, point mutations, etc) or an internal rearrangement system (e.g. Transposons, Insertion sequences or Inversion sequences).
3. Frequent genetic interchange may occur between strains of the species *M. ovipneumoniae*. Thus random genetic interchange between what were initially two strains of *M. ovipneumoniae* with markedly different genomes, could generate an

almost infinite number of strains which varied from their parental strains.

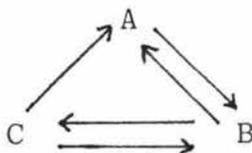
The first possibility, i.e. that a large number of different strains of *M. ovipneumoniae* are independantly maintained, is almost impossible to prove directly, but would gain plausibility if other explanations were excluded.

Stability of the *M. ovipneumoniae* genome

The stability of the *M. ovipneumoniae* genome was investigated by examining the REA patterns of a clone at intervals of (approximately) 133 generations. Three minor but nevertheless unequivocal changes were observed (Figures 10 through 18). These changes were non random in the sense that they were often found to be reversed, following further *in vitro* passage of the clone.

The three patterns (labelled A, B and C) differed in a few bands in the high molecular weight range (see Figure 17). It should be stressed that these differences were minor, indeed were at the limit of detection and in no way represent the large (usually total) differences seen in the REA patterns of independant isolates.

The interrelationship and "direction" of the changes observed are summarised in the following diagram:

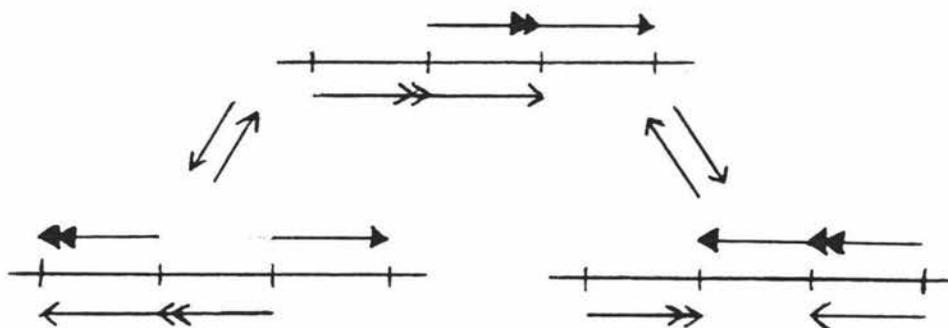


i.e. All are reversible except that we did not observe pattern A change directly to pattern C. The reversibility of the changes suggests that an internal rearrangement system operates in *M. ovipneumoniae*. The nature of such a

system was not investigated here. However, we suggest the following hypotheses to account for the observations:

1. Two inversion sequences may be present in the genome. When these invert they cause changes in the REA patterns. This should result in the occurrence of four reversible REA patterns. Only three were detected but perhaps the failure to see a fourth pattern was merely due to the relatively low number of clones examined.

Note: It is possible to further refine this hypothesis e.g. Two overlapping inversion sequences would explain the occurrence of only three patterns i.e. When one sequence inverts, it would result in the other inversion sequence being unable to invert, thus three genomic configurations, as illustrated in the diagram below might result.



2. A transposable element that can accurately excise and insert into a limited number of precise sites in the genome may be present in *M. ovipneumoniae*.

Non-reversed changes (which would be typical of "classical" type mutations) were not detected in the REA patterns after serial passage *in vitro* of the *M. ovipneumoniae* clone.

In vitro verses *in vivo* conditions

It is obvious that *in vitro* conditions are not exactly analogous to *in vivo* conditions, so different selection

pressures must exist. Thus for example, *in vitro* the concentration of growth promoting constituents (e.g. amino acids) will vary with time as will pH. These conditions are probably more stable *in vivo*, but nevertheless the organism growing *in vivo* will probably be exposed to even greater selection pressures e.g. The Mucocilliary escalator which can remove organisms from the lower respiratory tract, or the immune response. The lack of SPF sheep meant that we could not conduct an *in vivo* experiment to investigate the stability of the REA pattern of a strain of *M. ovipneumoniae* maintained long term *in vivo*. Such experiments have however been done with *Moraxella bovis* (Marshall *et al.*, 1985), and it was found that this organism changed more rapidly *in vitro* than *in vivo*. From this we infer that *M. ovipneumoniae* is not likely to be more stable *in vitro* than *in vivo*.

Can the changes observed following passage account for the large number of *M. ovipneumoniae* strains (as defined by REA)?

Our experiments looking for REA changes after passage were carried out over three months and involved a total of approximately 400 generations. The changes observed, though small, were nevertheless unequivocal. It would be quite plausible to argue that if the number of generations were increased by a factor of (e.g.) 100 fold and a similar amount of change occurred, that the changes would account for the number of strains seen in the field. However, the critical result is that the changes observed are not cumulative but were reversed and in our hands never gave more than 3 patterns. This being so, it is in fact arguable that *M. ovipneumoniae* strain 5 has an almost totally stable REA pattern, but with the proviso that certain "predictable" and reversible changes can occur in a limited number of high molecular weight bands.

No irreversible change was detected in any of the experiments so we conclude that genetic instability in the short to medium term does not account for the large number of

M. ovipneumoniae strains seen in the field, though of course evolutionary divergence in the long term at a rate too slow to be detected in our hands might and almost certainly does occur.

Does a changed REA pattern mean that protein changes have occurred?

As mentioned in Chapter 1, Chronic Non-Progressive pneumonia is a relatively long lasting disease (it can persist for up to 7 months, Gilmour *et al.*, 1982). This persistence could be explained if the organism changed antigenically. To investigate this, the total cellular proteins of clones showing REA patterns A, B and C, were examined by SDS-Polyacrylanide gel electrophoresis. (SDS-PAGE).

No changes in the proteins were seen, so either:

1. No changes occurred, or
2. Changes in the protein component were not detectable by SDS-PAGE. This technique separates proteins on the basis of size so if changes in antigenicity, but not in size occurred the differences would not be detected by the method used.

This latter possibility was not further investigated in the present work.

Genetic Interchange in *M. ovipneumoniae*

Two experimental approaches (or 3 if one can count attempts to demonstrate plasmids) were taken to attempt to demonstrate genetic interchange between *M. ovipneumoniae* isolates.

"Classical" antibiotic resistance crosses

Mutants resistant to Naladixic acid were mixed with mutants

resistant to Rifampicin and allowed to grow overnight. The number of "presumptive recombinants", i.e. clones resistant to both antibiotics, was not above the background level of double mutants. However, there remained the possibility that a small number of true recombinants were masked by the excessive "background". To investigate this, clones resulting from mixed cultures, including the "presumptive recombinants", were examined by REA. In all cases, their REA patterns were identical to one of their parent strains, which themselves showed no obvious similarities.

These experiments therefore failed to demonstrate any evidence of genetic interchange but do not exclude the possibility that *in vivo* conditions may allow genetic interchange to occur.

Investigation for the possible presence of extrachromosomal DNA

Four strains were examined for the presence of extrachromosomal DNA by the method of Eckhardt (1978). No extrachromosomal DNA was detected. It is however notoriously difficult to prove a negative hypothesis and it must be stated that extrachromosomal DNA may exist in *M. ovipneumoniae* but was not detected by the method used, which detects extrachromosomal DNA ranging between 2 and 150+ megadaltons. It is also possible that a mechanism which facilitates genetic interchange exists, but in the isolates examined was integrated within the genome.

Nevertheless, the conclusion that no mechanism of genetic interchange is demonstrable for *M. ovipneumoniae* is consistent with the finding of other investigations of the class Mollicutes, where despite some claims to the contrary, genetic interchange has not been unequivocally demonstrated.

Reason for the heterogeneity of REA patterns of *M. ovipneumoniae* - conclusions

Our results indicate that the heterogeneity seen in

M. ovipneumoniae is not likely to be the result of either:

1. Inherent instability of the *M. ovipneumoniae* genome, resulting in rapid genetic change, or
2. Frequent genetic interchange within the species, with a consequent change of the genome.

This leaves us with one original explanation, *viz* that a large number of stable but different strains of *M. ovipneumoniae* which evolved over a long time period are independantly maintained in the sheep population of New Zealand.

By using the Poisson distribution, and assuming an equal chance of finding any particular strain, we can estimate the minimum number of strains that must exist so that when 29 isolates of *M. ovipneumoniae* are randomly selected there will be a 95% probability that 2 or more are the same (i.e. a 5% probability that all will be different).

Pr (Getting 29 distinct strains from a population of X strains)

$$\begin{aligned}
 &= 1 \cdot \frac{x-1}{x} \cdot \frac{x-2}{x} \dots \frac{x-28}{x} \\
 &= \frac{x}{x} \cdot \frac{x-1}{x} \dots \frac{x-28}{x} \\
 &= \frac{x!}{(x-29)!} \cdot \frac{1}{x^{29}}
 \end{aligned}$$

Using Stirling's approximation ($\ln(x!) = x \ln x - x$ for large x), take logs and test for 5% region.

$$\begin{aligned}
 \ln(x!) - \ln(x-29)! - \ln(x^{29}) &= \ln 0.05 \\
 x \ln x - x - (x-29) \ln(x-29) - (x-29) - 29 \ln x &= \ln 0.05 \\
 x \ln x - x - (x-29) \ln(x-29) + (x-29) - 29 \ln x &= \ln 0.05 \\
 (x-29) \ln x - (x-29) \ln(x-29) &= 29 + \ln 0.05 \\
 (x-29) \ln\left(\frac{x}{x-29}\right) &= 29 + \ln 0.05 \\
 \ln\left(\frac{x}{x-29}\right) (x-29) &= 29 + \ln 0.05
 \end{aligned}$$

Take exponents of both sides

$$\left(\frac{x}{x-29}\right)^{(x-29)} = e^{(29 + \ln 0.05)}$$

But $e^{a+b} = e^a \cdot e^b$

$$\left(\frac{x}{x-29}\right)^{(x-29)} = e^{29} \cdot 0.05$$

Try Binomial using $\left(\frac{x-29}{x}\right)^{(29-x)} = \left(1 - \frac{29}{x}\right)^{(29-x)}$, but $\frac{29}{1}$ not small c.f. $(20-x)$. Therefore we cannot approximate. Thus the equation is solved numerically from here.

Thus if $x = 150$, then $\left(\frac{x}{x-29}\right)^{(x-29)} = e^{29} \cdot 0.05$

This means that if 29 isolates were randomly selected from a total of 150 different strains, then there would be a 95% probability that some must be the same (i.e. only a 5% probability that all would be different). Since we found none the same, this means that 150 must be the minimum number of strains that exist. Thus, with 95% certainty we can state that there are at least 150 different *M. ovipneumoniae* strains independantly maintained in the sheep population of New Zealand.

Is 150 a plausible minimum figure for the number of strains?

A possible answer to this question can be gained by considering epidemiological data from other systems.

For a disease to persist in a community, there must be either a continued supply of fresh susceptible hosts, or some form of maintenance host/reservoir in which the disease may reside until fresh susceptible hosts come into the community. The critical community size (i.e. the population size required to maintain a strain of disease without re-introduction of the disease) is dependant on a number of variables. The major, but not sole, criteria include:

- (1) The infectivity of the organism,
- (2) The ability of an organism to persist in infectious form in at least a proportion of those infected,
- (3) The birthrate i.e. the rate of recruitment of new susceptibles.

The ability of an organism to persist in an infectious form in the host is of critical importance. This can be illustrated by comparing the estimated critical community sizes for measles and chickenpox. Measles has no reservoir of infection, and must therefore be maintained by the newborn susceptibles. Its critical community size has been estimated at about 500,000 (Mims, 1982). Chickenpox, in comparison, can be maintained indefinitely in a community of less than 1000 individuals, due to causation of a persistent infection i.e. Chickenpox virus may remain dormant in the dorsal root ganglia of sensory nerves supplying the affected skin areas and may reactivate at a later stage and cause shingles, with consequent shedding of infectious virus particles (Mims, 1982).

M. ovipneumoniae also appears to cause a persistent infection in the sense that carriers exist i.e. *M. ovipneumoniae* can be isolated from 5 to 15% of normal, apparently healthy ewes showing no signs of CNP, which can thus act as reservoirs (Ionas, 1983). Thus we could reasonably expect that the critical community size for a single strain of *M. ovipneumoniae* would be much less than measles, and since the carrier rate is high, even less than chickenpox virus. There are approximately sixty million sheep in New Zealand (The New Zealand Yearbook, 1984), so even estimating the critical community size at the (possibly high) figure of 1000, it is possible for 60,000 different strains of *M. ovipneumoniae* to be maintained in the New Zealand sheep population. Thus

our requirements for the maintenance of a minimum of 150 strains appears to be easily met.

It has been suggested that genetic heterogeneity of a species is associated with its ability to colonise diverse habitats. For example *Acholeplasma axanthum* strains isolated from a variety of hosts and habitats show considerable genotypic heterogeneity (Stephens *et al.*, 1983; Razin *et al.*, 1983a). In contrast, species of strict host and tissue specificity are claimed to exhibit marked genetic homogeneity. For example *Mycoplasma pneumoniae*, which can only be isolated from the respiratory tract of man, show a high degree of genetic homogeneity (Chandler *et al.*, 1983; Razin *et al.*, 1983a).

Our studies indicate that if the above proves to be the general rule, then *M. ovipneumoniae* is an exception, i.e. *M. ovipneumoniae* is strict in its host and tissue specificity (it is found only in the respiratory tract of sheep and goats) and yet shows extreme genetic heterogeneity, even when compared to *A. axanthum*.

Our work here demonstrates that a large number of stable strains of *M. ovipneumoniae* are able to be maintained in the respiratory tract of sheep. It would be interesting to know if more than one strain may be maintained in a sheep at the one time. In this regard, there is a hint in the work of Jones (1982) that not only can two strains colonise the lung, but production of more severe disease may require two or more strains. This is presently being investigated, but remains outside the scope of the present thesis.

REA had been proposed as a tool in the study of the epidemiology of *M. ovipneumoniae* (Mew, 1982). The work in this thesis further validates this approach i.e. the REA patterns of different strains of *M. ovipneumoniae* were both different and stable enough to enable them to be used in an epidemiological study.

Appendix 1: *M. ovipneumoniae* growth and identification materials

1.1 FM4 Broth

This medium is the fourth of several media of Frey, Hanson and Anderson (1968)

NaCl	5.0g
KCl	0.4g
Mg ₅ O ₄ ·7H ₂ O	0.2g
Na ₂ HPO ₄ ·12H ₂ O	4.03g
KH ₂ PO ₄	0.1g
Glucose	10.0g
Peptone L S (Bacto)	10.0g
Yeast Autolysate (Albimi)	5.0g
NAD	0.1g
L-cystine HCl	0.1g
Eagles Vitamin Solution (100X)	25ml
Phenol Red (0.4%)	2.5ml
Penicillin	10 ⁶ units
Thallium Acetate	0.5g
Deionised water to	1000.0ml

Note: 1. The Thallium acetate was first dissolved in 10ml of deionised water and then added dropwise to the medium to prevent precipitation.

2. The basal medium was supplemented with 150ml of pig serum.

3. The pH was adjusted to pH 7.8 with 1.0N NaOH.

4. The medium was sterilised by first clarification through non-sterile 5.0, 0.45 and 0.2 μ m. Millipore cellulose acetate filters, and final filtration through a sterilised 0.2 μ m filter.

5. Stored at 4^oC.

Eagles Vitamin solution

D-biotin	20mg
Calcium pantothenate	20mg
Choline chloride	20mg
Folic acid	20mg
Riboflavin	2.0mg
Myo-Inositol	40mg
Niacinamide	20mg
Pyridoxine	20mg
Thiamine-HCl	20mg
Distilled water to	100ml

Stored in 25ml aliquots at -20°C .

1.2 FM4 agar

BHI (Difco)	0.74g
Agar	1.0g
Deionised water to	20.0ml

The BHI-agar was autoclaved at 121°C for 15 minutes. After it had cooled to approximately 50°C , it was added to 80ml of FM4 at 46°C and mixed.

4ml aliquots were pipetted into 50mm petri dishes. (Note: These petri-dishes have tight fitting lids to avoid desiccation of the agar during incubation of *M. ovipneumoniae*). The plates were dried at 37°C for 15 minutes and stored at 4°C .

1.3 Gel precipitin agar

Special Noble Agar	3.0g
NaCl	16.0g
Distilled water to	200ml

Sterilised by autoclaving at 121°C for 15 minutes. 20ml aliquots dispensed in 85mm petri dishes which were dried at 37°C for 15 minutes and stored at 4°C .

1.4 Antiserum to *M. ovipneumoniae* strain 5

Antigen was prepared by propagating *M. ovipneumoniae*, strain 5, in modified FM4 media, which contains neither swine serum nor animal peptone. These cells were centrifuged, washed twice with sterile PBS, and resuspended in 0.85% saline to give an approximate 10% solution.

0.5 ml of the antigen with an equal amount of Freund's complete adjuvant, was injected intramuscularly into rabbits. After one month, intramuscular booster inoculations of 0.5 ml of antigen were given at 14 day intervals. Rabbits were bled at intervals and the antibody titre was estimated by gel precipitin tests. Once a titre of 1:16 or greater was reached, further serial bleedings were pooled, heated at 56°C for 20 minutes, and stored at -20°C.

1.5 Source of *M. ovipneumoniae* strains

Strain 1 was isolated from the lungs of a New Zealand sheep by Dr J.K. Clarke in 1973.

Strain 5 was isolated from the lungs of a New Zealand sheep by Dr J.K. Clark in 1973.

Strain 10 was isolated from the lungs of an Australian sheep by Dr T.D. St George.

Strain L3C3 was isolated from the lungs of a New Zealand sheep by Dr J. K. Clarke in 1981.

Appendix 2: REA materials

2.1 Phosphate Buffered Saline (PBS)

NaCl	8.0g
KCl	0.2g
Na ₂ HPO ₄	1.15g
KH ₂ PO ₄	0.2g
Distilled water to	1000.0ml

The solution was autoclaved at 121^oC for 15 minutes.

2.2 Tris-EDTA solution

1M Tris-HCl pH 7.5	1.0ml
0.2M EDTA (disodium salt) pH 7.2	5.0ml
Distilled water to	10.0ml

Made up fresh from stock solutions before use

2.3 10% SDS

10% w/v Sodium Dodecyl Sulphate (BDH-specially pure).
Stored at room temperature.

Pronase

10mg/ml (Calbiochem B grade) in distilled water. Initially preincubated at 37^oC for 2 hours to remove DNAase activity.

Stored at -20^oC.

2.4 Phenol/Chloroform/Iso-Amyl Alcohol solution

Phenol redistilled under nitrogen and stored in the dark at -20^oC under Nitrogen. Both Chloroform and Iso-Amyl Alcohol were Reagent grade.

2.5 Saline Tris-EDTA (STE) Buffer (10X)

5M NaCl	20.0ml
1M Tris-HCl pH 7.5	50.0ml
0.2M EDTA (disodium salt) pH 7.2	5.0ml
Distilled water	25.0ml

Stored at room temperature.

2.6 Dialysis Tubing

10mm (flat width) dialysis tubing was cut into suitable lengths which were boiled in a 5% Sodium Carbonate solution. This solution was changed until no odour or colour could be detected. The dialysis tubing was then boiled in distilled water once; in 0.001M EDTA once, washed with distilled water, then boiled in distilled water and allowed to cool. This processed dialysis tubing was stored in distilled water at 4°C.

2.7 Tris-EDTA (TE) Buffer

1M Tris-HCl pH 7.5	40.0ml
0.2M EDTA (disodium salt) pH 7.2	20.0ml
Distilled water to	4000 ml

Made up fresh from stock solutions before use

2.8 Ethidium Bromide solution

2.5mg/ml in distilled water. Stored in the dark.

2.9 Calf Thymus DNA

50.0 µg/ml in distilled water. Stored in the dark at 4°C.

2.10 EcoRI buffer (5X)

1M Tris-HCl pH 7.5	50.0ml
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5M NaCl	5.0ml
1M MgCl_2	2.5ml
Bovine Serum Albumin (frV Sigma)	50.0ml
Distilled water to	100.0ml

Stored at 4°C.

2.11 EcoRI

4 units*/ μl . Stored in 50% Glycerol at -20°C.

*1 unit = Complete digestion of 1 μg of lambda DNA in 15 minutes at 37°C in a reaction volume of 50

2.12 Lambda (λ) DNA

325 μg / ml. Stored at 4°C.

2.12 "Running" Buffer

1M Tris-HCl pH 7.5	0.1ml
0.2M EDTA (Disodium salt) pH 7.2	0.05ml
Glycerol	2.0ml
10% SDS	0.05ml
Distilled water to	10.0ml

Stored at room temperature

2.14 Electrophoresis (E) Buffer (10X)

Trizma Base (Sigma)	96.88g
EDTA (Disodium salt)	7.44g
Sodium Acetate	8.20g
Glacial Acetic Acid to pH 7.8	
Distilled water to	2.0 litres

Stored at 4°C Diluted 1:10 with distilled water when required.

Appendix 3: SDS-PAGE materials

3.1 Bovine Serum Albumin (BSA) solution

A range of BSA solutions from 0 to 0.100mg prepared in 0.2M NaOH.

3.2 Coomassie Blue

Coomassie Blue G-250	100.0mg
95% Ethanol	50.0ml
85% (w/v) Phosphoric Acid	100.0ml
Distilled water to	1000.0ml

Filter through Whatman #1 filter paper. Store at room temperature in a dark bottle.

3.3 SDS sample buffer (4X)

β - mercaptoethanol	10.0ml
SDS	6.0g
Upper Tris Buffer	25.0ml
Distilled water to	100.0ml

Diluted 1:4 with protein sample

3.4 Bromophenol Blue tracking dye (10X)

Bromophenol Blue	0.05g
Glycerol	40.0ml
Distilled water to	50.0ml

Diluted 1:10 with protein sample prior to boiling

3.5 Acrylamide solution (Running Gel)

Acrylamide	30g
Methylene bis acrylamide	0.5g
Distilled water to	100ml

Acrylamide solution (Stacking Gel)

Acrylamide	30g
Methylene bis acrylamide	1.6g
Distilled water to	100ml

Acrylamide was added to distilled water and stirred until the solution returned to room temperature. The solution was filtered through Whatman #1 filter paper. Stored at 4°C.

3.6 Lower Tris Buffer (4X) pH 8.8

Trizma Base	18.17g
10% solution of SDS in water	4.0ml
12N HCl to	pH8.8
Distilled water to	100ml

Stored at 4°C

3.7 TEMED

NNN'N'-Tetramethylethylenediamine (Sigma 99%, liquid)

3.8 Upper Tris Buffer (4X) pH 6.8

Trizma Base	6.06g
10% solution of SDS in distilled water	4.0ml
12N HCl to	pH6.8
Distilled water to	100ml

Stored at 4°C

3.9 Tris-Glycine Buffer (pH 8.3)

Trizma Base	6.07g
Glycine	28.8g
SDS	2.0g
Distilled water to	2000ml

Stored at 4°C.

3.10 Coomassie Blue/Isopropanol stain

Isopropanol	250ml
Glacial acetic acid	100ml
Coomassie brilliant blue R-250	0.4g
Distilled water to	1000ml

Appendix 4: Materials for plasmid isolation4.1 Ficoll solution I

Ficoll	1.0g
Pronase (10mg/ml)	0.01ml
Bromophenol Blue	5.0mg
RNAase (DNAase free)	0.01mg
Tris Borate Buffer (1X) to	10.0ml

Stored at 4°C.

4.2 Tris Borate Buffer (10X) (pH = 8.2)

Trizma Base (Sigma)	108.0g
EDTA	9.3g
Boric Acid	55.0g
Distilled water to	1000.0ml

Diluted 1:10 with distilled water when required.

4.3 Ficoll solution II

SDS	0.02g
Ficoll	1.0g
Tris Borate Buffer (1X)	10.0ml

4.4 Ficoll solution III

SDS	0.02g
Ficoll	0.5g
Tris Borate Buffer (1X)	10.0ml

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