A Study of the Heterogeneity of *Mycoplasma ovipneumoniae* Isolates by Examination of their Proteins and Deoxyribonucleic Acid.

A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Microbiology at Massey University, New Zealand.

George Ionas

1989

ABSTRACT

Chronic non-progressive pneumonia is an important disease of three to ten month old sheep in New Zealand. The lesions invariably contain *M. ovipneumoniae* yet the disease, or at least its more severe manifestations, cannot be consistently produced by intranasal inoculation of isolates of this organism. This may be due to the existance of different strains of this organism. This *in vitro* study attempts to establish whether or not there is a large number of distinguishable strains of this organism.

Comparison of *M. ovipneumoniae* isolates by the metabolic inhibition test showed that they differed but did not allow the recognition of well defined groups. The heterogeneity of *M. ovipneumoniae* was then examined using SDS-PAGE of total proteins and it was found that all isolates derived from sheep on twenty different farms differed except for three isolated from one farm. Protein bands on SDS-PAGE gels could be common to all isolates, shared by some isolates, or unique to individual isolates.

Identification of surface proteins by partial proteolytic digestion of intact organisms indicated that about five proteins were present on the surface, some were common to all isolates, some were shared by several isolates and others were unique to one isolate.

Several proteins were investigated by "immuno-blotting". Most showed a serological relationship to one or more proteins of other isolates but antiserum to one such band reacted with only a proportion of the isolates. This band is a surface glycoprotein as indicated by proteolytic digestion and by periodic acid-silver staining. Antiserum to it could have the potential to divide *M. ovipneumoniae* isolates into two meaningful groups. This could best be done by using monoclonal antibodies, however this thesis pursues an alternative approach to comparing isolates namely the examination of the DNA of *M. ovipneumoniae*.

Examination of *M. ovipneumoniae* DNA, using digests produced by *Eco*RI, showed that isolates from sheep on different farms were totally distinct. This conclusion was extended to digests produced by a range of restriction endonucleases. However, some endonucleases gave only partial or no digestion. Such enzymes had cytosine-rich recognition sequences which suggested that *M. ovipneumoniae* may contain 5-methylcytosine. This possibility was

supported by using two endonucleases which cut the same sequence but which are, or are not, indifferent to cytosine methylation. The presence of 5-methylcytosine was then confirmed with one isolate by analysis of the DNA using HPLC. The DNA of this isolate had about 25% of its cytosine methylated.

Since 5-methylcytosine occurs in *M. ovipneumoniae* it is possible that the different restriction endonuclease patterns seen with different isolates may be due to differences in methylation rather than to differences in DNA sequence. To investigate this we undertook a comparison of the DNA of isolates using DNA-DNA hybridization. This confirmed that *M. ovipneumoniae* isolates were heterogeneous because they showed a range of homology of 70-100%. This is a wide range but can be accommodated within one species. Thus it confirms that *M. ovipneumoniae* is one species despite its heterogeneity and does not give support to the existence of two or more subspecies within the species.

The conclusion that the heterogeneity of the restriction endonuclease cleavage patterns depends on DNA sequences and not methylation was further confirmed by showing that adenine was not methylated. This was followed by digestion of DNA by *Dra*I which cleaves a sequence lacking cytosine (5'-TTT^{V}AAA-3'). Like other enzymes, this gave heterogeneous digestion patterns which indicated that methylation cannot account for the heterogeneity.

The remarkable heterogeneity of *M. ovipneumoniae* strains led us to ask the ultimate question in this context, namely "are isolates from the lung of one sheep heterogeneous?" Six lungs were investigated. Three contained four different strains, one contained three strains and two contained two strains. The four lungs containing three or four strains had large lesions and the other two had smaller lesions. We suggest that the classical view that one strain of a micro-organism colonizes a lesion to cause a disease may need to be modified, that is mixtures of micro-organisms of the same species may have a potentiating effect on pathogenicity.

ACKNOWLEDGMENTS

I am indebted to the Department of Microbiology and Genetics for providing the opportunity and facilities for these studies.

Department of Microbiology and Genetics

In particular I would like to thank:

My chief supervisor Dr J. K. Clarke for his excellent guidance and advice throughout the course of this study.

Professor D. F. Bacon, Dr M. F. Nulsen, Dr B. D. W. Jarvis and Mr L. J. H. Ward for their interest and discussions for these studies. I would also like to thank Dr R. J. Thornton for providing HPLC facilities, Mr R. H. Tucker and all other members of the academic and technical staff.

Department of Veterinary Pathology and Public Health

I would like to thank my second supervisor Dr R. B. Marshall for his advice and comments.

I would also like to thank:

The staff at the engineering workshop for the construction of some of the gel apparatus.

The staff of the central photographic unit.

Mr R. J. Jones for the excellent typing of this thesis.

Finally, my parents for their encouragement throughout this project.

TABLE OF CONTENTS

Page

TITLE PAGE i
ABSTRACT ii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF FIGURES xiv
LIST OF TABLES xxiv

INTRODUCTION:

Chronic Non-Progressive Pneumonia of Sheep in New Zealand:
The Disease and its Significance 1
Distinction Between Chronic and Acute Pneumonia 3
Nomenclature of the Disease 3
Correlation of *M. ovipneumoniae* with CNP 5
Experimental Transmission of CNP 6
Isolation of *M. ovipneumoniae* from Pneumonic Goat Lungs 9
Distinguishing Strains of *M. ovipneumoniae*:
A) Serology 10
B) SDS-PAGE 11
C) Identification of Proteins Exposed to the Outer Membrane of *M. ovipneumoniae*:
i. Identification of Surface Proteins by Fluorescent Labelling 12
ii. Identification of Surface Proteins by Proteolytic Digestion 13
D) Bacterial Restriction Endonuclease DNA Analysis (BRENDA) 14
Methylation of DNA 16
Extrachromosomal Elements in the Genus *Mycoplasma* 16

Page

Investigation of the Relationship of Micro-organisms Using DNA-DNA Hybridizations 17
Overall Objectives 17

CHAPTER ONE: **Examination of *M. ovipneumoniae* Isolates by the Metabolic Inhibition Test**

1.1 Introduction 19
1.2 Materials and Methods 19
1.2.1 Materials 19
1.2.2 Methods 20
1.2.2.i Quantal Titration of *M. ovipneumoniae* Strains 20
1.2.2.ii Production of Antisera 20
1.2.2.iii Metabolic Inhibition Test 20
1.2.2.iv Absorption of Antisera for use in Metabolic Inhibition Tests 21
1.3 Results 21
1.4 Discussion 25

CHAPTER TWO: **Separation and Comparison of *M. ovipneumoniae* Proteins by Iso-Electric Focusing (IEF) and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

A) A Comparison of Non-Ionic and Zwitterionic Surfactants for their Ability to Solubilize *M. ovipneumoniae* Membrane Proteins for use in IEF 27
A2.1 Introduction 27
A2.2 Materials and Methods 28
A2.2.1 Materials 28

Page

A2.2.2 Methods 29

A2.2.2.i A Spectrophotometric Determination of the Solublizing Ability of Non-Ionic and Zwitterionic Surfactants on a *M. ovipneumoniae* Strain 29

A2.2.2.ii An SDS-PAGE Comparison of Two Zwitterionic Surfactants on their Ability to Solublize Total *M. ovipneumoniae* Cells 30

A2.3 Results 30

A2.4 Discussion 34

B) Iso-Electric Focusing (IEF) of *M. ovipneumoniae* Proteins in Non-Denaturing Surfactants 35

B2.1 Introduction 35

B2.2 Materials and Methods 35

B2.2.1 Materials 35

B2.2.2 Methods 37

B2.2.2.i Sample Preparation 37

B2.2.2.ii Preparation of Iso-Electric Focusing Gels 38

B2.2.2.iii Iso-Electric Focusing of *M. ovipneumoniae* Protein Samples 39

B2.3 Results 41

B2.4 Discussion 44

C) A Comparison of *M. ovipneumoniae* Isolates by SDS-PAGE 45

C2.1 Introduction 45

C2.2 Materials and Methods 45

C2.2.1 Materials 45

Page

C2.2.2 Methods 49
C2.2.2.i The Collection, Isolation and Cloning of Mycoplasma Isolates Obtained from Pneumonic Sheep Lungs 49
C2.2.2.ii Antiserum Production 50
C2.2.2.iii Antigen Preparation for Gel Precipitin Test. 51
C2.2.2.iv Gel Precipitin Test 51
C2.2.2.v Preparation of a Standard Curve for the Estimation of Total Protein Content of *M. ovipneumoniae* 52
C2.2.2.vi The SDS-PAGE System 52
C2.3 Results 59
C2.4 Discussion 65

CHAPTER THREE: **Identification of Surface Proteins of *M. ovipneumoniae***

A) Identification of Surface Proteins of Intact *M. ovipneumoniae* Cells by Proteolytic Enzyme Digestion 66
A3.1 Introduction 66
A3.2 Materials and Methods 67
A3.2.1 Materials 67
A3.2.2 Methods 69
A3.2.2.i A Comparison of Three Proteolytic Enzymes to Determine their Ability to Selectively Remove the Surface Proteins from an Intact *M. ovipneumoniae* Strain 69
A3.2.2.ii Periodic Acid-Silver-Staining for the Detection of Glycoproteins of *M. ovipneumoniae* 69
A3.3 Results 70
A3.4 Discussion 75

Page

B) Investigations of Some *M. ovipneumoniae* Proteins by the "Immuno-Blot" Technique 77

B3.1 Introduction 77

B3.2 Materials and Methods 77

B3.2.1 Materials 77

B3.2.2 Methods 80

B3.2.2.i The Excision of *M. ovipneumoniae* Surface Proteins from SDS-PAGE Gels for the Production of Monospecific Antisera 80

B3.2.2.ii Antisera Production to Excised Surface Proteins from SDS-PAGE Gels 80

B3.2.2.iii Electrophoretic Transfer of Proteins from a SDS-PAGE Gel to a Nitro-cellulose Sheet ("Western-Blot") 81

B3.2.2.iv Goat Anti-Rabbit Horseradish Peroxidase Conjugate Enzyme Immunoassay 83

B3.2.2.v Photographing Visualized Bands 84

B3.3 Results 84

B3.4 Discussion 89

CHAPTER FOUR: **Investigation of the Heterogeneity of *M. ovipneumoniae* Isolates using Various Restriction Endonucleases Including a Comparison of Multiple Isolates from Individual Pneumonic Lungs**

4.1 Introduction 90

Page

4.2 Materials and Methods 91
4.2.1 Materials 91
4.2.2 Methods 94
A) Examining the Heterogeneity of *M. ovipneumoniae* Isolates from Sheep on Different Farms by using Restriction Endonucleases 94
4.2.2A.i Propagation and Preparation of *M. ovipneumoniae* Isolates for DNA Extraction 94
4.2.2A.ii DNA Extraction 95
4.2.2A.iii Spectrophotometric Determination of the Purity and Concentration of the DNA 96
4.2.2A.iv Digestion of DNA with ***Eco***R1 96
4.2.2A.v Gel Electrophoresis 97
4.2.2A.vi Photographing the Gel 99
B) Isolation of Multiple *M. ovipneumoniae* Isolates from Six Pneumonic Sheep and One Pneumonic Goat Lung 100
4.2.2B.i "Normal" Method of *M. ovipneumoniae* Isolation 100
4.2.2B.ii Isolation of *M. ovipneumoniae* Isolates Directly from FM4 Agar Plates and by Limit Dilution 100
4.2.2B.iii Cloning of Isolates 101
4.2.2B.iv Identification of Multiple Isolates Obtained from Individual Lungs as *M. ovipneumoniae* 101
4.3 Results 101
4.4 Discussion 136

Page

CHAPTER FIVE: **The Detection of Methylated Bases in the DNA of Some Isolates of *M. ovipneumoniae***

5.1 Introduction 139
5.2 Materials and Methods 141
5.2.1 Materials 141
5.2.2 Methods 143
A) Bacterial Restriction Endonuclease DNA Analysis to Detect Base Sequence Methylations 143
B) The Use of High-Performance Liquid Chromatography to Examine *M. ovipneumoniae* DNA for the Presence of 5-Methylcytosine 143
5.2.2B.i Preparation of Nucleoside Standards 143
5.2.2B.ii Enzymatic Digestion of *M. ovipneumoniae* DNA 143
5.2.2B.iii High-Performance Liquid Chromatography (HPLC) 144
5.3 Results 144
5.4 Discussion 150

CHAPTER SIX: **Examination of *M. ovipneumoniae* Strains for the Presence of Plasmids**

6.1 Introduction 157
6.2 Materials and Methods 157
6.2.1 Materials 157
6.2.2 Methods 159
A) The Detection of Small Plasmids 159

Page

B) The Detection of Large Plasmids 160
6.2.2B.i The Gel System 160
6.2.2B.ii Preparation of an Agarose Gel 160
6.2.2B.iii Propagation of Isolates 162
6.2.2B.iv Preparation of Samples 162
6.2.2B.v Electrophoresis Conditions 162
6.2.2B.vi Staining the Gel 162
6.2.2B.vii Gel Photography 163

6.3 Results 163
6.4 Discussion 165

CHAPTER SEVEN: **Investigation of the Relatedness of *M. ovipneumoniae* using DNA-DNA Hybridization**

7.1 Introduction 166
7.2 Materials and Methods 166
7.2.1 Materials 166
7.2.2 Methods 174

A) DNA-DNA Hybridization of *M. ovipneumoniae* Isolates and Other Members of the Class *Mollicutes* 174
7.2.2A.i Propagation of *M. ovipneumoniae*, *M. arginini*, *M. pneumoniae* and *A. laidlawii* strains 174
7.2.2A.ii DNA Extraction 174
7.2.2A.iii Purification of DNA by CsCl Density Gradient Ultra-centrifugation 175
7.2.2A.iv The Determination of:
a. The Tm of *M. ovipneumoniae* DNA and Other Micro-organisms 176
b. The Purity of the DNA by Measuring its Hyperchromatic Shift 176
7.2.2A.v Preparation of Radioactive ("Labelled") DNA Using the Nick-Translation Method 177

Page

7.2.2A.vi The Removal of Unincorporated dCTP [alpha-^{32}P] Following "Nick-Translation" Using a "Minispin Column" 178

7.2.2A.vii The Determination of the Specific Activity of Labelled DNA 179

7.2.2A.viii The Preparation of Unlabelled DNA for Hybridization 180

7.2.2A.ix DNA Reassociation 180

7.2.2A.x Calculation of the Concentration of Labelled and Unlabelled DNA to be Used in Hybridization Experiments 180

7.2.2A.xi The Separation of Single and Double Stranded DNA on Hydroxylapatite 183

B) Estimation of DNA Relatedness 184

7.3 Results 184

7.4 Discussion 196

CHAPTER EIGHT: **General Discussion** 198

APPENDIX I 206

APPENDIX II 210

APPENDIX III 213

BIBLIOGRAPHY 214

LIST OF FIGURES

Figure		Page
1	Colonies of *M. ovipneumoniae* incubated for six days at 37^{o}C	24
2	Lysis of *M. ovipneumoniae* by surfactants	32
3	Examination of solubilized and non-solubilized proteins following treatment of *M. ovipneumoniae* cells with Zwittergen 3-12 and 3-14	33
4	Preparation of an iso-electric focusing gel and assembly of apparatus	40
5	Separation of *M. ovipneumoniae* proteins by iso-electric focusing	43
6	Protein assay standard curve	53
7	Preparation of sodium dodecyl sulphate-polyacrylamide gels and assembly of apparatus	56
8	Identification of mycoplasmas as *M. ovipneumoniae* using the gel precipitin test	61
9	The total proteins of eight isolates of *M. ovipneumoniae* all derived from sheep on different farms and examined by SDS-PAGE	61
10	SDS-PAGE patterns of the total proteins of sixty *M. ovipneumoniae* isolates obtained from twenty different farms	62
11	Comparison of *M. ovipneumoniae* isolates with and without a particular protein band	64
12	Investigation of the reproducibility of SDS-PAGE protein separations using eight replicate cultures of one *M. ovipneumoniae* strain	64

Figure **Page**

13 A comparison of SDS-PAGE protein patterns of intact *M. ovipneumoniae* cell suspensions treated with three different proteolytic enzymes (pronase, papain or trypsin) at two different concentrations (5ug/ml or 50ug/ml) for varying times 72

14a A comparison of the total proteins of eight *M. ovipneumoniae* strains before and after exposure to 50ug/ml of trypsin for 10mins at 37^{o}C 73

14b An enlargment of Figure 14a which shows the region bounded by bands B and E 73

15 A comparison of eight *M. ovipneumoniae* strains before and after exposure to 50ug/ml of trypsin for 10mins at 37^{o}C. Stained with periodic acid-silver 74

16 Assembly of the gel holder to transfer proteins from an acrylamide gel to a nitrocellulose sheet 82

17 Transfer of the protein to the nitrocellulose sheet 82

18 Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band B 86

19 Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band C 86

20 Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band D 87

21 Eight *M. ovipneumoniae* isolates "blotted" with antisera raised to bands B, C and D 87

22 Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band E 88

23 Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band F 88

Figure **Page**

24 Preparation of agarose gels for bacterial restriction endonuclease DNA analysis 98

25 Bacterial restriction endonuclease DNA analysis of eight *M. ovipneumoniae* strains obtained from sheep on different farms. The DNA was cleaved using *Eco*RI 110

26 Bacterial restriction endonuclease DNA analysis of eight *M. ovipneumoniae* strains obtained from sheep on different farms. The DNA was cleaved using *Dra*I 110

27 Bacterial restriction endonuclease DNA analysis of eight *M. ovipneumoniae* strains obtained from sheep on different farms. The DNA was cleaved using *Hpa*II 111

28 Bacterial restriction endonuclease DNA analysis of eight *M. ovipneumoniae* strains obtained from sheep on different farms. The DNA was cleaved using *Msp*I 111

29a Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates which were recovered simultaneously from a single pneumonic sheep lung by the "normal" isolation method 112

29b Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates recovered from a single pneumonic sheep lung by selecting ten colonies derived from an inoculum of original lung material on FM4 agar 112

29c Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates recovered from a single pneumonic sheep lung by limit dilution 112

30 Bacterial restriction endonuclease DNA analysis of two *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 1) 113

Figure **Page**

31 Bacterial restriction endonuclease DNA analysis of two *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 2) 113

32 Bacterial restriction endonuclease DNA analysis of four *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 3) 114

33 Bacterial restriction endonuclease DNA analysis of four *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 4) 114

34 Bacterial restriction endonuclease DNA analysis of three *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 5) 115

35 Bacterial restriction endonuclease DNA analysis of four *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 6) 115

36 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Xba*I 116

37 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Bgl*II 116

38 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Hin*dIII 117

39 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Msp*I 117

40 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Dra*I 118

Figure **Page**

41 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Hpa*II 118

42 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Kpn*I 119

43 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Sal*I..... 119

44 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Bam*HI..... 120

45 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Sma*I 120

46 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Xho*I 121

47 Thirty isolates of *M. ovipneumoniae* from one sheep lung, showed four different DNA patterns when digested by *Eco*RI. Isolates representing each pattern were examined using *Hae*III 121

48 Thirty isolates of *M. ovipneumoniae* from one lung were assigned to four groups according to the BRENDA patterns observed. Within any one group the basic pattern was identical or showed only minor variations..... 122

Figure **Page**

49 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Xba*I 123

50 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Bgl*II 123

51 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Hin*dIII 124

52 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Msp*I 124

53 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Dra*I 125

54 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Hpa*II 125

55 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Kpn*I 126

56 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Sal*I 126

Figure **Page**

57 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Bam*HI 127

58 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Sma*I 127

59 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Xho*I 128

60 Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep and showing a similar, but not quite identical, pattern were examined by *Hae*III 128

61a SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered simultaneously from a single pneumonic sheep lung by the "normal" isolation method 129

61b SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered from a single pneumonic sheep lung by selecting ten colonies derived from an inoculum of original lung material on FM4 agar 129

61c SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered by ten parallel limit dilution titrations from a homogenate of single pneumonic lung lesion 129

62 SDS-PAGE examination of two *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e lung 1) 130

Figure **Page**

63 SDS-PAGE examination of two *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 2) 130

64 SDS-PAGE examination of four *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 3) 131

65 SDS-PAGE examination of four *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 4) 131

66 SDS-PAGE examination of three *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 5) 132

67 SDS-PAGE examination of four *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 6) 132

68 Gel precipitin test for the identification of a mycoplasma isolated from a pneumonic goat lung 133

69 A comparison of the total protein of an *M. ovipneumoniae* isolate from a pneumonic goat lung with three *M. ovipneumoniae* strains 133

70a Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates which were recovered simultaneously from a single pneumonic goat lung by the "normal" isolation method 134

70b Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates recovered from a single pneumonic goat lung by selecting ten colonies derived from an inoculum of original lung material on FM4 agar 134

Figure **Page**

70c Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates recovered from a single pneumonic goat lung by limit dilution 134

71a SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered simultaneously from a single pneumonic goat lung by the "normal" isolation method 135

71b SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered from a single pneumonic goat lung by selecting ten colonies derived from an inoculum of original lung material on FM4 agar 135

71c SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered by ten parallel limit dilution titrations from a single pneumonic goat lung 135

72 Demonstration of the activity of *Xho*I and its inability to cleave L6T3 DNA 147

73 BRENDA of *M. ovipneumoniae* strain L6T3 DNA digested with enzymes which discriminate between methylated and non-methylated adenine 147

74 BRENDA of *M. ovipneumoniae* strain L6T3 DNA digested with enzymes which discriminate between methylated and non-methylated cytosine 148

75 Further investigation of *M. ovipneumoniae* strain L6T3 DNA cleaved by *Msp*I and *Hpa*II for longer periods and/or increased concentrations 148

76a *M. ovipneumoniae* strain L6T3 DNA degraded to nucleosides and examined by HPLC 149

76b Standard mixtures of six nucleosides 149

76c A control for Figures 76a and 76b 149

77 Preparation of Eckhardt gels for the detection of large plasmids in *M. ovipneumoniae* lysates 161

Figure **Page**

78 Examination of eight *M. ovipneumoniae* strains for the presence of small plasmids 164

79 Examination of eight *M. ovipneumoniae* strains for the presence of large plasmids using the Eckhardt method 164

80 The increase in hyperchromatic shift as a measure of DNA purity 188

81 Determination of the midpoint (Tm) of thermal denaturation of the DNA from three mycoplasma species 189

82 A comparison of the midpoint of thermal denaturation (Tm) of DNA from *M. ovipneumoniae* strain L6I1 in two different buffers 190

83 Determination of the sonication period required to fragment DNA to approximately 700bp for use in DNA-DNA hybridizations 191

84 The effect of temperature on the hybridization of ^{32}P-DNA from *M. ovipneumoniae* strain L6I1 to an excess of unlabelled DNA from the same strain 192

85 Investigation of optimum conditions for separating single and double stranded DNA using hydroxylapatite 193

86 Investigation of the DNA homology of *M. ovipneumoniae* with different isolates of the same species and with other species of mycoplasma 194

LIST OF TABLES

Table		Page
I	Metabolic inhibition of eight *M. ovipneumoniae* strains tested against three antisera in the absence of complement	22
II	Metabolic inhibition of eight *M. ovipneumoniae* strains tested against three antisera in the presence of 7.5% complement	22
III	Metabolic inhibition of *M. ovipneumoniae* antisera cross-absorbed with heterologous *M. ovipneumoniae* strains	23
IV	Preparation of running and stacking gels	55
V	The number of *M. ovipneumoniae* strains and variants found in six sheep lungs	103
VI	The examination of four simultaneous isolates, from one lung (Lung 6), using a range of restriction enzymes	104
VII	The examination of four simultaneous isolates (from Lung 6), which showed only minor restriction pattern differences with *Eco*RI, with a range of restriction endonucleases	106
VIII	Variations in BRENDA patterns of thirty *M. ovipneumoniae* isolates derived from one pneumonic goat lung	109
IX	The degree of hybridization using ^{32}P-DNA probes for four *M. ovipneumoniae* strains with DNA from *M. ovipneumoniae*, *M. pneumoniae*, *M. arginini* and *A. laidlawii* strains	195

INTRODUCTION

Chronic Non-Progressive Pneumonia of Sheep In New Zealand: The Disease and its Significance.

Chronic non-progressive pneumonia (CNP) is a common disease of sheep in New Zealand and is most prevalent in the late summer/early autumn in sheep which are then three to nine months old. The disease is usually subclinical, but clinical disease can also occur and, at slaughter, extensive lung lesions are observed in these cases.

Although CNP is usually subclinical and on its own it is rarely fatal it is important economically in New Zealand for two reasons:

a) It causes the formation of pleural lesions in some lambs, which may lead to downgrading, or in severe cases to the rejection of carcasses for export (Dysart, 1976).

b) It diminishes weight gain in lambs (Harris and Alley, 1977).

The first point is widely accepted and can be measured in financial terms. The latter is difficult to quantify although it is plausable that a lamb with pneumonia will be less thrifty than one not so affected. Thus, St.George *et al*, (1971) and Carmichael *et al*, (1972) states, but cites no evidence, that CNP caused inadequate weight gain in lambs.

A study in New Zealand of the effect of CNP on weight gain was undertaken by Kirton *et al*, (1976). These workers examined 3243 lamb carcasses at slaughter over a five year period. They found that 60% of the lambs had CNP. However, the average carcass weight of lambs with CNP was significantly higher than normal lambs. This unexpected result did not lead Kirton *et al*, (1976) to conclude that CNP improved weight gain. Rather, they suggested that CNP did not affect weight gain.

An alternative hypothesis to explain Kirton's data was proposed by Harris and Alley, (1977). They suggested that the carcass weight difference should not be ignored and pointed out that most lambs contract CNP during their first summer and that the disease resolves over a

period of several months. Thus, most of the lambs without pneumonic lesions at slaughter would represent lambs which became infected with CNP earlier in the year followed by resolution of the lesions before slaughter. While the pneumonic sheep (which were heavier) had probably contracted the disease immediately prior to slaughter and were thus infected for a shorter period and probably at a less critical time in their life. These considerations allowed Harris and Alley, (1977) to conclude that the findings of Kirton *et al*, (1976) were consistent with the hypothesis that CNP has an adverse effect on the weight gain of lambs in New Zealand and hence is of economic importance.

As noted above not all cases of CNP resolve without sequelae. Thus, some lambs develop pleural adhesions and the carcasses of such animals are diverted from the main killing chains of the abbatoir to the "detain rail". Here the affected pleura is stripped from the carcasses and re-examined by meat inspectors prior to railing back into the mainstream of production. 816 700 (3.2%) of lambs killed in New Zealand during the 1974/75 season had pleural adhesions (Dysart, 1976) so the problem of dealing with large numbers of carcasses on a short detaining rail and the disruption of throughput of carcasses in such a labour-intensive industry is expensive though hard to quantify. This problem is made much greater by the fact that "lines of lambs" differ greatly in the prevalence of pleural adhesions, thus some flocks show no lesions whereas others exhibit a high prevalence and can slow the killing chain. Some persons in New Zealand regard this as a disaster second only to a defeat of the "All Blacks" by a Welsh rugby team. However, an even more severe problem arises: carcasses with pleurisy are not accepted in the United States, Canada or West Germany. So when slaughtered sheep of all ages are included, over two million such carcasses in the 1974/75 season were excluded from these markets. The United Kingdom will accept lamb and mutton which have minimal pleural lesions (Dysart, 1976) but reject carcasses with more widespread damage.

It has been estimated that $NZ1.8 million in revenue was lost to pleurisy in lambs in the 1974/75 season (Dysart, 1976). Due to a combination of inflation and the continued upgrading of standards for export carcasses, the present figure runs into many millions of dollars.

Distinction Between Chronic and Acute Pneumonia.

Acute pneumonia usually affects lambs in the early summer months and is characterized by a sudden onset of symptoms and rapidly progresses to cause the death of the animal. *Pasteurella haemolytica* is usually associated with acute pneumonic lesions (Downey, 1957) and can be clearly distinguished from chronic pneumonia by histopathological examination of lung lesions.

Acute pneumonia is characterized by intense congestion and varying degrees of red or grey consolidation of the ventral portion of one of both lungs. Cellular exudates are composed of neutrophils, macrophages and detached alveolar epithelial cells which many bacteria are closely associated with (Alley, 1975b). In contrast, chronic or "atypical" lesions are characterized by proliferation of lymphoid and epithelial tissue, with exudation of macrophages and to a lesser extent neutrophils (Alley, 1975b).

A similar, probably identical, disease to CNP has been described in sheep in other countries *viz* USA (Boidin *et al*, 1958; Barber and Fabricant, 1961; St.George and Carmichael, 1975), Britain (Mackay *et al*, 1963; Mackay and Nisbet, 1966; Jones *et al*, 1976), Turkey (Cottew *et al*, 1968), Kenya (Krauss and Wandera, 1970), Australia (Cottew, 1971; St.George *et al*, 1971; Carmichael *et al*, 1972; Sullivan *et al*, 1973a), New Zealand (Clarke *et al*, 1974; Alley *et al*, 1975b), Iceland (Friis *et al*, 1976) and Norway (Bakke and Nostvold, 1982).

Nomenclature of the Disease.

A veterinary pathologist and colleague, designated this chronic disease of New Zealand sheep as chronic non-progressive pneumonia (Alley, 1975a). This designation was derived from his observations, that chronic lung lesions are characterized by the presence of macrophages and lymphocytes and is readily distinguished from acute (bacterial) pneumonia which elicites a neutrophil responce. CNP can also be differentiated from progressive pneumonia of sheep because the disease fails to progress and ultimately resolves.

A bewildering number of names have been assigned to this disease. Some of these names have merit, but we have selected CNP as the designation. Other names for what is probably the same disease are listed below and their merits are considered.

Summer pneumonia (St.George *et al*, 1972)
Hogget pneumonia
Enzootic pneumonia (Salisbury, 1957)
Atypical pneumonia (Stamp and Nisbet, 1963)
Proliferative exudative pneumonia (Gilmour *et al*, 1979)
Proliferative interstitial pneumonia (Carmichael *et al*, 1972)
Sheep pulmonary adenomatosis (Mackay *et al*, 1966)

The designation "summer pneumonia" was derived from the observation that in Australia CNP reaches its peak prevalence in lambs slaughtered in the summer months (St.George *et al*, 1972). It can be criticised on two grounds i.e. it is not the only pneumonia to occur in summer, even in Australia, and in New Zealand the disease reaches its peak prevalence not in summer but in the late summer and early autumn months.

"Hogget pneumonia" is a correct designation in the sense that it affects six to eight month old lambs (i.e. hoggets). However, again it is not the only type of pneumonia seen in such animals so the name is ambiguous.

The designation "enzootic pneumonia" correctly implies that the disease is enzootic in New Zealand (Salisbury, 1957). However, there are other causes of pneumonia in New Zealand sheep such as *Pasteurella haemolytica* and while this is also an "enzootic" pneumonia it is readily distinguishable from CNP.

The designation "atypical pneumonia" was derived from human pathology and is used in contrast to "typical" pneumonia which is due to bacteria. As applied to lambs (Stamp and Nisbet, 1963) "atypical" is unsatisfactory since the "atypical" lesions outnumber the "typical" by about two orders of magnitude. Furthermore, "atypical" pneumonia in humans has come to be applied mainly, if not exclusively, to disease caused by *M. pneumoniae*, whereas in sheep the aetiology of "atypical" pneumonia has not been unequivocally established although *M. ovipneumoniae* undoubtably plays a major role.

The designation "proliferative exudative pneumonia" was applied by Gilmour *et al*, (1979) to lesions produced by inoculating a lung homogenate from sheep with "atypical pneumonia" into the bronchi of normal sheep. It is a descriptive term for the lesions produced and left open the question as to whether or not the lesion represented experimental transmission of "atypical" pneumonia.

The descriptive name "proliferative interstitial pneumonia" has merit but could also be applied to the disease caused by progressive pneumonia virus (PPV) i.e. it does not distinguish between chronic lesions which are, or are not progressive.

The term "sheep pulmonary adenomatosis" is confusing because it has sometimes been used to indicate a disease which is probably identical to CNP, but more recently it has come to by applied to a disease of sheep transmitted by a virus (Mackay *et al*, 1966; Wandera, 1970). It follows that this term is not now a synonym for CNP.

Correlation of *M. ovipneumoniae* with CNP.

The experimental transmission of ovine pneumonia by the intratracheal inoculation of pneumonic lung tissue suspension was described by St.George *et al*, (1971). A cytopathic agent was isolated in cell cultures inoculated with the ground lung suspension. The agent was able to be grown in cell-free liquid medium i.e. Hanks medium plus lactalbumin hydrolysate, yeast extract and foetal calf serum. The colonies of the organism isolated on solid medium were tiny, colourless and round with no "centre". They were similar to *M. pneumoniae* colonies. Further examination showed that the agent was probably a mycoplasma i.e. it passed through a 0.22u pore-size filter and grew in the presence of thallium acetate, streptomycin and penicillin.

Carmichael *et al*, (1972) isolated two species of mycoplasma from the lungs of a Queensland (Australia) flock of sheep with CNP. One mycoplasma, which formed centred colonies on solid medium, was identified as *M. arginini* (biotype C) and was also isolated from the nasal passages of normal and pneumonic sheep. The second mycoplasma (biotype Y-98) was recovered from the nasal cavities, trachea and bronchi of pneumonic lambs and was similar to the organism isolated by St.George *et al*, (1971). This organism formed "vacuolated" centreless colonies and fermented glucose. It was serologically different from mycoplasmas recovered from other animal species and was also antigenically

distinct from twelve ovine and caprine mycoplasma serotypes as shown by metabolic-inhibition tests. The name *M. ovipneumoniae* was proposed by Carmichael *et al*, (1972).

Nine strains of glycolytic mycoplasmas isolated from the respiratory tract of apparently healthy sheep, pneumonic sheep and sheep exhibiting pulmonary adenomatosis in Scotland, were compared with the Queensland strain (Y-98) by Jones *et al*, (1976). All nine mycoplasma strains were compared by polyacrylamide-gel electrophoresis, gel precipitin, metabolic and growth inhibition tests. They gave similar results indicating that they were related to each other and to the Queensland Y-98 strain, therefore they were regarded as belonging to the same species. However, metabolic and growth inhibition tests showed some intraspecific differences with the "apparent polarization" of strains isolated from sheep with and without "pulmonary adenomatosis". Jones *et al*, (1976) also pointed out that the serological relationship of *M. ovipneumoniae* to other members of the genus *Mycoplasma* has been insufficiently investigated. So using his strains and the Queensland strain (Y-98), he tested them using the metabolic inhibition test against hyperimmune sera to thirty-three named mycoplasma species or sub-species and six serogroups of bovine or caprine origin. Jones *et al*, (1976) confirmed that his strain should be considered as a distinct species with the name *M. ovipneumoniae*.

M. ovipneumoniae strains have now been reported to be isolated from pneumonic lesions, healthy lungs and also from the nasal cavities of sheep from: Australia (Furlong and Cottew, 1973; Cottew, 1971); New Zealand (Clarke *et al*, 1974, Alley *et al*, 1975b); England (Leach *et al*, 1976); USA (St.George and Carmichael, 1975, Brogden *et al*, 1988); Scotland (Jones *et al*, 1976); Iceland (Friis, 1976); Norway (Bakke and Nostvold, 1982); Spain (Garcia *et al*, 1988); Senegal (Konte and Breard, 1988) and Ireland (Malone *et al*, 1988).

Experimental Transmission of CNP.

The experimental transmission of CNP to caesarean-derived, colostrum-deprived and conventional lambs was described by St.George *et al*, (1971). They isolated a mycoplasma (subsequently designated *M. ovipneumoniae*) from a pneumonic lung homogenate and propagated the culture *in vitro* for six passages before inoculating sheep and producing lesions which the authors claimed were similar to the field disease.

Sullivan *et al*, (1973b) isolated a mycoplasma from a pneumonic lung and inoculated day-old Merino lambs either by exposing them to an aerosol or by intravenous inoculation. Other

lambs were exposed to the aerosol-infected lambs. Post-mortem examination of the infected lambs found that proliferative interstitial pneumonia had developed. They also found that the in-contact lambs also developed a similar pneumonia. They concluded that aerosol exposure and administration by direct contact demonstrates the infectivity of the organism *via* the respiratory route. However, they could not recover the mycoplasma from the infected lambs and attributed this to technical problems.

Foggie *et al*, (1976) inoculated colostrum-deprived specific pathogen free lambs endobronchially with a Scottish isolate of *M. ovipneumoniae* and produced mild lesions in three out of six lambs. They were able to recover *M. ovipneumoniae* from the lungs of all infected animals but not from the controls.

Jones *et al*, (1978), used a lung suspension prepared from the lesions of naturally occurring "atypical pneumonia" and inoculated sheep endobronchially. They found that the lung homogenate included *P. haemolytica* (several serotypes), *M. ovipneumoniae* and *E. coli*. The lesions that developed in the inoculated animals were indistinguishable from atypical pneumonia. High titres of *P. haemolytica* and *M. ovipneumoniae* were recovered from the lungs. In a parallel experiment Jones *et al*, (1978) used a second pneumonic lung suspension containing *P. haemolytica* (serotype A2), *M. ovipneumoniae* and *M. arginini*. This suspension, containing the combined cloned cultures of the three organisms isolated was endobronchially inoculated into sheep. The sheep showed clinical symptoms of the respiratory diseases and the lesions were similar as those observed in field cases of "atypical pneumonia". *M. ovipneumoniae* was recovered from the inoculated animals but the recovery of *P. haemolytica* and *M. arginini* was less consistant. They concluded that mycoplasmas and *P. haemolytica* may be the aetiological agents involved in atypical pneumonia.

In further transmission experiments, Gilmour *et al*, (1979) inoculated specific pathogen free and conventially reared lambs. The lambs were inoculated *via* the endobronchial route with individual strains of *M. ovipneumoniae*, combination of *M. ovipneumoniae* and *P. haemolytica* and *P. haemolytica* alone. They concluded that the administration of individual *M. ovipneumoniae* strains produced mild pneumonic lesions but an increased severity of the lesions was noted when *M. ovipneumoniae* and *P. haemolytica* were administered together i.e. about 70% of the lambs exhibited "proliferative exudative pneumonia". They further concluded that *M. ovipneumoniae* becomes established in the

lung, but that *P. haemolytica* does so less readily and that individual strains of *M. ovipneumoniae* did not vary significantly in their pathogenicity.

In New Zealand Alley and Clarke (1979) inoculated twenty five-month-old conventially reared lambs with a pneumonic lung homogenate using an intranasal aerosol. Seventeen (85%) of the lambs developed CNP indistinguishable from field cases. In a parallel experiment a further twenty lambs were inoculated intranasally with a cloned culture of *M. ovipneumoniae*, four (20%) of the experimental lambs showed lesions which were less severe than the natural disease. This result suggests that *M. ovipneumoniae*, though involved in CNP, may not on its own be capable of causing the lesions seen in the more severe field cases.

In further experiments (Alley and Clarke, 1980) in which sheep inoculated with pneumonic lung were treated with penicillin, mild lesions were produced and *M. ovipneumoniae* was recovered from them. They concluded that a penicillin sensitive organism, *viz* a bacterium, plays a role in the pathogenesis of field cases of CNP. In the same laboratory, Brian (1980) showed that lung homogenates treated with digitonin, which inactivates mycoplasmas but not bacteria, failed to transmit the disease and more recently Jones *et al*, (1982b) produced CNP using intratracheal inoculation of *M. ovipneumoniae* alone. However, they state that *P. haemolytica* should be considered as an important exacerbating factor of the morbidity and of the severity of "chronic ovine pneumonia".

An unusual experiment which is of particular interest in the possible interpretation of the results of this thesis was conducted by Jones *et al*, (1982a) who used clones of *M. ovipneumoniae* to produce pneumonic lesions in specific pathogen free lambs. A mixture of six *M. ovipneumoniae* strains were used to exclude the possibility that any one strain could be of low virulence. Three days after intratracheal inoculation of the lambs with the mixture, *P. haemolytica* (serotype A2) was also administered to the lambs. In a parallel experiment a number of sheep were also inoculated with one of each of the six *M. ovipneumoniae* strains used in the combined inoculant. This was followed three days later with a *P. haemolytica* intratracheal inoculation. They found that multiple clones of *M. ovipneumoniae* used as an inoculum produced major lesions in 53% of the lambs. In contrast, lambs inoculated with any one single strain of *M. ovipneumoniae*, only 17% showed lesions. The re-isolation rate of *M. ovipneumoniae* was highest in the mixed clone inoculation group of lambs. Jones *et al* (1982a) concluded that multiple clones of

M. ovipneumoniae were more effective than individual *M. ovipneumoniae* strains and with the combination of *P. haemolytica*, were able to produce the disease.

From the experimental transmission results obtained by Jones *et al*, (1982a) we can speculate that if multiple clones of *M. ovipneumoniae* act as an exacerbating factor, then it should be possible to isolate multiple *M. ovipneumoniae* strains from a single lung with severe CNP. A section of this thesis (chapter four) is concerned with evidence that multiple *M. ovipneumoniae* strains are present within a single pneumonic lung.

Isolation of *M. ovipneumoniae* from Pneumonic Goat Lungs.

The increase in economic importance of goat-farming for fibre production in New Zealand has led to an elevated awareness of diseases in goats. *M. ovipneumoniae*, *M. arginini*, *M. mycoides* subspecies *mycoides* and *M. mycoides* subspecies *capri* have been isolated from the respiratory tract of goats and it is thought that they may be involved in causing chronic pneumonia (Cottew *et al*, 1968; Barber and Yedloutschnig, 1970; Erno *et al*, 1978; Livingston and Gauer, 1979; Cottew and Yeats, 1981; Goltz *et al*, 1986; Nascimento *et al*, 1986; Jones and Wood, 1988).

Notwithstanding the above, *M. ovipneumoniae* is the mycoplasma which most frequently is associated with caprine chronic pneumonic (Livingston and Gauer, 1979; Goltz *et al*, 1986; Jones and Wood, 1988).

Goltz *et al*, (1986) provided the first evidence that *M. ovipneumoniae* is pathogenic for goats, causing pneumonia and pleuritis. They conducted a survey of 175 goat lungs which exhibited lesions and found that 36.0% of the lungs yielded *M. ovipneumoniae*, 4.6% yielded *M. arginini*, 8.0% had both *M. ovipneumoniae* and *M. arginini* and 5.1% yielded a mixture of other mycoplasmas (i.e.*M. mycoides* subspecies *mycoides* and other and other, unidentifiable, species). Goltz *et al*, (1986) were also able to experimentally transmit *M. ovipneumoniae* to goats and reisolated the same organism from the lung. In a parallel experiment Goltz *et al*, (1986) also inoculated goats with *M. arginini*, but no lung lesions developed and they failed to reisolate the organism. They concluded that *M. ovipneumoniae* was pathogenic for goats causing pneumonia and pleuritis, while *M. arginini* was apparently not a primary pathogen.

Jones and Wood (1988), while examining cases of caprine pleuropneumonia in Oman, examined twenty-one goat lungs and three of the six cases of acute pneumonia yeilded *M. ovipneumoniae* isolates. They also found that *M. ovipneumoniae* was present in eight of the twenty-one goat lungs examined. They concluded that *M. ovipneumoniae* was associated with both chronic and acute forms of pneumonia.

A small portion of this thesis (see chapter five) is devoted to the isolation and study of *M. ovipneumoniae* isolates obtained from the lungs of a New Zealand goat exhibiting a disease designated CNP by Dr M. R. Alley, who coined this name for the sheep disease (Alley, 1975a).

Distinguishing Strains of *M. ovipneumoniae*.

A) Serology.

The subcomittee on the taxonomy of mollicutes (1979) has proposed minimal standards for the description of new species of the Class *Mollicutes*. These standards are based on cultural, biochemical and antigenic characteristics. The first two of these standards are used to identify the species but the serological characteristics may be used to identify either interspecies of intraspecies differences.

Serological tests which examine the antigens of a mycoplasma, e.g. agar gel immunodiffusion, counter immuno-electrophoresis and the complement fixation test, are useful for examining similarities and differences between mycoplasma species. Thus Ball and Todd (1978) used both double immunodiffusion and counter-immunodiffusion tests to examine the relationship among *M. ovipneumoniae*, *M. dispar* and *M. hyopneumoniae*. They concluded that cross-reactions had occurred between these three species. However, we are concerned with intraspecies differences and hence the serological tests most likely to be useful are the metabolic inhibition and growth inhibition tests since these are relatively strain specific (Ball and Todd, 1978).

Jones *et al*, (1976) is the only group of workers to compare strains of *M. ovipneumoniae* by the metabolic inhibition and growth inhibition tests. They examined ten ovine mycoplasma strains against antisera produced to five of the strains. They found that all strains cross-reacted to some degree but nevertheless showed antigenic differences between some

strains tested and claimed to distinguish two groups of *M. ovipneumoniae*, which roughly corresponded to isolates from sheep with and without CNP.

Some preliminary work in this laboratory (Clarke, personal communication) showed that isolates of *M. ovipneumoniae* in New Zealand are serologically related but not identical. This confirms the conclusion of Jones *et al*, (1976), but unlike Jones, Clarke found no consistent distinction between strains derived from pneumonic lungs and other isolates. Furthermore, I was able to show that nasal isolates, colonize the lung frequently (Ionas *et al*, 1985). The present investigation started with some preliminary work on the serology of *M. ovipneumoniae* but clearly a method which is more definitive in its ability to distinguish strains than the metabolic inhibition test or the growth inhibition test using polyclonal antibody would be advantageous.

B) SDS-PAGE.

Fowler *et al*, (1963) were the first to suggest that the electrophoretic seperation of proteins of pleuropneumonia-like organisms, as mycoplasmas were then called, could be used as a method for identifying the species of isolates. Razin and Rottem (1967) were the first workers to apply the technique to several different mycoplasmas and they concluded that isolates of different species gave patterns which are totally, or at least substantially unrelated. In contrast, different isolates of one species gave identical or closely similar patterns. These conclusions have been confirmed and extended to many different mycoplasmas by subsequent workers (Razin, 1968; Zola *et al*, 1970; Forshaw, 1972; Daniels and Meddins, 1973; Gois *et al*, 1974; Jones *et al*, 1976; Asa *et al*, 1979; Chandler *et al*, 1982).

Jones *et al*, (1976) applied this technique to ten isolates of *M. ovipneumoniae* and concluded that there was "a very close resemblance between all ten strains". Jones *et al*, (1976) stressed the similarity of patterns and did not comment on the existance of any differences.

Mew *et al*, (1985), in this laboratory, used SDS-PAGE to examine the total proteins of eight *M. ovipneumoniae* isolates obtained from the respiratory tract of sheep on different flocks in New Zealand. We found that most of the protein bands were common to all the isolates examined, but nevertheless each isolate differed by a number of minor protein bands, suggesting that each was unique. These differences, detected in the protein patterns of

M. ovipneumoniae, prompted us to extend the work and in this thesis we examine a larger number of *M. ovipneumoniae* isolates which were recovered from sheep on different farms and also included a comparison of isolates from sheep from one farm (see chapter two).

C) Identification of Proteins Exposed to the Outer Membrane of *M. ovipneumoniae.*

In the course of this present investigation to assign *M. ovipneumoniae* isolates into biologically meaningful groups, it became clear that *M. ovipneumoniae* isolates obtained from the respiratory tract of sheep were extremely heterogeneous by several criteria, including the examination of total proteins by SDS-PAGE. For this reason we decided to attempt to examine the surface proteins of *M. ovipneumoniae* since it is these proteins which are of more immunological importance. Although several techniques can be used to identify surface proteins, we used two approaches: (a) fluorescent compounds to preferentially label the surface of intact *M. ovipneumoniae* cells and (b) proteolytic enzymes to digest and hence selectively remove the proteins from the outside of the membrane of intact *M. ovipneumoniae* cells.

i. Identification of Surface Proteins by Fluorescent Labelling.

The essential properties of a chemical reagent for selectively labelling proteins exposed on the outer membrane surface are:

a) It must not pass through the membrane.

b) The reagent must react with the membrane under normal physiological conditions i.e. temperature, pH and tonicity, without disrupting the cell.

c) The label must be detectable in small amounts i.e. in practise, fluorescent or radioactive compounds are used.

d) The label should react non-selectively with all surface proteins.

It is not certain that any compound totally fulfills these criteria.

Maddy (1964) suggested that isothiocyanates do not penetrate the membrane. Ionas (1983) exposed intact *M. ovipneumoniae* to fluorescein isothiocyanate (FITC) to preferentially label

the proteins exposed to the outer membrane for a short period of time. I concluded that some selective labelling had occurred, as not all of the protein bands (seen by Coomassie Blue staining) were stained with the FITC label. However, too large a number of FITC-labelled bands were stained to be accounted for by surface protein staining alone so I concluded that the label had to some degree entered the membrane and labelled some internal proteins.

The selective labelling of surface proteins with either fluorescent or radioactive probes has been well documented with mammalian cells and micro-organisms (Maddy, 1964; Dockter, 1979; Schmidt-Ullrich *et al*, 1973). However, little has been described on the use of such compounds on mycoplasmas (Amar *et al*, 1974; Horowitz *et al*, 1987). Dockter (1979) labelled the surface proteins of intact human erythrocytes using 3-azido-(2,7)-naphthalene disulphonate (ANDS) and Maddy (1964) with 4-acetamide, 4'-isothiocyanostilbene-2, 2' disulphonic acid which is also a fluorescent probe. Both of these compounds are claimed by the above authors to be unable to cross membranes and thus must label the surface proteins only. Much effort was made at the early stage of the present thesis work, to apply this approach to allow the identification of the surface proteins of *M. ovipneumoniae*. This work was unproductive and is not recorded outside this introduction. We found that ANDS, although able to label bovine serum albumin and hence to allow its detection at nanogram levels, did not label the surface of intact *M. ovipneumoniae* cells for reasons which we did not determine. Other photochemical probes e.g. diazonium salts (Amar *et al*, 1974) have failed to selectively label the outer membrane proteins of mycoplasma cells because in our hands these compounds were found to enter the cells i.e. proteins other than surface proteins were labelled.

ii. Identification of Surface Proteins by Proteolytic Digestion.

The identification of proteins exposed on the outer membrane of intact mycoplasmas by proteolytic digestion has been well documented (Amar *et al*, 1974; Hu *et al*, 1977; Leith *et al*, 1983; Kahane *et al*, 1985; Klinkert *et al*, 1985; Hu *et al*, 1982; Riethman *et al*, 1987; Horowitz *et al*, 1987).

Amar *et al*, (1974) has done extensive work to identify the surface proteins of *A. laidlawii* and *M. hominis*, using several proteolytic enzymes i.e. pronase, papain and trypsin. They concluded that pronase (20ug/ml) in contact with cells for 10mins was the least suitable proteolytic enzyme because it caused the greatest damage to the cells and hence digested

the internal proteins. They found that low concentrations of trypsin and papain were able to remove proteins from the surface of *A. laidlawii* and *M. hominis*, but a small amount of leakage from the cell was noted. They attributed this to the lysis of a small percentage of cells rather than to an increased permeability of all cells. Trypsin was found to be preferable to pronase and papain because it had a lesser tendancy to lyse the cells.

The present research applies Amer's method to identify surface proteins of *M. ovipneumoniae*.

D) Bacterial Restriction Endonuclease DNA Analysis (BRENDA).

The ability of restriction endonucleases to recognize and cleave specific nucleotide sequences in double stranded DNA has led to the development of a technique to assess the identity or relatedness of micro-organisms. Much work has been done on viruses e.g. Skare *et al*, (1975) and bacteria e.g. Marshall *et al*, (1981). In general the technique is most useful for distinguishing between isolates of organisms with a small genome.

The technique is suitable for mycoplasmas because the genome of about 700 to 800 kilobases (Christansen, 1987) means that there will not be an excessive number of cleavage bands. This allows a comparison between strains. The number of cleavage sites is governed by the endonuclease used and a six-base "cutter" will normally produce fewer fragments than a four-base "cutter". The low GC ratio of mycoplasma DNA in association with a six-base "cutter" which recognizes a sequence containing a high proportion of cytosine and guanosine in their recognition sites (i.e. *Sma*1, 5'-CCC$^{\nabla}$GGG-3'), will also reduce the band number.

Chan and Ross (1984), used BRENDA to compare two mycoplasma species (*M. hyopneumoniae* and *M. flocculare*) which were indistinguishable using biochemical or serological methods. However, they were able to distinguish between them by examining their restriction cleavage patterns.

Although BRENDA has many advantages for discriminating between organisms, there are a number of potential problems, both technical and scientific. The DNA must be of suitable purity and neither sheared nor degraded. Also it is not possible to distinguish between degrees of relatedness especially in the sense that no numerical values can be assigned to the results. This is unlike DNA-DNA hybridizations. Nevertheless, BRENDA has been

successfully used to identify strains of viruses (Skare *et al*, 1975) and bacteria (Marshall *et al*, 1981).

More relevantly, other workers (Razin *et al*, 1983a,b,c) have examined mycoplasma, ureaplasma and acholeplasma DNA cleavage patterns following digestion with a range of restriction endonucleases. The cleavage patterns obtained with *M. gallisepticum* and *M. genitalium* isolates, unlike *Acholeplasma*, showed minimal or no variation within the species (Razin *et al*, 1983c), so they suggested that mycoplasmas of strict host and tissue specificity exhibited marked genetic homogeneity. Similarly, the restriction pattern for five *M. pneumoniae* strains which differed in virulence and adherance capacity, exhibited similar DNA cleavage patterns, indicating a high degree of genetic homogeneity (Razin *et al*, 1983b). Thus the cleavage patterns could apparently be used to identify a mycoplasma species because of the heterogeneity of the patterns. In contrast, Razin *et al*, (1983c) found that isolates of *A. axanthum* differed markedly in their DNA cleavage patterns. In contrast, when they examined nine ureaplasma serovars, digested with a variety of restriction endonucleases, they were able to divide the ureaplasma serovars into just two distinct groups and hence showed the usefulness of BRENDA as a powerful tool in distinguishing between mycoplasma strains.

BRENDA was used to distinguish between isolates of *M. ovipneumoniae* obtained from different flocks of sheep in New Zealand (Mew *et al*, 1985). We found that the DNA cleavage patterns obtained following digestion of eight isolates of *M. ovipneumoniae* with the restriction endonuclease *Eco*RI were all totally different. This marked heterogeneity was in marked contrast to the results obtained with *M. gallisepticum* and *M. genitalium* (Razin *et al*, 1983c) and *M. pneumoniae* (Razin *et al*, 1983b), which as noted above gave homogeneous patterns within each species.

BRENDA has also been used as an epidemiological tool to examine the colonization by *M. ovipneumoniae* of the nasal tract of a flock of sheep (Ionas *et al*, 1985). Within this one flock it was possible to identify seven "groups" of *M. ovipneumoniae*, where each group contained a markedly different BRENDA pattern. Ionas *et al*, (1985) were also able to follow the colonization of the nasal cavity of each lamb within the same flock and found that one *M. ovipneumoniae* strain would apparently be lost and replaced with another strain. We also found that at slaughter approximately 90% of the *M. ovipneumoniae* isolates obtained from the lungs were the same as the strain which was present in the nasal cavity of the same lamb three weeks before slaughter. This suggests that the nasal and lung isolates of

M. ovipneumoniae do not represent independant populations.

These points adequately indicate that BRENDA patterns can be used to identify strains of *M. ovipneumoniae* and a major part of this thesis applies and extends this conclusion.

Methylation of DNA.

This topic arose late in the present investigation and is dealt with in detail later. It is relevant because methylation affects the ability of restriction enzymes to cleave DNA.

DNA may be methylated to some degree and in general, cytosine-methylation occurs in eucaryotic DNA (Razin, 1985) whereas the DNA of procaryotes usually has adenine-methylation (Razin, 1985). In contrast to the above statements however, Razin and Razin (1980) examined hydrolysed DNA from *Mycoplasma hyorhinis* using high pressure liquid chromatography and found that about 5.8% of the cytosine residues were 5-methylcytosine. This is an important observation which impacted on the present work, but a detailed consideration of the consequences is postponed to chapter five. It should be noted however, that since restriction endonucleases are affected by methylation, it is possible that identical DNA sequences, methylated differently, could give different cleavage patterns.

Extrachromosomal Elements In the Genus *Mycoplasma*.

Extrachromosomal elements are common in procaryotes but there are few reports of extrachromosomal DNA in mycoplasmas. However, Zouzias *et al*, (1983) were able to isolate a plasmid from *Mycoplasma hominis* by CsCl density gradient centrifugation and showed, by electron microscopy, that the plasmid was a covalently closed circle. Harasawa and Barile (1983) conducted a survey to investigate the presence of extrachromosomal DNA in several isolates of each of several mycoplasma species. They found two plasmids. One was from an isolate of *M. hominis* which confirms the work of Zouzias *et al*, (1983) and the other was from a mycoplasma of unidentified species.

There has been no report of the examination of *M. ovipneumoniae* isolates for the presence of extrachromosomal elements so we examine a number of isolates of this species for the presence of plasmids and the results are reported in chapeter six.

Investigation of the Relationship of Micro-organisms using DNA-DNA Hybridizations.

The technique of comparing organisms by DNA-DNA hybridizations is simple in principle. A radioactive DNA probe (Rigby, 1977) is mixed with an excess of non-radioactive DNA from a range of isolates of the same species or a range of different species. Following incubation in standard conditions and separation of single and double stranded DNA, the extent of hybridization can be evaluated numerically to give a measure of the relatedness of different organisms (Brenner and Falkow, 1971; Brenner *et al*, 1982). This technique has been used to compare different species of mycoplasmas (Reich *et al*, 1966; Somerson *et al*, 1966; Peterson and Pollock, 1969; Askaa and Erno, 1976; Sungino *et al*, 1980; Thomas and Dierks, 1980; Chandler *et al*, 1982; Christiansen and Emo, 1982; Yogev and Razin, 1986).

DNA-DNA homology has been adopted by the Subcommittee on the Taxonomy of *Mycoplasmatales* (1972) as part of a minimal standard for the description of new species of the order *Mycoplasmatales*. DNA homology techniques have also been used, albeit less extensively, to compare isolates of the same mycoplasma species (Somerson *et al*, 1966). Thus, Somerson *et al* (1966) used DNA-RNA homology techniques to determine the genetic relatedness of the nucleic acids of eight mycoplasma isolates which were serologically classified as *Mycoplasma hominis*. The eight isolates each showed more homology with themselves than with any of the other seven i.e. each isolate was different at least to a small extent. We apply this approach to *M. ovipneumoniae* (see chapter seven).

Overall Objectives.

In brief, this thesis addresses the topics listed below. However, some e.g. serology, were not investigated in depth whereas others e.g. the methylation of DNA, its consequences for BRENDA and DNA-DNA hybridizations represent a major part of the investigation.

i) Comparison of *M. ovipneumoniae* isolates using a metabolic inhibition test.
ii) Separation and comparison of the proteins of *M. ovipneumoniae* isolates by IEF and SDS-PAGE.
iii) Identification of surface proteins of *M. ovipneumoniae*
iv) Investigation of the heterogeneity of *M. ovipneumoniae* isolates using various restriction endonucleases including a comparison of multiple isolates from individual pneumonic lungs.
v) Detection of methylated bases in the DNA of some isolates of *M. ovipneumoniae*.

vi) Examination of *M. ovipneumoniae* strains for the presence of extrachromosomal elements.

vii) Investigation of the relatedness of *M. ovipneumoniae* isolates using DNA-DNA hybridizations.

CHAPTER ONE

Examination of *M. ovipneumoniae* Isolates by the Metabolic Inhibition Test.

1.1 Introduction.

M. ovipneumoniae is associated with chronic non-progressive pneumonia (CNP), which is an economically important disease of sheep in New Zealand. The organism can be recovered from virtually 100% of CNP lung lesions, but can also be recovered from the nasal tract of some sheep and the colony morphology of isolates can vary (see below). These considerations suggest the possibility that isolates of the organisms may not be identical. If so, the most important variation from the point of view of immunity, is serological variation between isolates from pneumonic lungs. Some such variation has been reported (Jones *et al*, 1976) for isolates in the United Kingdom.

This section examines several isolates using the metabolic inhibition (MI) test to investigate whether or not isolates recovered from the lungs of New Zealand sheep are serologically homogeneous.

1.2 Materials and Methods.

1.2.1 Materials

1) **FM4 Medium**: see appendix

2) **Pooled Guinea-Pig serum**
   Stored in aliquots at -70^{o}C. This was a source of complement.

3) **Phosphate Buffered Saline**: see appendix

1.2.2 Methods

1.2.2.i Quantal Titration of *M. ovipneumoniae* Strains.

Eight *M. ovipneumoniae* strains were each propagated in 50ml of FM4 Medium and stored at -70^{o}C in 2ml aliquots. An aliquot from each frozen *M. ovipneumoniae* culture was thawed and serial 10-fold dilutions in FM4 Medium were prepared and incubated at 37^{o}C. The end-point was recorded as the highest dilution which produced a colour change in the FM4 Medium (i.e. a colour change from red to yellow corresponding to a drop of at least one pH unit).

1.2.2.ii Production of Antisera.

Rabbit antisera was raised against three *M. ovipneumoniae* strains (1, 5 and 10) as described in chapter two - section C2.2.2.ii. The antisera were heated to 56^{o}C for 30mins to inactivate complement before use.

1.2.2.iii Metabolic Inhibition Test.

MI tests were performed in disposable plastic, flat-bottomed micro-titration plates (Linbro^{TM}). The plates and their lids were sterilized with ultra-violet radiation using a 30 watt germicidal lamp (Philips^{TM}) at a distance of 0.5m for 30mins.

50ul of FM4 Medium was dispensed into ten micro-titre wells and 150ul into two further wells which were used as a control. 50ul of an initial 1:4 dilution of antiserum was used to prepare serial 2-fold dilutions in FM4 Medium in a row of ten micro-titre wells. 50ul of a mycoplasma culture containing 10^{4}ccu* was added to all the wells and the volume of each well was adjusted to 200ul by the addition of FM4 Medium. This gave a dilution of 1:40 of the antibody in the first well. MI tests, in the presence of complement, were achieved by supplementing the FM4 Medium with complement to give a final concentration of 7.5% guinea-pig serum per well. Note that in a standard complement titration, using 1% sensitized sheep red blood cells, the guinea-pig serum had an end-point of 1:320. Thus, 7.5% guinea-pig serum represents a 24 MCD_{50}. Each *M. ovipneumoniae* culture tested by MI was assayed to confirm the calculated titre.

The micro-titre plates were incubated at 37^{o}C in a humidified container. The results were read when a 10^{4} dilution of a test strain (not incubated in antiserum) changed colour. The endpoint was was then determined as being the first well which exhibited a colour change (i.e. a pH drop of one unit).

1.2.2.iv Absorption of Antisera for use in Metabolic Inhibition Tests.

M. ovipneumoniae strains 1, 5 and 10 were propagated in 100ml of FM4 Medium. The cells were centrifuged at 15 500g for 15mins, washed twice in PBS and made up to a 50% suspension in PBS. 0.1ml of each mycoplasma suspension was placed into separate 1.5ml Eppendorf microfuge tubes and 0.4 ml of non-homologous antisera to total *M. ovipneumoniae* strains was added. Each microfuge tube was mixed gently and then incubated for 14hrs at 4^{o}C. The mycoplasma cells were then separated from the antisera by centrifugation at 15 500g for 5mins. The serum was collected and re-centrifuged to ensure that all the mycoplasma cells were removed (i.e. until no cellular deposit appears at the base of the tube). The serum was then heated to 56^{o}C, tested for sterility and used in a MI test in the presence of 7.5% complement as described in Section 1.2.2C.

*Note: One colour changing unit (ccu) was defined as the highest dilution of a mycoplasma suspension that produced a colour change in the medium.

1.3 Results.

MI tests with eight *M. ovipneumoniae* strains in the absence of complement gave low titres (<40) in almost all cases (Table I). However, in the presence of 7.5% guinea-pig serum (i.e. complement) the antisera gave higher titres (Table II).

Each of the three mycoplasma antisera tested (in the presence of complement) inhibited all eight *M. ovipneumoniae* strains, although the titres varied.

Three *M. ovipneumoniae* strains (1, 5 and 10) were used to cross-absorb antisera prior to use in a MI test. The results (compared with non-absorbed antisera) are shown in Table III.

Table I: Metabolic Inhibition of Eight *M. ovipneumoniae* Strains Tested Against Three Antisera in the Absence of Complement.*

Test Strain of *M. ovipneumoniae*	**Metabolic Inhibition Titres of Antisera to:** Strain 1	Strain 5	Strain 10
1	<40	<40	<40
2	<40	<40	<40
4	80	<40	<40
5	<40	<40	<40
10	<40	<40	<40
37E	<40	<40	<40
MPP74	<40	80	<40
L3/C3	<40	<40	<40

*Note: This table represents two experiments which gave identical results.

Table II: Metabolic Inhibition of Eight *M. ovipneumoniae* Strains Tested Against Three Antisera in the Presence of 7.5% Complement.*

Test Strain of *M. ovipneumoniae*	**Metabolic Inhibition Titres of Antisera + 7.5% Complement to:** Strain 1	Strain 5	Strain 10
1	160	40	80
2	80	160	40
4	320	320	80
5	80	320	40
10	320	320	320
37E	40	40	80
MPP74	40	320	80
L3/C3	160	160	40

*Note: These are the results of one typical experiment. In repeated experiments the results were substantially unaltered, although 2-fold changes in titre occurred in a few cases.

Table III: Metabolic Inhibition of *M. ovipneumoniae* Antisera Cross-Absorbed with Heterologous *M. ovipneumoniae* Strains.*

Antibody to Strain	Antibody Absorption Strain	Antibody Tested With Test Strain	Absorbed Antibody Titre	Non-Absorbed Antibody Titre
1	5	1[a]	40	80
1	10	1[a]	320	320
1	5	10[b]	40	80
1	10	5[b]	320	320
5	1	5[a]	<40	40
5	10	5[a]	320	320
5	1	10[b]	<40	40
5	10	1[b]	320	320
10	1	10[a]	40	80
10	5	10[a]	<40	40
10	1	5[b]	40	80
10	5	1[b]	<40	40

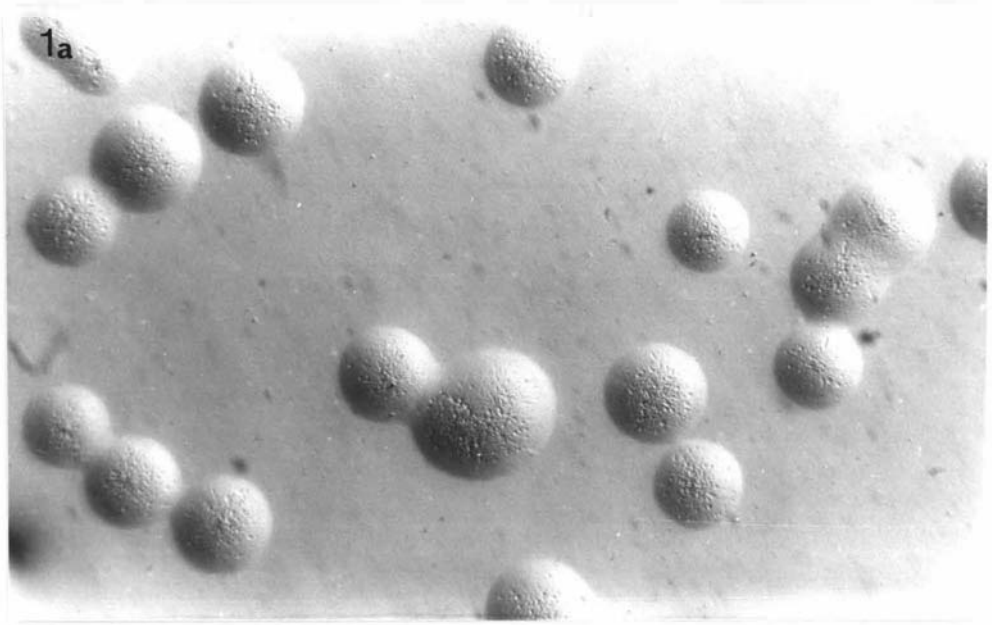
*Note: Antisera was cross-absorbed before being tested with either (a) homologous or (b) heterologous mycoplasma strains.

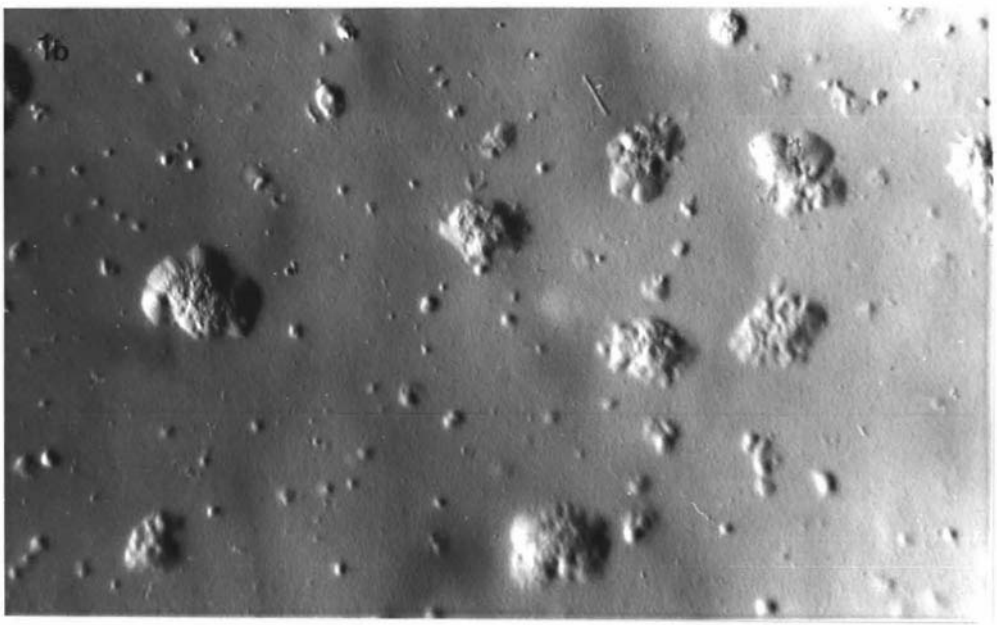
Figure 1 (a and b)

Colonies of two lung isolates of *M. ovipneumoniae* incubated for six days at 37^{o}C. These represent the extreme range of colony morphology, but the vast majority give colonies which resemble strain 5 (Figure 1a) rather than strain 1 (Figure 1b). Note the circular outline and pitted appearance of typical *M. ovipneumoniae* colonies in Figure 1a.

Technical Note:

Magnification 120x


1a


1b

1.4 Discussion.

Antibody prepared against whole organisms caused metabolic inhibition of *M. ovipneumoniae* in some instances but, in the absence of complement, the titre was invariably low (Table I). However, in general, complement was needed for metabolic inhibition by antibody of *M. ovipneumoniae*. A complement concentration corresponding to 7.5% fresh guinea-pig serum was used routinely because preliminary work (data not shown) indicated that a complement level of greater than 7.5% (24 MCD_{50}) did not increase the metabolic inhibition titre.

Metabolic inhibition clearly demonstrates antigenic differences in different isolates of *M. ovipneumoniae*. For example, compare antisera prepared against strains 1 and 5 (Table II). Antiserum to strain 1 showed a 2-fold preference (titre of 160) for strain 1 when compared with strain 5 (titre of 80) whereas antiserum to strain 5 showed an 8-fold preference (titre of 320) to strain 5 when compared to strain 1 (titre of 40). On its own this result might be taken to denote the existance of two related, but nevertheless distinct, groups of *M. ovipneumoniae*. It might be tempting to denote these as serovars 1 and 2 however, such a distinction would not be helpful and indeed would be confusing. Thus, antibody to all three strains (1, 5 and 10) inhibited all eight strains tested, but the titres obtained (Table II) did not allow the delineation of any clearly defined groups and, as noted above, all strains are related but nevertheless the organisms are serologically heterogeneous.

The simplest possibility is that two antigens are involved: One representing a group-specific antigen and another (possibly several others) representing strain-specific antigens. This led us to attempt to increase the discriminating capacity of the antigen by cross-absorption. However, in most instances a 2-fold drop in titre occurred with both homologous and heterologous strains following absorption.

Because of these results, which reflect the results of other workers (Jones *et al*, 1976), we decided to concentrate on a different approach, namely to examine the proteins of *M. ovipneumoniae* isolates using Sodium Dodecyl Sulphate Polyacrylamide-Gel Electrophoresis (SDS-PAGE) techniques with the possibility in mind that this may allow us to distinguish individual isolates and perhaps to detect grouping of isolates when a significant number are compared. We do not wish to imply that further refinements of serology have no potential to assist progress, indeed the production of monoclonal antibody might assist

serological studies. However, such an approach was not available to us during the course of this study.

Another approach is to examine the serology of *M. ovipneumoniae* using antisera to individual proteins in a "Western Blot". We return to this topic in the latter part of chapter three.

CHAPTER TWO

Separation and Comparison of *M. ovipneumoniae* Proteins by Iso-Electric Focusing (IEF) and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

A) Comparison of Non-Ionic and Zwitterionic Surfactants for their Ability to Solubilize *M. ovipneumoniae* Membrane Proteins for use in IEF.

A2.1 Introduction.

Metabolic inhibition tests using polyclonal antibody demonstrated that *M. ovipneumoniae* is not a serologically homogeneous species. However, metabolic inhibition tests, even with absorbed sera, raised against whole organisms do not have the potential to distinguish groups or serovars of *M. ovipneumoniae*. Because of this, several approaches were considered.

a) Production of monoclonal antibody to *M. ovipneumoniae*.
b) Examination and comparison of the total proteins (or their surface proteins only) of *M. ovipneumoniae*.

Since the technology for monoclonal antibody production was not available to us we adopted the second approach. Proteins can be examined and compared in at least two ways: iso-electric focusing (IEF) of solublized (undenatured) total proteins or by SDS-PAGE, which involves the denaturing of proteins by heat and SDS.

SDS-PAGE is a relatively simple technique and applies to all proteins because heating in SDS is a potent means of solublizing even membrane proteins. We returned to this technique (see later in this chapter) but there is a potential advantage in separating undenatured proteins e.g. antibody production to native proteins would reflect the "natural" immune response to *M. ovipneumoniae* while the response to SDS-heat treated proteins may or may not when used as immunogens produce antibody to the native protein. With this in mind this section investigates the solublization of *M. ovipneumoniae* proteins by various surfactants and compares the total undenatured protein of several isolates by using the solublized extracts.

A surfactant to be used for iso-electric focusing should be either non-ionic or zwitterionic so that it does not contribute any nett charge to the proteins. This will allow any proteins to migrate in a gel in the presence of an electric field until the protein reaches a position in the gel which corresponds to its iso-electric point where the protein will have no nett charge and hence remain fixed.

Five non-denaturing surfactants were chosen and compared for their ability to solublize the proteins of *M. ovipneumoniae*. Two surfactants; Tween 20 and Triton X-100 are non-ionic, while the remaining three surfactants; CHAPS, Zwittergen 3-12 and Zwittergen 3-14 are zwitterionic (i.e. have no nett charge). The ability of these agents to solublize intact *M. ovipneumoniae* cells was examined initially by spectrophotometric determination of the drop in optical density. The two best solublizing agents were then chosen and examined in more detail for their ability to solublize all the proteins of *M. ovipneumoniae*. This was done by comparing the sediments and the supernatants of lysates by SDS-PAGE.

A2.2 Materials and Methods.

A2.2.1 Materials

1)**FM4 Medium**: see appendix

2)**Phosphate Buffered Saline**: see appendix

3)**Surfactants:** The following were made to a concentration of 12%

a. Tween 20 (Polyoxyethylene sorbitan monolaurate) (Bio-Rad™, Bio-Rad Laboratories, Richmond, California, USA).
b. Triton X-100 (Octyl phenoxy polyethoxyethanol) (Sigma™, Sigma Chemical Company, St Louis, Missouri, USA).
c. CHAPS (3-[(3-Cholamidopropyl) dimethyl-ammonio]-1-propane-sulphate) (Sigma™, Sigma Chemical Company, St Louis, Missouri, USA).
d. Zwittergen 3-12 (3-Dodecyldimethylammonium-1-propane sulphonate) (Serva™, Serva Feinbiochemica, Heidelberg, West Germany).
e. Zwittergen 3-14 (3-Tetradecyldimethylammonium-1-propane sulphonate) (Serva™, Serva Feinbiochemica, Heidelberg, West Germany).

4)**Protein Assay**: see section C2.2.2.v.

5)**SDS-PAGE**: see section C2.2.2.vi.

A2.2.2 Methods

A2.2.2.i A Spectrophotometric Determination of the Solubilzing Ability of Non-Ionic and Zwitterionic Surfactants on a *M. ovipneumoniae* Strain.

M. ovipneumoniae strain 5 was propagated in 300ml of FM4 Medium, the cells were collected by centrifugation, washed twice in PBS and resuspended in PBS to give a 10% suspension. A 12% solution of two non-ionic surfactants (Tween 20 and Triton X-100) and three zwitterionic surfactants (CHAPS, Zwittergen 3-12 and Zwittergen 3-14) were prepared and from these serial 2-fold dilutions of each surfactant in PBS were made. 100ul of a 10% *M. ovipneumoniae* cell suspension was added to 200ul of each dilution. A PBS control was included. The suspensions/lysates were mixed for 60sec, placed in 1ml glass cuvettes with a 10mm light path and their optical densities at 500nm were measured using a Cecil™ CE559 spectrophotometer meter.

A2.2.2.ii An SDS-PAGE Comparison of Two Zwitterionic Surfactants on their Ability to Solublize Totai *M. ovipneumoniae* Cells.

A 734ul aliquot of a 10% *M. ovipneumoniae* cell suspension (15mg/ml) in PBS was placed in a 1.5ml Eppendorf microfuge tube. 66ul of a 12% solution of either Zwittergen 3-12 or Zwittergen 3-14 was added to the *M. ovipneumoniae* cell suspension, giving a total cell suspension volume of 0.8ml with a final surfactant concentration of 1%. The microfuge tube containg the cell suspension and surfactant were mixed thoroughly and centrifuged at 15 500g for 3mins. The supernatant was removed and placed in a fresh microfuge tube and recentrifuged to ensure that all unsolublized cellular material was sedimented (no deposit was observed in the second centrifugation step). The sediment was resuspended in PBS to the original volume of the cell suspension (0.8ml).

Using SDS-PAGE (see section C2.2.2.vi), the supernatant, sediment and total *M. ovipneumoniae* proteins were compared. A constant sample volume (7.34ul) of each fraction was loaded on to the gel and electrophoresed.

A2.3 Results.

The ability of two non-ionic and three zwitterionic surfactants to solublize an intact *M. ovipneumoniae* cell suspension (as indicated by a drop in optical density) at various surfactant concentrations is shown in Figure 2. The two non-ionic surfactants; Tween 20 and Triton X-100 at low (0.25%) surfactant concentrations decreased the optical density by 12.8% and 38.5% respectively. At a concentration of 4% this increased to 29.2% for Tween 20 and to 53.8% for Triton X-100.

CHAPS showed a poor ability to solublize the intact mycoplasma cell suspension. At concentrations of less than 3.25% its ability to solublize the cell suspension was inferior to Triton X-100. However, at higher surfactant concentrations its ability to solublize the cell increased somewhat but it was inferior to the other zwitterionic surfactants.

Zwittergen 3-12 caused a 59.6% drop in the optical density of the cell suspension, even at a low (0.25%) surfactant concentration and at a much higher surfactant concentration (4%) Zwittergen 3-12 marginally increased its solublizing efficiency i.e. a 72% drop in the optical density.

The third zwitterionic surfactant (i.e. Zwittergen 3-14) caused an approximate 91% decrease in the optical density of the cell suspension at a surfactant concentration of 0.25%. At 0.5% the optical density drop increased to approximately 95%. At higher surfactant concentrations (i.e. at 4%) it reached 97%. Clearly, Zwittergen 3-14 is a potent solublizer and is markedly superior to the other four surfactants tested. However, a decrease in the optical density is not a direct measure of solubilization so Zwittergen 3-12 and 3-14 were used to solublize an *M. ovipneumoniae* suspension and the resulting preparation was centrifuged. Little or if any sediment was visible, however the supernatant was removed and the sediment was resuspended to its original volume. Aliquots of these and the total lysate (at equivalent concentrations) were examined by SDS-PAGE, the results are shown in Figure 3.

Figure 2

Lyses of *M. ovipneumoniae* by surfactants. Two non-ionic surfactants (Tween 20 and Triton X-100) and three zwitterionic surfactants (CHAPS, Zwittergen 3-12 and Zwittergen 3-14) were compared for their ability to solublize a 10% *M. ovipneumoniae* cell suspension as measured by a decrease in optical density.

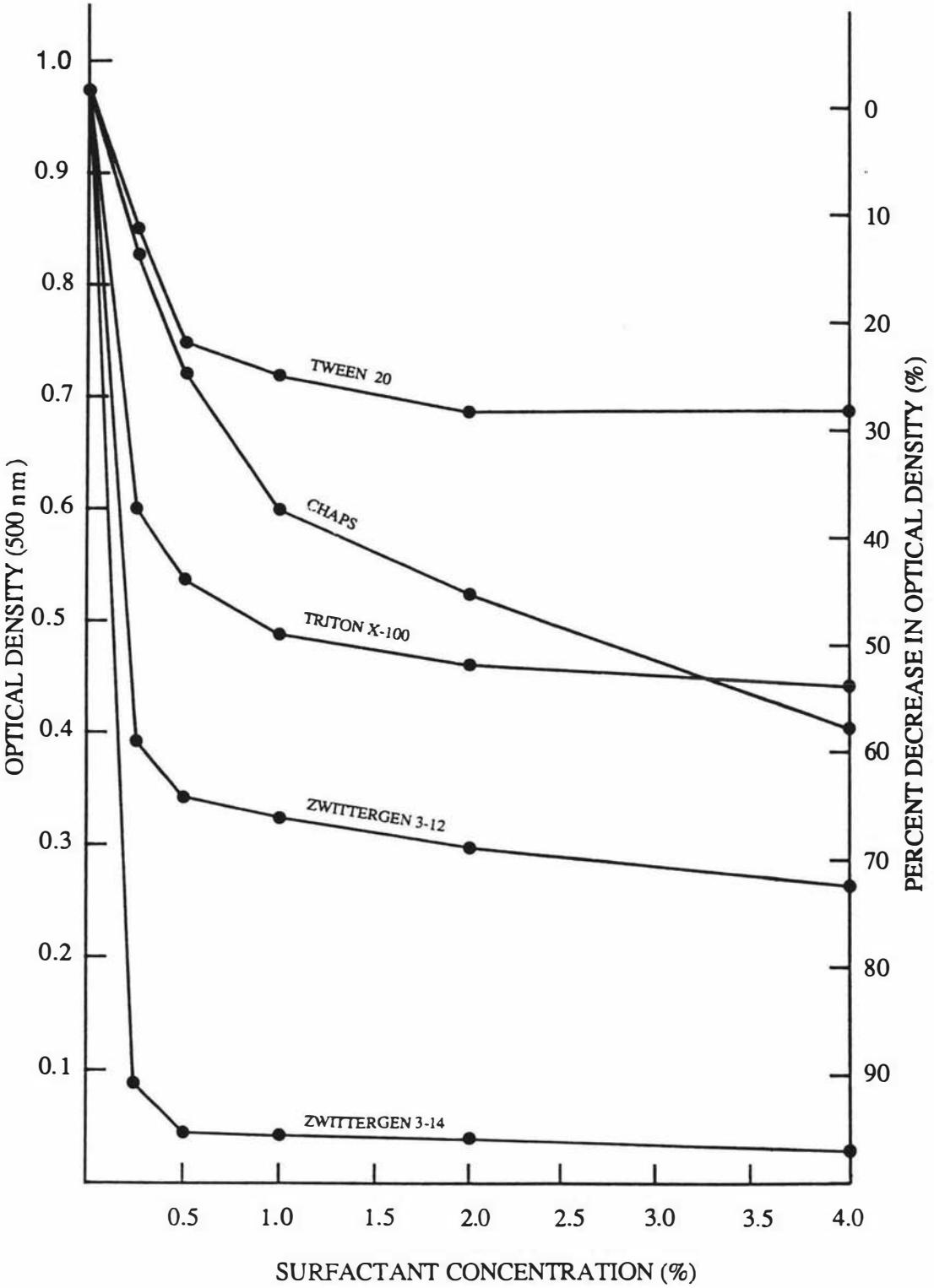
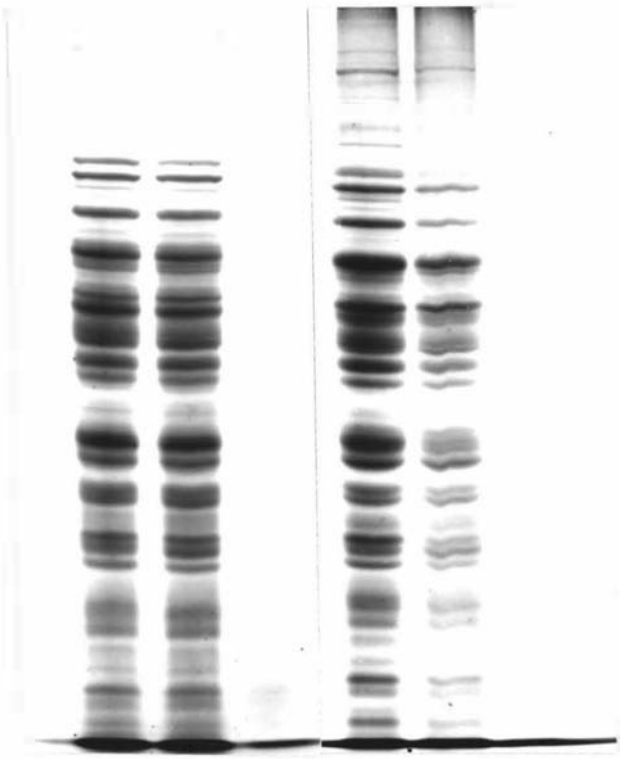

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
OPTICAL DENSITY (500 nm)
0
10
20
30
40
50
60
70
80
90
PERCENT DECREASE IN OPTICAL DENSITY (%)
TWEEN 20
CHAPS
TRITON X-100
ZWITTERGEN 3-12
ZWITTERGEN 3-14
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
SURFACTANT CONCENTRATION (%)

Figure 3

Examination of solubilized and non-solubilized proteins following treatment of *M. ovipneumoniae* cells with Zwittergen 3-12 and 3-14.

M. ovipneumoniae was treated with Zwittergen 3-12 and Zwittergen 3-14. Following centrifugation, the supernatant and deposit fraction were compared with the total proteins of *M. ovipneumoniae* using SDS-PAGE. Lane 1: total protein of *M. ovipneumoniae* strain 37E; Lane 2: supernatant fraction solublized by Zwittergen 3-12; Lane 3: sediment remaining after Zwittergen 3-12 solublization; Lane 4: total protein of *M. ovipneumoniae* strain 37E; Lane 5: supernatant fraction solublized by Zwittergen 3-14; Lane 6: sediment remaining after Zwittergen 3-14 solublization.

Note that both surfactants, especially Zwittergen 3-14, solubilized all protein.



A2.4 Discussion.

As estimated by optical density measurements, Tween-20, Triton X-100 and CHAPS caused only a partial clearing of a turbid suspension. However, by this criterion Zwittergen 3-12 and 3-14 were potent solublizing agents at even low solublizing concentrations of 0.25%. Although the drop in optical density was up to 97% when *M. ovipneumoniae* was solubilized by Zwittergen 3-12, no significant deposit was observed following centrifugation. More significantly however, all the fractions seen when *M. ovipneumoniae* was lysed by SDS-PAGE (Figure 3, lane 1) were present in the supernatant of *M. ovipneumoniae* lysed by Zwittergen 3-12 (Figure 3, lane 2) and furthermore, the trace sediment when solubilized by SDS-PAGE (Figure 3, lane 3) indicated that no major protein remained unsolubilized by Zwittergen 3-12. The same applies to Zwittergen 3-14 which however, left even less unsolubilized protein (Figure 3, lane 6). Consequently, Zwittergen 3-14 is a suitable agent to solublize *M. ovipneumoniae* proteins for iso-electric focusing.

B) Iso-Electric Focusing (IEF) of *M. ovipneumoniae* Proteins in Non-Denaturing Surfactants.

B2.1 Introduction.

Iso-electric focusing (IEF) was used to separate the proteins of eight *M. ovipneumoniae* isolates solublized with a non-denaturing surfactant (Zwittergen 3-14). Seven of these isolates were obtained from different farms in New Zealand and the other strain was isolated in Australia and kindly supplied by Dr T.D. St.George in 1973.

B2.2 Materials and Methods.

B2.2.1 Materials

I. For Sample Preparation.

1)**FM4 Medium**: see appendix

2)**Phosphate Buffered Saline**: see appendix

3)**Solubilzing Mixture** (2x)

Zwittergen 3-14 ultrapure (Serva)	0.2g
Dithiothretol (Sigma)	0.04g
Glycerol	0.9ml
Distilled Water to	10.0ml

ii. For Preparation of Iso-Electric Focusing Gels.

1)**Acrylamide Solution**

Acrylamide (Serva)	29.1g
Distilled Water to	100.0ml

2)**N,N-Methyiene Bis Acrylamide Solution**

N,N-Methylene Bis Acrylamide	0.09g
Distilled Water to	100.0ml

Both the acrylamide and the N,N-Methylene Bis Acrylamide stock solutions were filtered through a 0.45u Millipore^{TM} filter before being stored in a dark bottle at 4^{o}C.

3)**Ammonium Persulphate Solution**

Ammonium Persulphate	0.05g
Distilled Water to	1.0ml

This solution was made immediately before use.

4)**Ampholine^{TM} pH 3.5-10.0 (LKB Bromma)**

This solution was stored at 4^{o}C

5)**Electrode Solutions**

a.Anode (+) 1M H_3PO_4

Phosphoric Acid (Analar)	2.8ml
Distilled Water to	50.0ml

b.Cathode (-) 1M NaOH

Sodium Hydroxide (Analar)	2.0g
Distilled Water to	50.0ml

The cathode electrode solution was placed into a Buchner flask and heated to 40^{o}C in a waterbath for approximately 30mins while under vacuum.

Note: This degassing procedure removes dissolved CO_2 from the cathode solution which would otherwise effervesce around the electrode when the current flows. This interfers with the constant wattage through the gel and results in "skewed" protein bands.

6)**Fixing Solution**

Trichloroacetic Acid (Analar)	34.5g
Sulphosalicylic Acid (Analar)	10.4g
Distilled Water to	300.0ml

The solution was stored at room temperature.

7)**Staining Solution**

Trichloroacetic Acid (Analar)	34.5g
Sulphosalicylic Acid (Analar)	10.4g
Coomassie Brilliant Blue R250	0.35g
Distilled Water to	300.0ml

The solution was heated to 60^{o}C for 20mins with intermittent stirring to solublize the Coomassie Brilliant Blue. It was freshly prepared and used immediately to stain the electrofocused proteins.

8)**Destaining Solution**

Ethanol (Analar)	500.0ml
Acetic Acid (Analar)	160.0ml
Distilled Water to	2000.0ml

This solution was stored at room temperature and could be re-used if passed through a column of activated charcoal.

9)**Preserving Solution**

Ethanol (Analar)	500.0ml
Acetic Acid (Analar)	160.0ml
Glycerol	30.0ml
Distilled Water to	2000.0ml

This solution was stored at room temperature.

B2.2.2 Methods

B2.2.2.i Sample Preparation.

Eight *M. ovipneumoniae* isolates were propagated in 150ml FM4 Medium, the cells were collected by centrifugation, washed twice and resuspended in PBS to give an approximate 10% suspension. A 1ml aliquot of the resuspended cells were sonicated for a total of 5mins at 30sec intervals in a 4^{o}C waterbath using a MSE 150 ultrasonic disintegrator at 150 watts. A 100ul aliquot of lysate was assayed for protein concentration as described in section C2.2.2.v.

0.6ml of lysed *M. ovipneumoniae* cells were transferred to a 1.5ml Eppendorf microfuge tube and 0.6ml of solublizing mixture (2x concentrated) was added. This was thoroughly mixed, centrifuged at 15 500g for 10mins (to remove any cell debris) and stored at -20^{o}C until required.

B2.2.2.ii Preparation of Iso-Electric Focusing Gels.

A clean glass plate measuring 12.5cm x 26.0cm x 0.3cm (WxLxT) was placed flat on the bench and 2ml of distilled water was placed in the centre of the glass plate at one end. A sheet of thin plastic (Gelbond^{TM}) was pressed on to the glass surface starting at the wetted end and air bubbles trapped between the two surfaces were expelled using a rubber roller. A second glass plate (12.5cm x 25.0cm x 0.3cm) with two (12.5cm x 25.0cm x 0.05cm) Teflon^{TM} side spacers permanently adhered to either edge of one side of the glass plate was lowered on to the plastic sheet. The two glass plates were clamped together with bulldog clips and placed on a levelled surface ready for the addition of acrylamide.

The acrylamide gel was prepared in a 100ml Buchner flask from stock solutions:

Acrylamide Solution	3.5ml
N,N-Methylene Bis Acrylamide Solution	3.5ml
LKB 1818 Ampholine^{TM}	1.5ml
Distilled Water to	20.5ml

This solution was placed under vacuum at 37^{o}C and degassed for about 15 to 20mins with occassional swirling of the gel solution. Following degassing, 0.5ml of Ammonium Persulphate Solution was added to the gel solution and mixed without forming air bubbles. The gel solution was immediately injected between the glass plates using a 20ml syringe fitted with a flexible tube (5cm long and with an internal diameter of about 0.3cm) placed against the 0.05cm gap. Care was taken to avoid air bubbles when casting the gel. If bubbles were present then the preparation was discarded and a new one prepared. Polymerization took approximately 60mins to complete at room temperature. Then the unpolymerized gel solution (exposed to air) was removed by tilting the glass plates at a 45^{o} angle and letting the unpolymerized gel solution drip into a sink. The original bottom glass plate was separated and the Gelbond^{TM} plastic sheet with the gel adhered to it was peeled

from the upper glass plate. The gel was used immediately but can be stored at 4^{O}C in a humidified container for up to 3 days.

B2.2.2.iii Iso-Electric Focusing of *M. ovipneumoniae* Protein Samples.

Electrophoresis of *M. ovipneumoniae* proteins was carried out using a flat bed 2117-301 Multiphore^{TM} electrophoresis unit (LKB). 5ml of kerosine was spread over the cooling plate and the temperature of the cooling plate was reduced to 10^{O}C using a Multitemp^{TM} thermostatic circulator (LKB). The polyacrylamide gel (bound to the Gelbond^{TM} plastic sheet) was placed on the surface of the kerosene wetted cooling plate without trapping air bubbles (these cause differential cooling which results in skewed bands). Strips of filter paper of 0.6cm wide and 25cm long (1cm shorter than the gel) were cut and soaked in the appropriate solutions (anode - 1M H_3PO_4 and cathode - 1M NaOH). The electrodes were positioned on the gel approximately 0.3cm from the (long) edge and were 0.5cm short at each end from the ends of the gel. Note that it is important to ensure that the electrodes did not protrude beyond the end of the gel. If this occurs, it allows wetting of the edge of the Gelbond^{TM} and electrical shorting of the system will result.

Whatman N^{O}1 filter paper was cut into rectangular pieces 0.5x1.0cm and positioned on the gel's surface approximately one to two centimetres from the cathode. 20ul equivalent to 80ug of solubilized protein was applied to the filter paper.

The electrofocusing lid was positioned so that the two platinium wires were in contact with the electrode wicks. The terminals were then connected to a constant power supply unit (LKB 2197). An extra electrophoresis safety lid was positioned over the electrofocusing lid. The pressure bar kept the platinum electrodes in constant contact with the anode and cathode wicks (see Figure 4).

The voltage was set at 2000 volts (max), current set at 50 milliamps (max) and the power was set at 25 watts (max). Note that the power is initially limiting, but as the run proceeds the current diminishes and the voltage increases and then voltage finally becomes limiting.

M. ovipneumoniae proteins were electrophoresed for 30mins and the filter paper rectangles were removed. The gel was then electrophoresed for a further 30mins to sharpen the

Figure 4

Preparation of an iso-electric focusing gel and assembly of apparatus.

a) Using a roller a thin plastic sheet (Gelbond^{TM}) was securely stuck on to a glass plate (12.0cmx26.0cmx0.3cm) wetted with about 2ml of water.

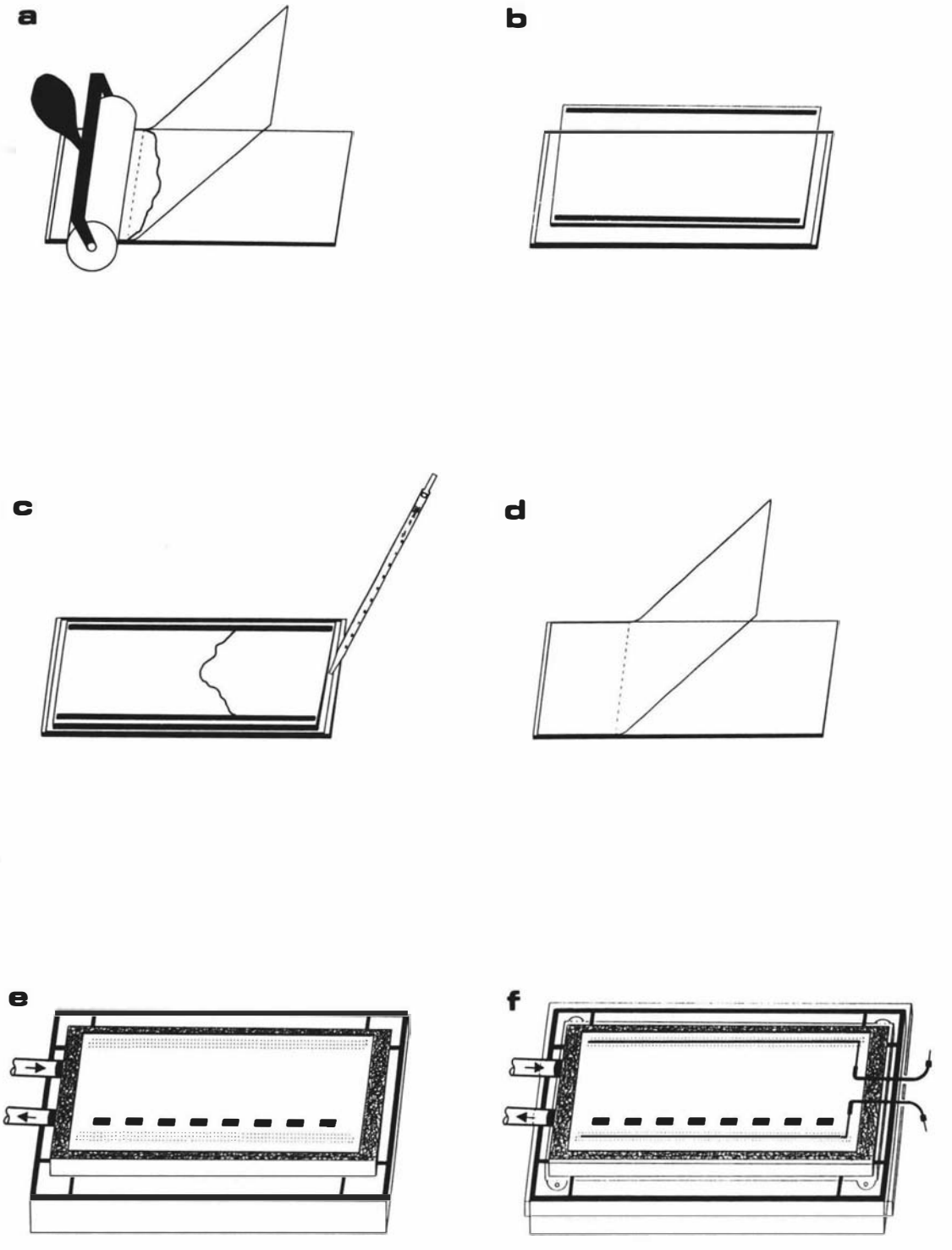
b) A second glass plate (12.0cmx25.0cmx0.3cm) containing two Teflon^{TM} side-spacers (0.5cm in thickness) was lowered on to the plastic sheet. This formed a chamber (12.0cmx25.0cmx0.05cm) to cast the gel. The glass plates were clamped together with "bulldog" clips (not shown for the sake of clarity).

c) Approximately 20ml of as yet unpolymerized gel solution containing ampholines was popetted between the plastic sheet and the upper glass plate.

d) Following polymerization (about 60mins) the upper glass plate was removed and the plastic sheet with the polymerized gel was peeled from the lower glass plate.

e) The plastic sheet with the gel adhering to it was positioned on a cooling plate coated with a thin film of kerosene. Two electrode wicks saturated with the appropriate buffer were positioned at opposite sides of the gel. Pieces of filter paper (0.5cmx1.0cm) soaked with 20ul (80ug) of solubilized protein were applied to the cathode end of the gel surface.

f) A lid was positioned so that it's two electrodes were placed in contact with the wicks. A second lid was positioned over the initial lid to conceal any exposed electrodes. Electrophoresis was conducted with the voltage set at 2000 volts (max), current set at 25 milliamps (max) and power set at 25 watts (max). After 30mins of electrophoresis, the filter paper soaked with 20ul of solubilized protein was removed and the gel was electrophoreses for a further 30mins.


a
b
c
d
e
f

protein bands. Using a LKB surface electrode (Multiphor Electrode 2117-111) the pH gradient was measured at various intervals. The pH at the anode end of the gel was pH 3.5 and pH 10.0 at the cathode end of the gel, these pH values corresponded to the pH range of the Ampholines used. The two electrode wicks were then carefully removed without damaging the gel and the gel then placed in 300ml of fixing solution for 30mins. The Fixing Solution was discarded and the gel washed in 300ml of Destaining Solution for 5mins. The Destaining Solution was also discarded and the gel was immersed in 300 ml of preheated ($60^{o}C$) Staining Solution. A lid was placed over the container and the gel was left to stain for 10mins. The Staining Solution was discarded and the gel washed with several changes of Destaining Solution until the background was clear. The gel surface was then wiped with a ball of cotton wool to remove any undissolved coomassie-blue crystals*. The gel was then placed in Preserving Solution for 30mins and then (still attached to the Gelbond™ support) in a fume hood overnight. This allowed partial drying so next day the gel's surface was sticky and a layer of plastic (LKB preserving sheet) was placed over its surface. Air bubbles between the gel and plastic sheet were removed by using a photographic roller. The IEF gel was used either as a photographic negative or stored as a permanent record.

* Note:

Unlike typical 10% SDS-PAGE gels, the 4% acrylamide gel tends to allow the adherence of any undissolved coomassie-blue crystals.

B2.3 Results.

Using a zwitterionic surfactant (1% Zwittergen 3-14), eight *M. ovipneumoniae* isolates were compared by iso-electric focusing as shown in Figure 5.

The solubilization of total mycoplasma protein was apparently almost complete, however a trace of precipitated protein appeared on the gels surface at the site where the samples were applied (Figure 5, arrow A). The proteins were separated according to their charge an a pH 3.5 to pH 10.0 gradient. The protein bands separated from all eight *M. ovipneumoniae* isolated and were located between pH 4.5 to pH 7.5. Many protein bands were clearly resolved indicating that little if any protein degradation had occurred.

A comparison of the eight *M. ovipneumoniae* isolates reveals that each isolate is distinct in the sense that no two isolates exhibit the same protein profile. About fifteen to eighteen

proteins are shown photographically, but up to twenty-three proteins were seen by visual examination in each protein profile. Some protein bands are common to all isolates examined, in particular two major protein bands were common. They were located at the pH 5.0 and pH 7.5 region as indicated by the arrows in Figure 5 (arrows C and B respectively).

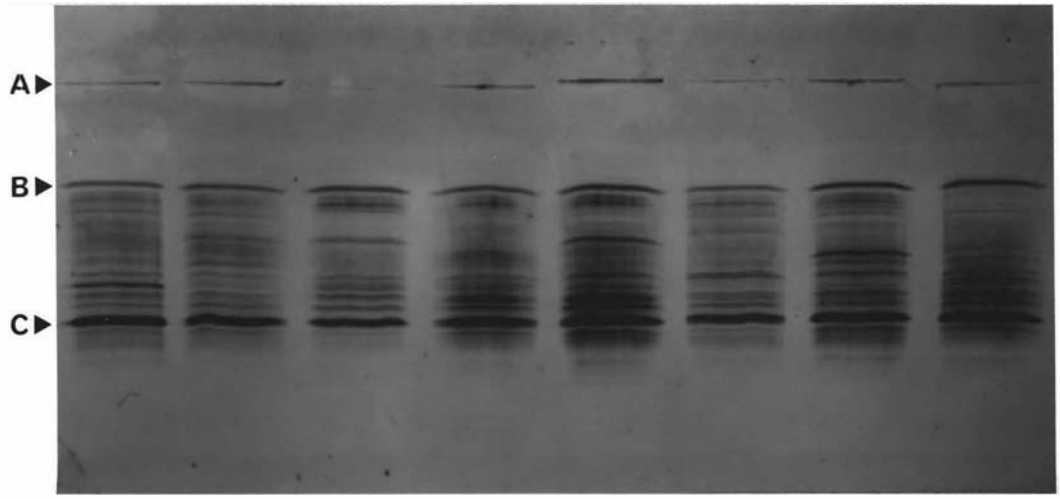
Figure 5

Separation of *M. ovipneumoniae* proteins by iso-electric focusing.

Eight *M. ovipneumoniae* strains obtained from sheep on different farms were solubilized with a non-denaturing surfactant (Zwittergen 3-14) and the proteins were separated by iso-electric focusing in a pH 3.5 to pH 10.0 polyacrylamide gel gradient.

The samples are (from left to right); Lane 1, strain 1; Lane 2, strain 2; Lane 3, strain 4; Lane 4, strain 5; Lane 5, strain 10; Lane 6, strain 37E; Lane 7, strain MPP74; Lane 8, strain L3/C3.

Note that while some bands are common to all isolates (arrow B and C), no two tracks are identical. Arrow A represents a trace of precipitated proteins at the site where the samples were applied.


A
B
C

B2.4 Discussion.

The solubilization of the proteins of *M. ovipneumoniae* by Zwittergen 3-14 allowed us to separate them by IEF. However, some problems arose. Attempts (not recorded here) to include Zwittergen 3-14 in the gel, so as to ensure that all solubilized proteins were retained in solution, were not entirely successful. Zwittergen 3-14 had two effects; it interfered with gel polymerization which made the gel almost impossible to handle and furthermore, frequently bands were wavy and skewed (results not shown) and hence unsuitable for comparing isolates. For these reasons all experiments recorded here were undertaken with gels which did not contain Zwittergen 3-14. This caused a small precipitate to appear at the site of application of the specimen (Figure 5, arrow A) and hence we could not be confident (rather the reverse) that all the proteins, including the important membrane proteins, were represented in the banding pattern.

Despite these difficulties the proteins from eight strains of *M. ovipneumoniae*, examined by IEF (Figure 5), show that while there are many common bands, including two prominent bands near the extremities of the gel (pH 5.0 and pH 7.5), each isolate had a pattern which did not exactly match any other isolates. This indicates that the proteins of *M. ovipneumoniae* are heterogeneous. However, this result (which was obtained with difficulty rather late in this research programme) did not give any clear indication that the isolates could be assigned to a limited number of groups.

For these reasons we did not further pursue the use of IEF to attempt to sub-divide *M. ovipneumoniae* isolates into groups but decided to concentrate on the technically simpler technique of SDS-PAGE which, although it has the disadvantage of denaturing proteins, has the advantage of allowing all proteins (including membrane proteins) to be solubilized, separated and compared. The next section examines many isolates of *M. ovipneumoniae* by SDS-PAGE.

C) A Comparison of *M. ovipneumoniae* Isolates by SDS-PAGE.

C2.1 Introduction.

When examined by metabolic inhibition, strains of *M. ovipneumoniae* are all related in the sense that extensive cross-reactions occur, but are heterogeneous in the sense that antibody titers vary considerably when different isolates are compared. Therefore, *M. ovipneumoniae* isolates could not be assigned into well defined groups by using the metabolic inhibition test (see chapter one). Iso-electric focusing (IEF) of native proteins also did not allow isolates of *M. ovipneumoniae* to be assigned into groups.

SDS-PAGE, unlike IEF, separates proteins according to molecular size rather than charge. It also allows solubilization of all proteins and remains unaffected by amino acid substitutions which will alter the iso-electric point. The use of polyacrylamide gel electrophoresis has been used by other researchers (Razin and Rottom, 1967; Razin, 1968; Zola *et al*, 1970 and Gois *et al*, 1973) as a means of identification and classification of mycoplasma isolates, though not of *M. ovipneumoniae*.

This section examines the use of SDS-PAGE as a tool to identify individual *M. ovipneumoniae* isolates and investigates its potential for assigning them into recognizable groups. Initially, eight *M. ovipneumoniae* strains were examined. However, because of the heterogeneity seen, this was extended to include a further sixty *M. ovipneumoniae* isolates obtained from three sheep on each of twenty different farms. This allows comparisons to be made between isolates on different farms and also between isolates from sheep within one farm.

C2.2 Materials and Methods.

C2.2.1 Materials

I. For Isolation of Mycoplasma Isolates.

1)**FM4 Medium**: see appendix

2)**Eagles Vitamin Solution**: see appendix

3)**"Modified" FM4 Medium**: see appendix

4)**FM4 Agar**: see appendix

ii. For Antiserum Production.

1)**"Modified" FM4 Medium**: see appendix

2)**0.15M Saline**: see appendix

3)**Freunds Complete Adjuvant** (Difco)

iii. For Preparation of Antigen for Gel Precipitin Test.

1)**Phosphate Buffered Saline**: see appendix

2)**Tris-EDTA Solution**: see appendix

3)**Triton X-100** (Sigma)

4)**FM4 Medium**: see appendix

iv. For Gel Precipitin Test.

1)**Noble Agar**

Noble Agar (Difco)	3.0g
NaCl	16.0g
Distilled Water to	200.0ml

This was autoclaved at 121^{o}C for 15mins and dispensed in 20ml aliquots into standard size petri dishes.

v. For the Preparation of a Standard Curve for the Estimation of Total Protein Content of *M. ovipneumoniae*.

1)**Coomassie Blue Reagent**

Coomassie Blue G-250	0.1g
95% Ethanol	50.0ml
85% (w/v) Phosphoric Acid	100.0ml
Distilled Water to	1000.0ml

Coomassie Blue G-250 was dissolved in a mixture of ethanol and phosphoric acid and diluted to 1000ml with distilled water. The dye reagent was filtered through two layers of Whatman $N^{o}1$ filter paper and stored at room temperature in a dark bottle.

2)**Bovine Serum Albumin** (1mg/ml)

Bovine Serum Albumin	10.0mg
0.2M NaOH to	10.0ml

Made fresh with every batch of Coomassie Blue Reagent.

vi. For the Preparation of SDS-PAGE Gels.

1a)**Running Gel Solution**

Acrylamide	30.0g
Methylene-bis-Acrylamide	0.5g
Distilled Water to	100.0ml

1b)**Stacking Gel Solution**

Acrylamide	30.0g
Methylene-bis-Acrylamide	1.6g
Distilled Water to	100.0ml

Acrylamide was dissolved in 70ml of distilled water and allowed to return to room temperature. Methylene-bis-Acrylamide was dissolved in the acrylamide solution and the volume was made to 100ml. Both the running gel and the stacking gel acrylamide solutions

were filtered through a single layer if Whatman N^{o}1 filter paper. Both solutions were stored at 4^{o}C for not longer than six weeks.

2a)**Lower Tris Buffer** (pH 8.8)

Trizma Base (Sigma)	18.17g
10% Solution of SDS in Distilled Water	4.0ml
12N HCl to pH 8.8	
Distilled Water to	100.0ml

2b)**Upper Tris Buffer** (pH 6.8)

Trizma Base (Sigma)	6.06g
10% Solution of SDS in Distilled Water	4.0ml
12N HCl to pH 6.8	
Distilled Water to	100.0ml

For both of the above solutions the Trizma Base was added to 70ml of distilled water and the reaction adjusted to the required pH with 12N HCl. The SDS was then added and the volume was made up to 100ml.

3)**Ammonium Persulphate Solution**

Ammonium Persulphate	0.1g
Distilled Water to	1.0ml

This solution was made fresh before use.

4)**Tris-Glycine Reservoir Buffer** (pH 8.3)

Trizma Base (Sigma)	6.07g
Glycine	28.8g
SDS	2.0g
Distilled Water to	2000.0ml

This solution was stored at room temperature.

5)**SDS-Sample Buffer** (x4)

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

This solution was diluted 4-fold when added to the protein sample.

6)**Bromophenol Blue Tracking Dye** (x10)

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

The tracking dye was diluted 10-fold in the solubilized solution prior to boiling.

7)**Phosphate Buffered Saline**: see appendix

8)**Coomassie Blue Stain**

Isopropanol	250.0ml
Glacial Acetic Acid	100.0ml
Coomassie Brilliant Blue R-250	0.4g
Distilled Water to	1000.0ml

The stain was filtered through two layers of Whatman $N^{O}1$ filter paper before being stored at room temperature.

C2.2.2 Methods.

C2.2.2.i The Collection, Isolation and Cloning of Mycoplasma Isolates Obtained from Pneumonic Sheep Lungs.

1) Collection of Sheep Lungs.

Pneumonic sheep lungs exhibiting non-progressive pneumonia (CNP) were collected from the abbatoir and placed in individual plastic bags to prevent cross-contamination. The lungs were labelled and photographed to record the lesions.

2) Isolation of Mycoplasmas from the Lungs.

Pneumonic lung specimens were aseptically cut into small pieces and fragments were placed into bottles containing 3ml of FM4 Medium to give an approximate 20% suspension. The suspension was shaken and a 0.3ml aliquot was transferred into a fresh bottle containing about 2.7ml of FM4 Medium. The original lung suspension was discarded and the inoculated media was incubated at 37^{o}C. The inoculated media was examined daily for a pH (colour) change, i.e. from red to yellow. When this was observed, 0.3ml was transferred to a bottle containing 2.7ml of FM4 Medium. However, if no colour change was observed after seven days 0.3ml was passaged. The samples were incubated for a further seven days and if still negative, the sample was frozen at -70^{o}C except for a 0.05ml aliquot which was spotted onto a FM4 agar plate and incubated at 37^{o}C for seven days.

The plates were examined for colonies after three, five and seven days using a plate microscope at 88x magnification. Colonies were removed from the positive plates using a pasteur pipette. Individual colonies with a small plug of agar, were inoculated into 3ml of FM4 Medium and incubated at 37^{o}C. When the culture has grown in the FM4 Medium it was cloned.

3) Cloning of Isolates.

A 10ml aliquot of culture was passed through a 0.45u filter to remove any cell aggregates and ten-fold serial dilutions of the filtrate was prepared in FM4 Medium. 0.05ml of each dilution was transferred to petridishes (Falcon^{TM}, 5.0cm x 0.9cm style with tight fitting lid) containing FM4 agar, incubated at 37^{o}C and examined using a plate microscope after three to seven days for the presence of colonies. Isolated colonies near the limiting dilution were selected and cloned. This was repeated thrice.

C2.2.2.ii Antiserum Production.

0.1ml of M. ovipneumoniae (strain 5) was propagated for eight passages in 10ml aliquots of "Modified" FM4 Medium and incubated at 37^{o}C. When the final passage culture exhibited a colour change from pH 7.8 to approximately pH 6.8, it was added to 150ml of "Modified" FM4 Medium and incubated at 37^{o}C on a rotary shaker at 125rpm. Following the growth of this culture a 0.05ml aliquot of culture was spotted on to BHI agar (to exclude the possibility

of bacterial contamination). A further 0.05ml of culture was spotted on FM4 Agar to check that the culture produced confluent growth, typical of *M. ovipneumoniae*.

The remaining suspension of *M. ovipneumoniae* was centrifuged at 15 500g for 20mins and the resulting pellet was resuspended in 0.15M saline and re-centrifuged. The pellet was resuspended in 0.15M saline to give an approximate 10% suspension. This was stored at -20^{o}C.

0.5ml of the antigen was mixed with an equal volume of Freund's Complete Adjuvant, 0.5ml was injected intramuscularly in each of the hind legs of New Zealand White rabbits (after a pre-immunization bleeding). After four weeks, 0.5ml of antigen was injected into each hind leg. This was repeated at weekly intervals for a further four weeks. The rabbits were then test bled at regular intervals and re-inoculated until the antibody titre was sufficiently high i.e. 1/16 dilution of the antibody gave a detectable line in a gel precipitin test. The rabbit was then bled by cardiac puncture and the serum collected was heated at 56^{o}C for 30mins and stored at -20^{o}C.

C2.2.2.iii Antigen Preparation for Gel Precipitin Test.

300ml of FM4 Medium was inoculated with a *M. ovipneumoniae* culture and incubated at 37^{o}C on a rotary shaker set at 125rpm. Following the growth of the culture (pH approximately 6.8) the cells were centrifuged at 15 500g for 20mins, the supernatant discarded and the deposit was washed twice in PBS. The deposit was resuspended in 1.5ml Tris-EDTA Solution, sonicated (using a Soniprep^{TM} MSE 150watt ultrasonic disintegrator) for 2 x 10secs in an ice bath and Triton X-100 was added to a final concentration of approximately 2%. This antigen was stored at -20^{o}C.

C2.2.2.iv Gel Precipitin Test.

The central well was filled with 50ul of antiserum prepares to *M. ovipneumoniae* strain 5 and the peripheral wells were filled with 50ul of different antigen suspensions (as prepared in section C2.2.2.iii). *M. ovipneumoniae* strain 5 antigen suspension and FM4 Medium were used as positive and negative controls.

C2.2.2.v Preparation of a Standard Curve for the Estimation of Total Protein Content of *M. ovipneumoniae*.

The procedure used in the preparation of a standard curve was a modification of the technique used by Bradford (1976). Briefly, a range of Bovine Serum Albumin (BSA) solutions from 0 to 100ug/0.1ml were prepared in 0.2M NaOH and were heated to 100^{o}C for 3mins, simulating the SDS treatment used in solubilizing total *M. ovipneumoniae* proteins.

The BSA solutions were cooled to room temperature, 5ml of Coomassie Blue Reagent was dispensed to each 0.1ml dilution and mixed by inversion. 3ml aliquots of the BSA-Coomassie Blue Reagent were placed into glass cuvettes and their absorbances were measured at 595nm using a Cecil^{TM} CE599 spectrophotometer. The absorption values were recorded (there was a linear relationship between absorbance and concentration) and used as a calibration curve to determine unknowns (Figure 6).

C2.2.2.vi The SDS-PAGE System.

General

The method used to electrophoretically separate proteins according to their molecular size, was basically the SDS-discontinuous system used by Laemmli (1970). However, since several minor, but significant technical changes were made, the system is described in some detail.

A) The Gel System

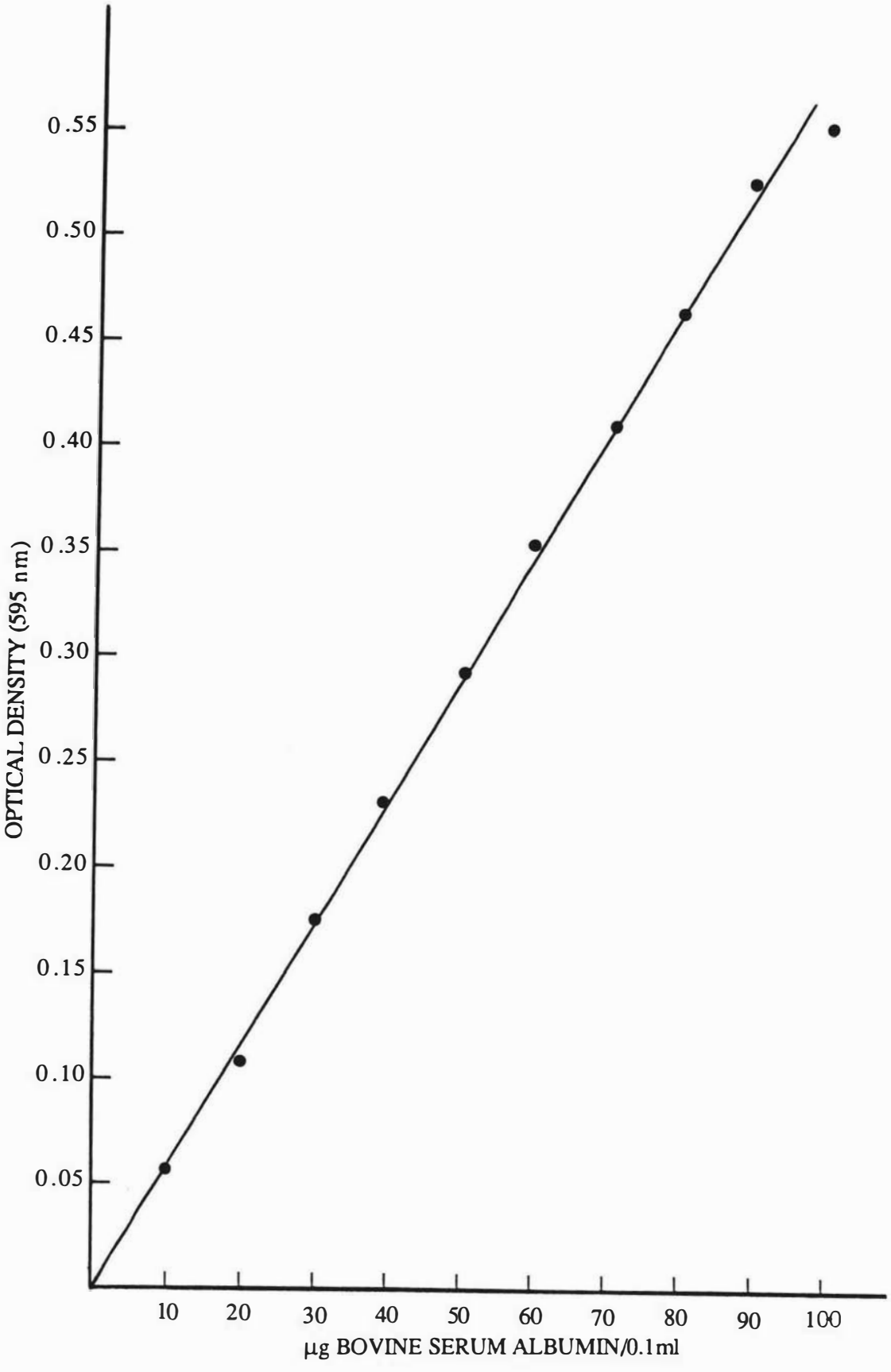
1. Gel Apparatus

A gel solution was polymerized between two glass plates and placed vertically between an upper and lower reservoir (negative and positive electrodes respectively) containing the appropriate buffers (see Figure 7f).

Figure 6

Protein assay standard curve.

A typical standard curve prepared using bovine serum albumin with a modified version of the Bradford coomassie blue dye binding method (see materials and methods). The standard curve (determined for each batch of reagent) was used to determine the protein concentration of *M. ovipneumoniae* lysates.


OPTICAL DENSITY (595 nm)
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
10
20
30
40
50
60
70
80
90
100
µg BOVINE SERUM ALBUMIN/0.1ml

2. Preparation of the Glass Plates

Two rectangular pieces of plate glass measuring 17cm x 13cm x 0.55cm were cut. From one of these plates a section of glass was removed (see Figure 7a) to allow contact between the upper reservoir buffer and the gel.

A high degree of cleanliness is required to produce good separation of proteins, hence after each use each plate was washed in detergent (5% Alconox^{TM}) and thoroughly rinsed with distilled water. If, however, the glass plates still had a grease film, they were soaked overnight in chromic acid (5g sodium dichromate dissolved in 5ml distilled water and 100ml of concentrated H_2SO_4 was added slowly while stirring). The perspex spacers and comb were cleaned with 75% ethanol, rinsed in water and dried.

3. Assembly of Apparatus

A glass plate with the cut segment was placed flat on the bench and a small "thread" of petroleum jelly was extruded from a syringe and placed along the base and sides of the glass plate, approximately 2mm from the edge. The spacers (i.e. 13.5cm x 1.2cm x 0.15cm) were pressed into place, starting from the bottom spacer and then the side spacers (see Figure 7a). Another "thread" of petroleum jelly was placed on top of the positioned spacers, approximately 2mm from the edge and a second glass plate was lowered on to the spacers ensuring that the spacers did not move out of position. It was important to avoid petroleum jelly spreading to where it would be in contact with the gel solution. The two glass plates were then clamped together on a perspex stand (see Figure 7b) with two "bulldog" clips on each side and were then ready for the gel solution to be added.

B) Preparation of SDS-PAGE Gels

Table IV: Preparation of Running and Stacking Gels.

Final Acrylamide Concentration	Running Gel[1] 7.5%	Running Gel[1] 10.0%	Stacking Gel 4.5%
Lower Tris Buffer	5.0ml	5.0ml	-
Upper Tris Buffer	-	-	2.5ml
Running Gel Solution	5.0ml	6.7ml	-
Stacking Gel Solution	-	-	1.5ml
Distilled Water	10.0ml	8.3ml	6.0ml
Ammonium Persulphate Solution	0.1ml	0.1ml	0.05ml
TEMED[2]	0.01ml	0.01ml	0.01ml

1 The above volumes were used for 11.5cm x 11.0cm x 0.15cm gels which required an approximate volume of 19.0ml.

2 N,N,N[1], N[1] Tetramethylethylenediamine

1. The Running Gel.

An acrylamide gel (7.5% or 10%) was prepared from stock solutions (at room temperature in the order listed in Table IV and mixed thoroughly after each addition to ensure even polymerization. Immediately after preparation the gel solution was poured between the glass plates (see Figure 7b). When the desired gel length (approximately 11cm) was attained distilled water was immediately pipetted (with care) on to the top of the mixture (see Figure 7c). This water had two functions: It allowed bubbles on the gel surface to escape, thus leaving the surface flat and secondly it gave anaerobic conditions which are required for complete polymerization. Polymerization took approximately 45 to 60 mins and was complete when an interphase appeared between the gel and the water.

2. The Stacking Gel

The stacking gel was prepared in the order listed in Table IV. The distilled water overlaying the polymerized gel was decanted and the surface of the gel was rinsed with 1-2ml of freshly prepared (unpolymerized) stacking gel solution (see Figure 7d). More of the stacking gel solution was then added to fill the remaining space between the glass plates i.e. approximately 5ml. A perspex comb was inserted into the stacking gel between the glass plates (see Figure 7e) and the acrylamide solution was left to polymerize for 5-10 mins. The

Figure 7

Preparation of sodium dodecyl sulphate-polyacrylamide gels and assembly of apparatus.

a) Position of the two glass plates and spacers.

b) The glass "sandwich" was placed vertically on a stand and approximately 20ml of as yet unpolymerized running gel solution was poured between the two glass plates.

c) A layer of distilled water (to assist polymerization) was placed on the running gel and left for 45mins.

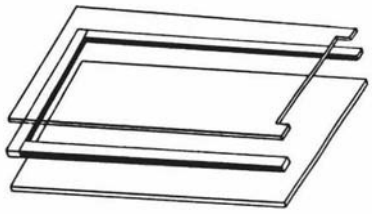
d) The distilled water was poured from the surface of the polymerized gel and approximately 5ml of stacking gel solution was poured on top of the polymerized running gel.

e) Before polymerization of the stacking gel solution occurred, a "comb" was inserted between the glass plates and into the stacking gel solution, to cast sample application wells. The "comb" was withdrawn from the polymerized stacking gel after 5mins. The glass "sandwich" was removed from the gel stand and the bottom perspex spacer was removed.

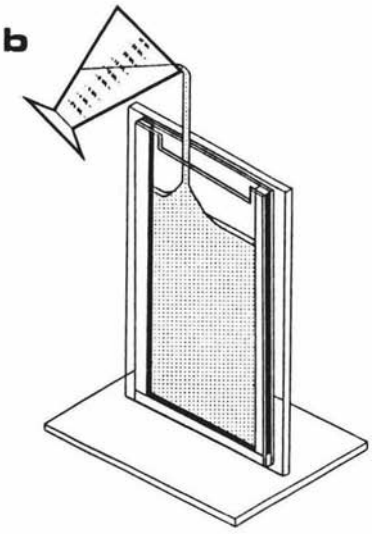
f) The gel "sandwich" was then placed on the gel electrophoresis apparatus (the notched glass plate facing the apparatus) and tris-glycine reservoir buffer was added to almost fill both tanks (the upper reservoir being the cathode and the lower reservoir being the anode). The samples were electrophoresed at 15mA until the dye (present in the lysate) had passed through the stacking gel and then at 10mA through the running gel (for approximately 4hrs).

Note that for the sake of clarity "bulldog" clips holding the gel "sandwich" to the gel stand and the electrophoresis apparatus have been omitted.

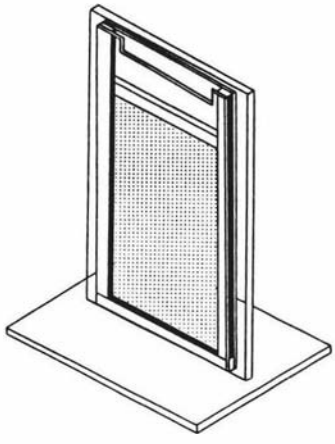
a



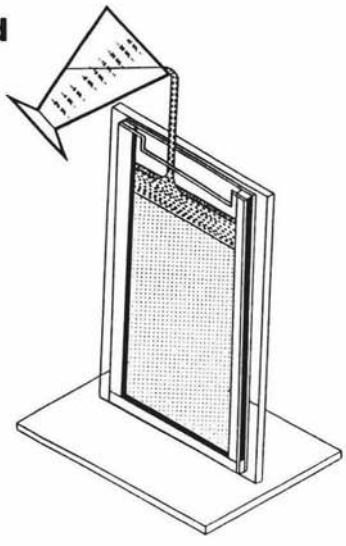
b



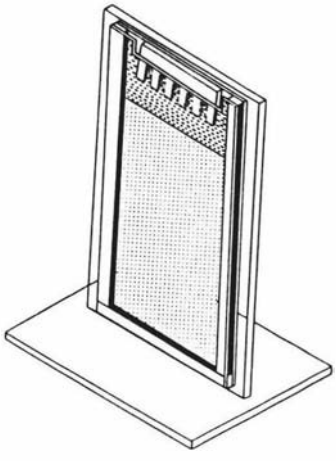
c



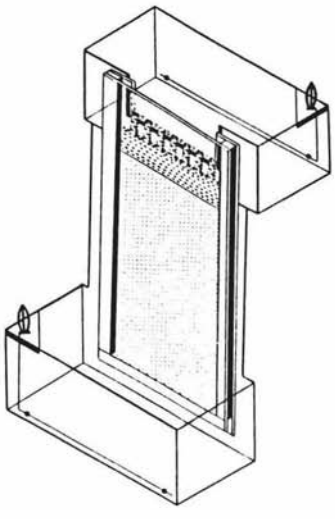
d



e



f



comb was then carefully removed from between the glass plates, ensuring that the "walls" of the wells were not disturbed and each well was rinsed with Tris-Glycine Reservoir Buffer.

The plates were removed from the stand and the exterior surfaces were wiped dry and the positions of the wells were outlined with a marker-pen (this made the location and application of protein samples into the cast well easier). The bottom spacer was carefully removed from between the glass plates, to allow contact between the gel and the anode.

A "thread" of petroleum jelly was placed between the upper (cathode) and lower (anode) reservoir tanks on the electrophoresis apparatus (see Figure 7f). The glass assembly was then clamped with "bulldog" clips and Tris-Glycine Reservoir Buffer was added to the upper and lower reservoirs. Air bubbles trapped at the base of the gel were displaced using a syringe, containing Tris-Glycine Reservoir Buffer, fitted with a bent needle.

C) Preparation and Application of *M. ovipneumoniae* Proteins for SDS-PAGE.

1. Extraction of Total Proteins.

300ml of each *M. ovipneumoniae* isolate was grown in FM4 Medium as described in section B2.2.2.i. The culture was then centrifuged at 15 500g for 10mins at 4^{o}C. The resulting deposit was washed twice in 0.15M PBS and resuspended to give an approximate 10% suspension. The cells were then placed in an ice bath and sonically disrupted (using five 10sec intervals) at 20kc/s using a MSE Soniprep^{TM} 150watt ultrasonic disintegrator. The lysed suspension was stored at -70^{o}C until required.

2. Protein Assay of *M. ovipneumoniae* Lysates.

A 0.1ml aliquot of sonically disrupted cells were diluted 1/3 and 1/10 in 0.2M NaOH and were heated at 100^{o}C for 3mins to solubilize the protein. The diluted solutions were cooled to room temperature and each dilution was further diluted 1/10 in 0.2M NaOH to give a 1/30 and 1/100 dilution. 0.1ml from each of the four dilutions was mixed with 5ml of Coomassie Blue Reagent and placed into glass cuvettes. The absorbance was measured at 595nm and the protein concentration was determined from a standard curve (see Figure 6).

3. SDS-Solubilization of *M. ovipneumoniae* Proteins for PAGE.

A 0.1ml aliquot of sonically disrupted cells, 25ul of SDS-Sample Buffer and 12.5ul of Bromophenol Blue Tracking Dye was mixed in a 1.3cm x 10.0cm Kimax^{TM} tube and heated to 100^{o}C for 3mins to denature and solubilize the proteins. The solution was cooled to room temperature and centrifuged at 4000g for 15mins before application to the gel.

4. Application of Samples to the Gel.

80ug of protein solubilized in SDS-Solution containing Bromophenol Blue Tracking Dye was added to each well. Glycerol present in the tracking dye ensured that the samples settled to the bottom of the well.

5. Electrophoresis of Proteins through the Gel.

The protein samples were electrophoresed through the stacking gel using a current of 15 milliamps (approximately 60mins). When the tracking dye migrated to the stacking gel/running gel interface the current was decreased to 10 milliamps and maintained until the tracking dye was about 0.5cm from the bottom edge of the running gel (approximately 4.5hrs).

6. Staining the Gel.

After electrophoresis the gel was removed from the electrophoresis apparatus and the side spacers were removed from the gel. The glass plates were prized apart using a spatula and a reference mark was made on the gel by removing the bottom left corner. The glass plate with the adhering gel was inverted (gel side down) over a container of Coomassie Blue Stain and using a spatula a corner of the gel was carefully separated from the plate. The gel then peeled off the glass surface under gravity and was left to stain overnight.

7. Destaining the Gel.

The gel was destained in 200ml aliquot of 10% v/v acetic acid. The aliquots were replaced several times until the gel became transparent except for the protein bands.

8. Gel Photography.

Following the destaining procedure the gel was soaked in distilled water to remove the acetic acid from the gel (acetic acid vapours tend to corrode the surface coating of the camera lens). The gel was then placed on an illumination box fitted with an opal-white screen and photographed through an orange filter on 12.5cm x 10.0cm Tech Pan (Kodak^{TM}) film.

9. Densitometry of Protein Bands.

Gels were scanned using a LKB 2222 UltroScan XL Laser Densitometer. All results obtained i.e. position of bands, height of bands, integrations of areas beneath a peak and relative areas beneath peaks, proved useful in differentiating between *M. ovipneumoniae* isolates.

C2.3 Results.

Sixty mycoplasma isolates were obtained from sixty specimens of pneumonic lungs which represented three sheep from each of twenty different farms. These isolates were glycolytic, all gave "vacuolated" centreless colonies on 1% agar (see Figure 1a) and produced lines of identity with a standard strain of *M. ovipneumoniae* in a gel precipitin test (see Figure 8) using antiserum to the standard strain. We conclude that all sixty represent isolates of *M. ovipneumoniae*.

The SDS-PAGE protein banding patterns of eight original *M. ovipneumoniae* strains is shown in Figure 9. In each protein pattern, about fifty to sixty bands are visible and the majority of these bands are common to all isolates however, each isolate has a number of bands not shared by other isolates which means that the overall pattern of bands of each isolate is unique. This uniqueness in principle allows each of the eight isolates to be differentiated from the seven other isolates examined.

When the sixty *M. ovipneumoniae* isolates (Figures 10a, b, c and d) were examined, it was found that isolates from three different sheep on one farm (1a, 1b and 1c) gave indistinguishable patterns (Figure 10a). However, in no other case was the banding pattern of any isolate identical to that of any other isolate. This applied to isolates from sheep on

one farm as well as for isolates from sheep on different farms i.e. fifty-eight different patterns were seen when the sixty isolates were compared.

About half of the isolates showed a prominant protein band, approximately 2/3 down the gel, with a molecular weight of about 40 000 [Figure 10b, compare lanes 8c and 9c (* = band present) with 9a and 9b (band absent)]. To confirm and illustrate this, eighteen *M. ovipneumoniae* isolates were selected. Half of these had and half had not, the band on the original gel. Fresh cultures of each were prepared and were again examined by SDS-PAGE. The isolates were placed in two groups of nine and the results are shown in Figure 11. This confirms the reproducibility of the banding difference even following further passage of the organism and re-extraction of the proteins. In addition, to demonstrate the reproducibility of the banding patterns of any one isolate, eight replicate cultures of *M. ovipneumoniae* strain 5 were processed separately and compared by SDS-PAGE. This result is shown in Figure 12.

Figure 8

Identification of mycoplasmas as *M. ovipneumoniae* using the gel precipitin test. The central well contains antiserum to a standard strain of *M. ovipneumoniae*. The peripheral wells contain the following antigens: Well 1, strain 1; Well 2, strain 2; Well 3, strain 4; Well 4, strain 5 (homologous strain); Well 5, strain 10; Well 6, strain 37E; Well 7, strain MPP74; Well 8, strain L3/C3.

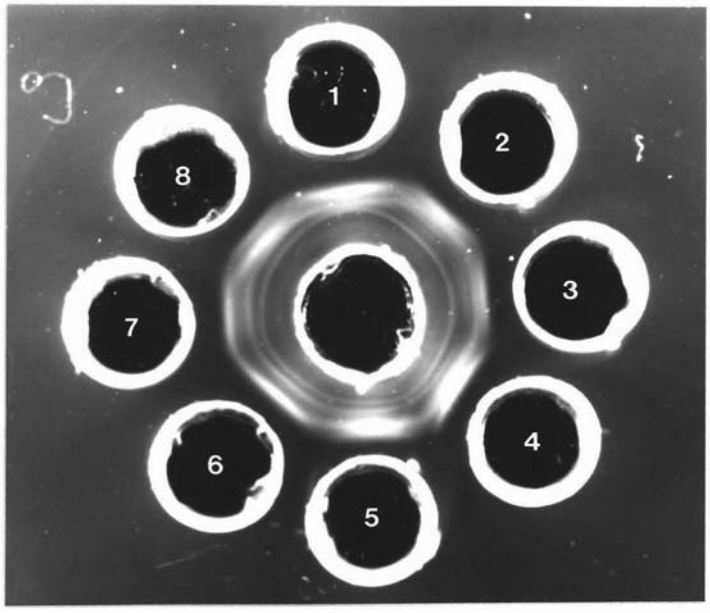
Medium controls (not shown here) gave no precipitate with the antiserum.

Figure 9

The total proteins of eight isolates of *M. ovipneumoniae* all derived from sheep on different farms and examined by SDS-PAGE. The samples are (from left to right); Lane 1, strain 1; Lane 2, strain 2; Lane 3, strain 4; Lane 4, strain 5; Lane 5, strain 10; Lane 6, strain 37E; Lane 7, strain MPP74; Lane 8, strain L3/C3. Note that although there are a large number of common bands, no two isolates give identical banding patterns i.e. each lane is unique.

Technical Note:

A 10% gel was used with a protein loading of 80ug/lane.


1
2
3
4
5
6
7
8

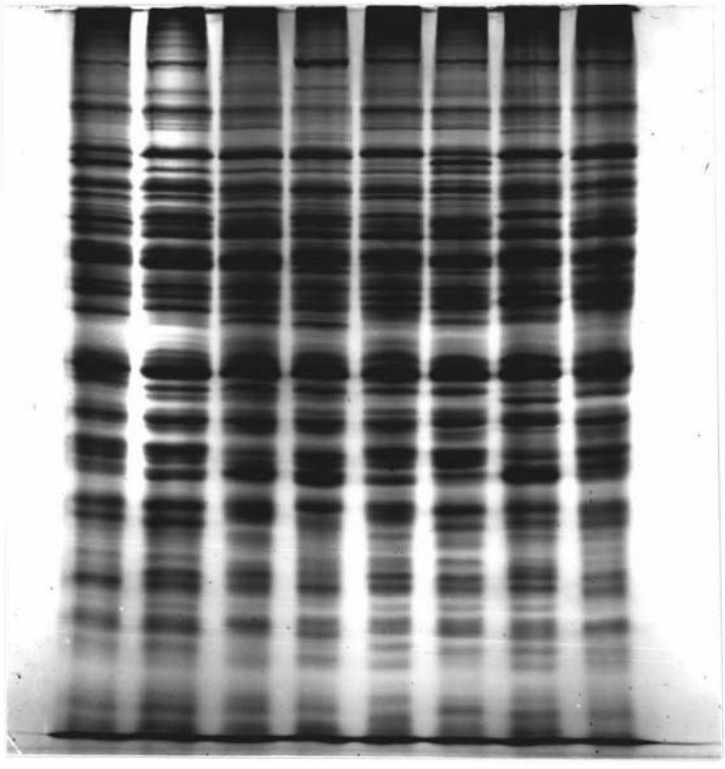


Figure 10 (a, b, c and d)

SDS-PAGE patterns of the total proteins of sixty *M. ovipneumoniae* isolates obtained from twenty different farms are shown. The isolates from one farm carry the farm number plus a letter (a, b or c).

Figure 10a: Lane 1, 1a; Lane 2, 1b; Lane 3, 1c; Lane 4, 2a; Lane 5 ,2b; Lane 6, 2c; Lane 7, 3a; Lane 8, 3b; Lane 9, 3c; Lane 10, 4a; Lane 11, 4b; Lane 12, 4c; Lane 13, 5a; Lane 14, 5b; Lane 15, 5c.

Figure 10b: Lane 1, 6a; Lane 2, 6b; Lane 3, 6c; Lane 4, 7a; Lane 5 ,7b; Lane 6, 7c; Lane 7, 8a; Lane 8, 8b; Lane 9, 8c; Lane 10, 9a; Lane 11, 9b; Lane 12, 9c; Lane 13, 10a; Lane 14, 10b; Lane 15, 10c.

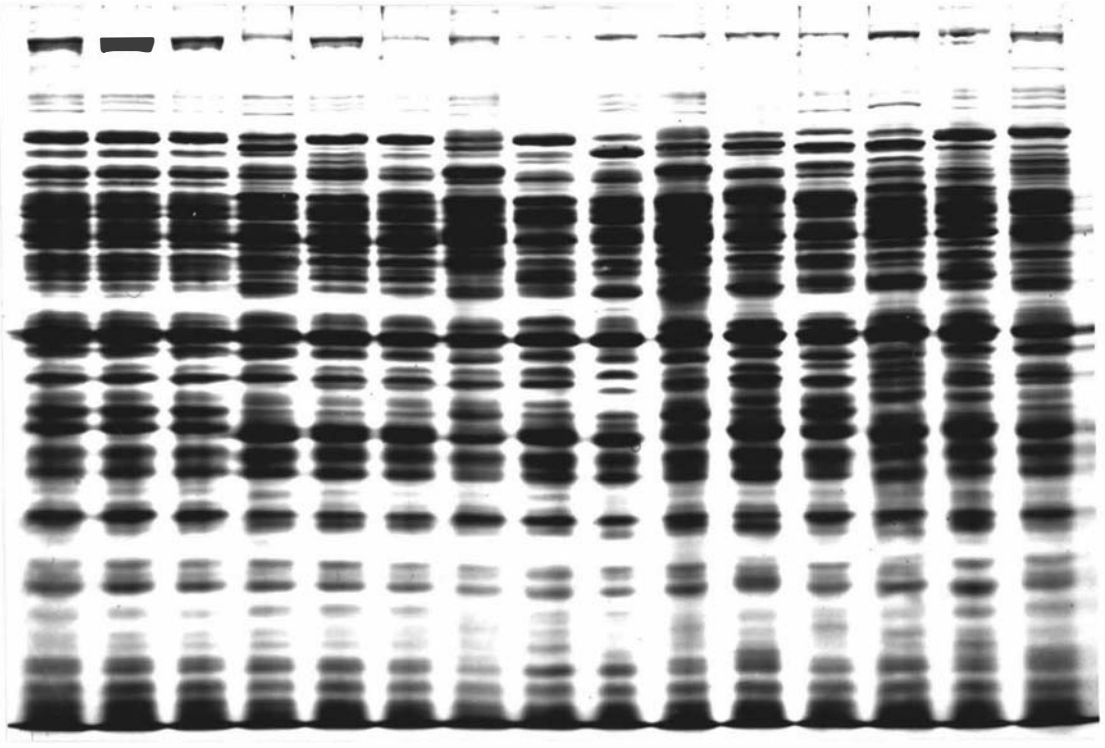
Figure 10c: Lane 1, 11a; Lane 2, 11b; Lane 3, 11c; Lane 4, 12a; Lane 5 ,12b; Lane 6, 12c; Lane 7, 13a; Lane 8, 13b; Lane 9, 13c; Lane 10, 14a; Lane 11, 14b; Lane 12, 14c; Lane 13, 15a; Lane 14, 15b; Lane 15, 15c.

Figure 10d: Lane 1, 16a; Lane 2, 16b; Lane 3, 16c; Lane 4, 17a; Lane 5 ,17b; Lane 6, 17c; Lane 7, 18a; Lane 8, 18b; Lane 9, 18c; Lane 10, 19a; Lane 11, 19b; Lane 12, 19c; Lane 13, 20a; Lane 14, 20b; Lane 15, 20c.

Patterns 1a, 1b and 1c are identical, however apart from this, each pattern is unique. One prominent band (just below the middle of the pattern) is present, at least as a major protein, in only about half of the isolates [e.g. compare 8c and 9c (band present) and with 9a and 9b (band absent)]. This is further illustrated in Figures 10c and 10d.

Technical Note:

A 10% gel was used with a protein loading of 80ug/lane.



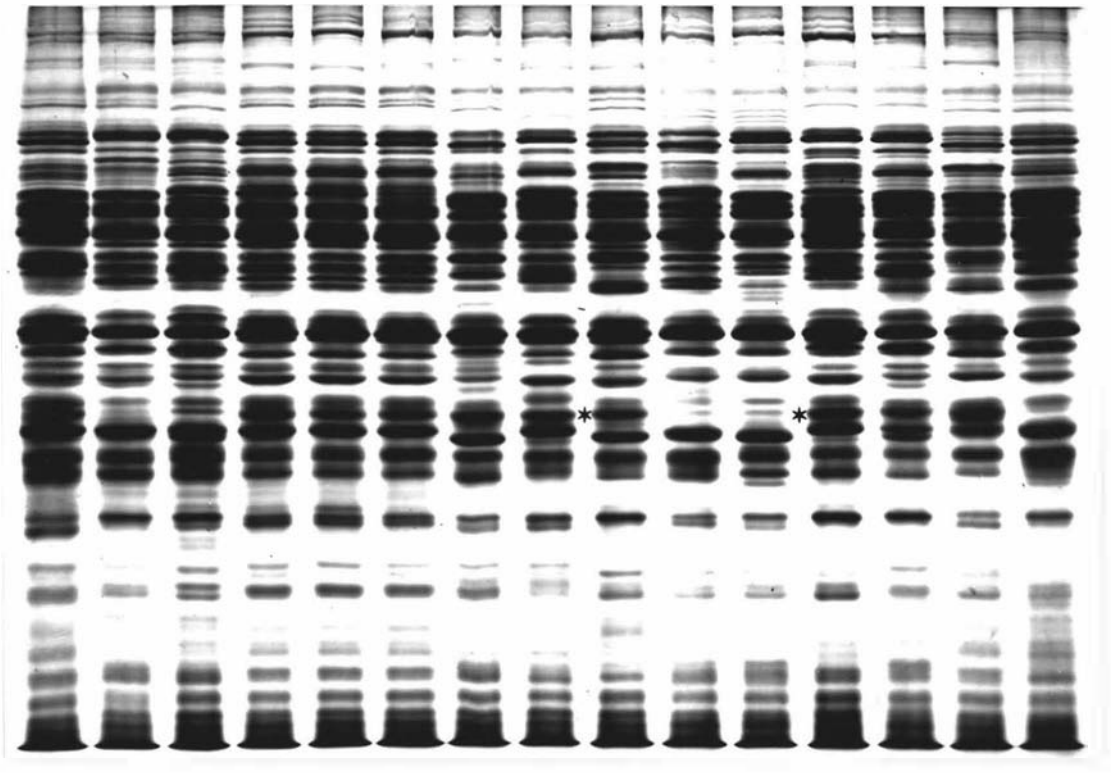
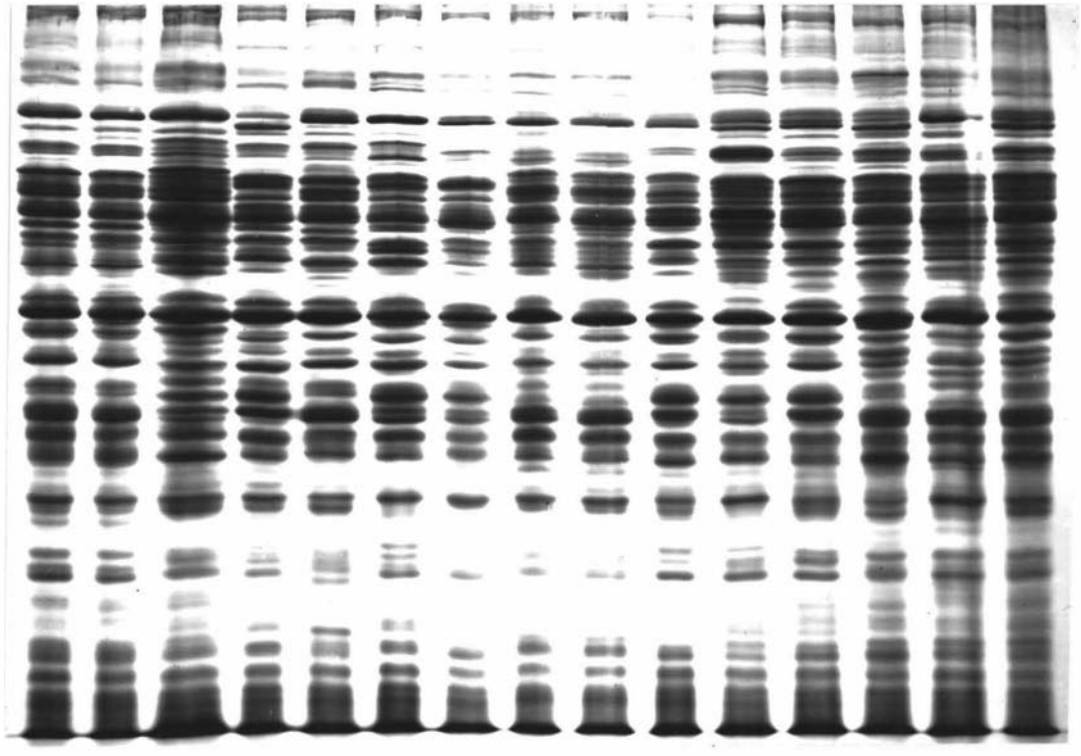


Figure 10 (continued)



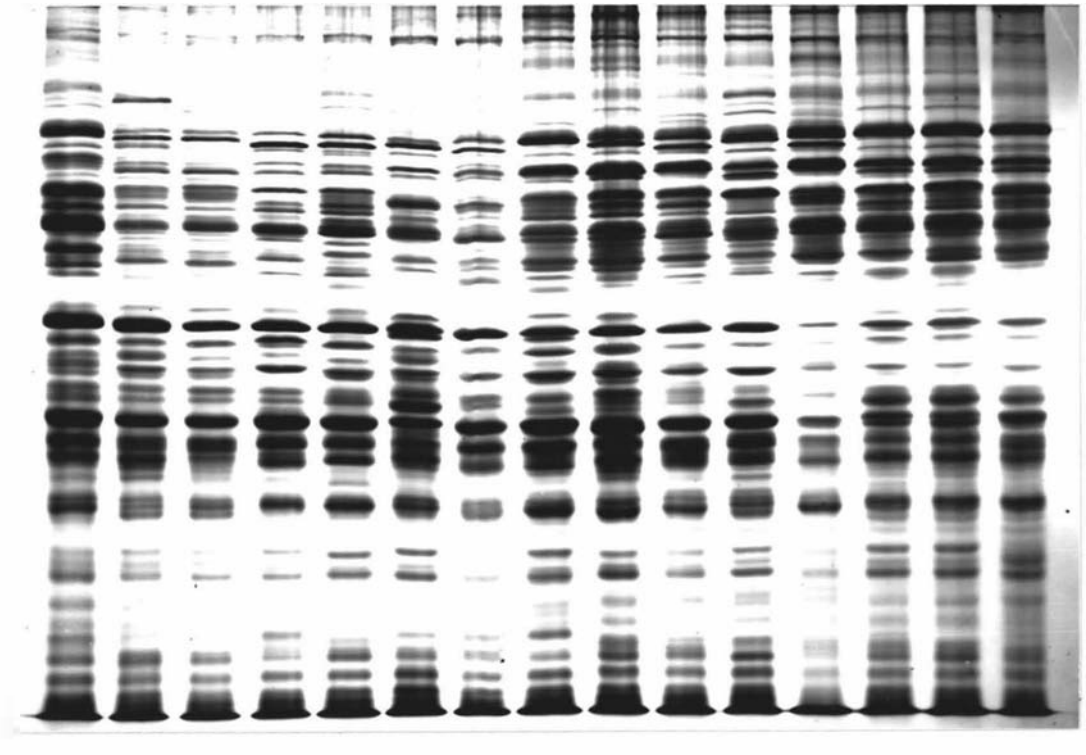


Figure 11

Comparison of *M. ovipneumoniae* isolates with and without a particular protein band.

Two lots of nine *M. ovipneumoniae* isolates were chosen for the presence (identified by an arrow) or absence of a prominent protein band. These isolates were related from Figures 10a, b, c and d. New lysates were prepared from fresh cultures and their proteins were re-examined. Lane 1, 1c; Lane 2, 4c; Lane 3, 7a; Lane 4, 8b; Lane 5 ,9c; Lane 6, 10b; Lane 7, 12a; Lane 8, 14c; Lane 9, 17c; Lane 10, 2a; Lane 11, 3c; Lane 12, 5c; Lane 13, 6b; Lane 14, 7a; Lane 15, 10c, Lane 16, 15a; Lane 17, 16b; Lane 18, 19a; Lane 19, molecular weight markers.

Note that the nine isolates on the left have the band (arrowed) while in the nine isolates on the right the band is absent. Also note that no two lanes are identical.

Technical Note:

A 10% SDS-PAGE gel was used with a protein loading of 60ug per lane.

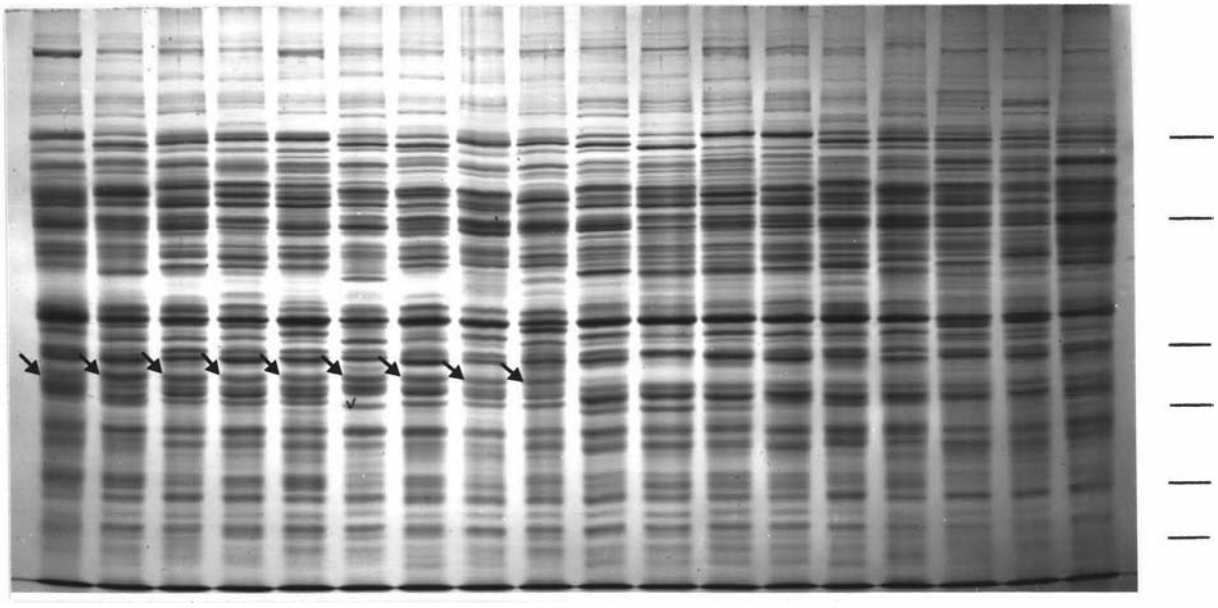
Figure 12

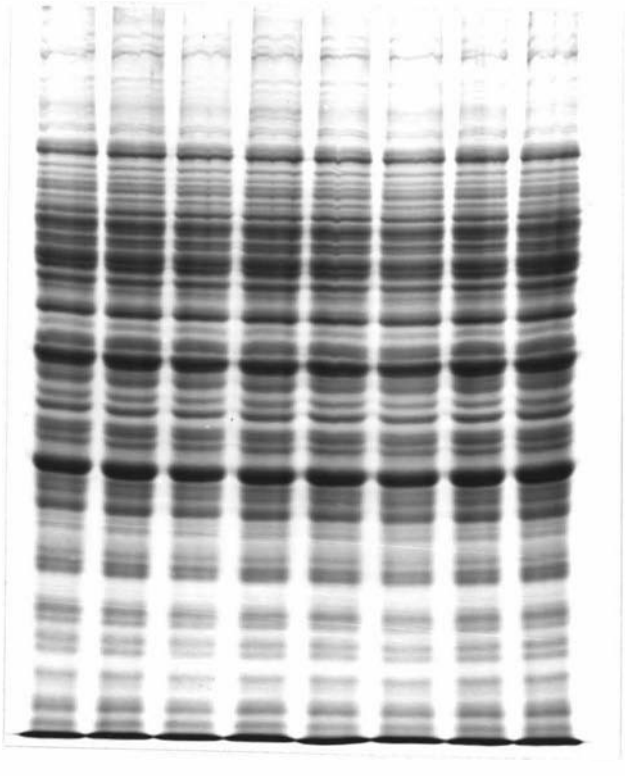
Investigation of the reproducibility of SDS-PAGE protein separations using eight replicate cultures of one *M. ovipneumoniae* strain.

Note that all tracks are identical.

Technical Note:

A 10% SDS-PAGE gel was used with a protein loading of 60ug per lane.





C2.4 Discussion.

Before placing emphasis on SDS-PAGE banding pattern differences in isolates, it is necessary to unequivocally show that banding patterns from one isolate are constant. A constant pattern must be seen not only when replicate samples of the same extract are compared but also when extracts are prepared from replicate cultures of the same isolate. Figure 12 shows that the latter condition was met. The following discussion relies on that observation.

One of the original aims of this thesis was to attempt to assign *M. ovipneumoniae* isolates to serological or biological groups. This requires a method of distinguishing isolates but also requires that the difference between isolates would conform to a pattern which would allow "groups" to be defined. With this in mind the banding patterns were examined and the most obvious conclusion is that isolates show extreme heterogeneity i.e. only three isolates of sixty were identical and these came from sheep on one farm. This allows isolates to be distinguished but not grouped. Despite this heterogeneity most protein bands were common to all isolates, however some bands were present in a proportion of isolates and absent in others. One such band (Figure 11, see arrow) was present in about 50% of the isolates. Since this is a constant result it could be argued that *M. ovipneumoniae* isolates can be divided into two groups. However, this approach is unlikely to recommend itself as an effective method of grouping *M. ovipneumoniae* isolates for several reasons: it would require high resolution gels; the presence or absence of the band noted in about half of the isolates does not correlate with the presence or absence of any other bands which casts doubt on its significance and finally and perhaps critically, the presence or absence of the band has not, or at least has not yet been correlated with any biological property. If, for example, it was shown to represent a surface protein and antibody to it caused metabolic inhibition its significance would be greater. In the next chapter we attempt to identify surface proteins.

CHAPTER THREE

Identification of Surface Proteins of *M. ovipneumoniae*.

A) Identification of Surface Proteins of Intact *M. ovipneumoniae* Cells by Proteolytic Enzyme Digestion.

A3.1 Introduction.

Examination of *M. ovipneumoniae* by SDS-PAGE and IEF showed, as would be expected, that the organism produces many proteins, but less expected, that the protein profiles of almost all isolates differed in at least a few bands although most protein bands were common to all isolates. Neither these results, nor the earlier serological results enabled us to assign the isolates to a limited number of biological meaningful groups.

Considered from the point of view of immunity to the organism, it seems probable that the many common protein bands represent mainly internal proteins whereas some or perhaps most of the variable bands may represent different surface proteins which could account for the large number of different strains, as demonstrated by SDS-PAGE results.

Consequently, this section of the work was undertaken to identify surface proteins of *M. ovipneumoniae*. Two approaches were taken *viz* (a) selectively label the proteins of intact mycoplasmas by fluorescent dyes, which were claimed to be unable to penetrate membranes, and (b) examine the selective removal of proteins from intact *M. ovipneumoniae* isolates which were exposed for periods up to 30mins to various proteolytic enzymes. Much work on the former proved to be non-productive because in our hands an excessive number of proteins were labelled, so that it was concluded that labelling was not confined to surface proteins or none at all were labelled (with ANDS). This work is not reported here. Thus the identification of surface proteins was undertaken by examining the action of proteolytic enzymes on washed intact cells.

Following an initial investigation, we used one enzyme (trypsin) to identify surface proteins of eight *M. ovipneumoniae* strains. These strains were examined by convential staining

using Coomassie Blue and also by staining gels with periodic acid-silver stain which although not totally specific for glycoproteins, stains these preferentially.

A3.2 Materials and Methods.

A3.2.1 Materials

i. For the Comparison of Three Proteolytic Enzymes to Determine their Ability to Selectively Remove the Surface Proteins from an Intact *M. ovipneumoniae* Strain.

1)**FM4 Medium**:see appendix

2)**FM4 (Protein-Free) Medium**

As for FM4 Medium, however, Swine serum, Bacto Peptone (Difco), Yeast Autolysate (Pfizer) and 0.4% Phenol Red were ommitted.

3)**Enzyme Inhibiting Solution**

Trizma Base (Sigma)	6.0g
β-mercaptoethanol	10.0ml
Bromophenol Blue	0.02g
Glycerol	16.0ml
SDS	6.0g
12N HCl to pH 6.8	
Distilled Water to	100.0ml

This solution was stored at 4^{o}C until required.

4)**Proteolytic Enzymes**

i. Pronase (Sigma Type XIV)
ii. Papain (BDH)
iii. Trypsin (Difco)

Each enzyme was made to a concentration of 200ug/ml in FM4 (Protein-Free) Medium. These stock solutions were stored at -20^{o}C and diluted to the required concentration.

ii. For Periodic Acid-Silver Staining in the Detection of Glycoproteins of *M. ovipneumoniae*.

1)**Fixing Solution**

Isopropyl Alcohol	75.0ml
Acetic Acid (Glacial)	30.0ml
Distilled Water to	300.0ml

2)**7.5% Acetic Acid**

3)**0.2% Aqueous Periodic Acid**

4)**Ammoniacal Silver Stain**

Ammonium Hydroxide (Conc)	4.2ml
Sodium Hydroxide (0.36%)	63.0ml
Silver Nitrate (19.4%)	12.0ml
Distilled Water to	300.0ml

The ammonium hydroxide and sodium hydroxide solutions were mixed with 200ml of distilled water. The silver nitrate solution was added dropwise while the solution was vigorously agitated. A transient brown precipitate formed but slowly disappeared with continual mixing. This solution was prepared immediately prior to use.

5)**Developing Solution**

Citric Acid	15.0mg
Formaldehyde	57.0ul
Methanol (Analytical Grade)	30.0ml
Distilled Water to	300.0ml

This solution was prepared immediately prior to use.

6)**Reaction Terminating Solution**

Ilford^{TM} Hypan Rapid Fixer	333.0ml
Distilled Water to	1000.0ml

A3.2.2 Methods.

A3.2.2.i A Comparison of Three Proteolytic Enzymes to Determine their Ability to Selectively Remove the Surface Proteins from an Intact *M. ovipneumoniae* Strain.

An aliquot of *M. ovipneumoniae* strain 5 was inoculated into 2000ml of FM4 Medium and incubated at 37^{o}C. When the culture indicated a pH decrease of one pH unit (i.e. pH 7.8 to pH 6.8) the cells were centrifuged at 15 500g for 15mins at room temperature and washed twice in FM4 (Protein-Free) Medium to remove proteins present in the growth medium (FM4 Medium). The cells were suspended in FM4 (Protein-Free) Medium to give a 10% suspension and placed in an ice bath. A 100ul aliquot was assayed for protein concentration (see section C2.2.2.v), using FM4 (Protein Free Medium) as a blank. The cell suspension was then diluted to a concentration of 20mg/ml and 1ml aliquots were transferred to each of twenty-four 1.3cmx10.0cm glass Kimax^{TM} tubes and placed into a 37^{o}C waterbath. All three enzymes (pronase, papain and trypsin) were diluted to two concentrations i.e. 10ug/ml and 100ug/ml in FM4 (Protein-Free) Medium and warmed to 37^{o}C. 1ml of each enzyme was added to the *M. ovipneumoniae* cell suspension. The mixtures were incubated for the following times: 1, 3, 10 and 30mins. The reaction was terminated by the addition of 2ml of Enzyme Inhibiting Solution at 100^{o}C. The lysed cell suspension was thoroughly mixed and left at 100^{o}C for 3mins. The lysates were cooled, centrifuged at 5000g for 10mins and examined by SDS-PAGE (see section C2.2.2.vi).

A3.2.2.ii Periodic Acid-Silver Staining for the Detection of Glycoproteins of *M. ovipneumoniae.*

Following SDS-PAGE, the gel was placed into 300ml of fixing solution and agitated gently overnight. The gel was then soaked in 7.5% Acetic Acid for 30mins, placed in 0.2% periodic acid for 60mins at 4^{o}C and washed in several changes of distilled water for 3hrs. The gel was then placed in the Ammoniacal Silver Stain, agitated continuously for approximately 30mins, washed for 2mins in distilled water and placed in the Developing Solution. The gel was gently agitated to ensure that it remained submerged. In approximately 5mins the major glycoproteins and polysaccharides started to appear and after 20mins most of the bands were visible. The developing reaction was terminated by the addition of photographic fixer. The stained gel was washed in several changes of distilled water and photographed.

A3.3 Results.

Intact mycoplasma cells were exposed to one of two concentrations (5ug/ml or 50ug/ml) of three proteolytic enzymes (pronase, papain and trypsin) for periods of up to 30mins. Enzyme action was then terminated by SDS and heat and the total proteins were separated on a 10% acrylamide gel by SDS-PAGE. The results are shown in Figures 13a, b, c, d, e and f, which also includes untreated cells for comparison.

At the higher concentrations, pronase and papain (Figure 13b and 13d respectively) caused major changes in the banding pattern. Thus, most proteins, especially those with higher molecular weights, disappeared and were replaced by fragments of lower molecular weight. This suggests that most proteins, including non-surface proteins, were affected because of cell lysis. In contrast, exposure of intact *M. ovipneumoniae* cells to the lower concentrations of papain and trypsin (Figure 13c and 13e respectively), did not visibly alter the banding pattern. However, this pattern was altered by weak (5ug/ml) pronase and strong (50ug/ml) trypsin in an identical manner (Figure 13a and 13f respectively). Thus, several protein bands (see arrows) were greatly diminished in intensity while most other bands were unaffected. Since trypsin, unlike pronase, did not cause cell lysis, even when used at a higher concentration for 30mins, it was used to investigate and compare *M. ovipneumoniae* strains.

The total protein of these eight isolates of *M. ovipneumoniae* with or without exposure to trypsin (50ug/ml) for 10mins, are compared in Figure 14. Following preliminary results, the acrylamide gel concentration was reduced from 10% to 7.5%, to allow better separation of the middle to high molecular weight proteins.

The results (Figure 14) indicate that four protein bands (A, B, C and D), common to all eight *M. ovipneumoniae* strains were either totally removed or greatly diminished in intensity, when the bands are compared with untreated proteins.

One protein (band E) is present in only two *M. ovipneumoniae* strains (1 and 37E) and was diminished in staining intensity, but not totally removed by trypsin treatment.

A number of different protein bands, each of which are unique to one *M. ovipneumoniae* strain and which are removed (or at least diminished) by trypsin treatment of intact cells were also identified (e.g. band F, Figure 14).

In parallel experiments, the same eight intact *M. ovipneumoniae* strains, with and without trypsin treatment, were electrophoresed using SDS-PAGE and stained using a periodic acid-silver stain. The results (Figure 15) show that three of the four common surface protein bands, previously identified by coomassie blue staining (Figure 14, bands B, C and D), were also stained by the periodic acid-silver stain. This stain, although not totally specific, preferentially stains glycoproteins (see general discussion). Of the four surface proteins, only one (band A) did not stain with the periodic acid-silver stain, suggesting that it is a non-glycosylated surface protein. One surface protein (band E), present in only two of the eight *M. ovipneumoniae* strains and identified previously by coomassie blue staining (Figure 14, band E), also stained with the periodic acid-silver stain. Only major common bands, or bands close to them, can be unequivocally equated between the periodic acid-silver stained and coomassie blue stained gels. However, with this proviso, our results indicate that unique bands, i.e. bands present in only one of the eight isolates removed by trypsin, were not stained by the periodic acid-silver stain thus they probably do not represent glycoproteins.

Figure 13 (a, b, c, d, e and f)

A comparison of SDS-PAGE protein patterns of *M. ovipneumoniae* cell suspensions treated with three different proteolytic enzymes (pronase, papain or trypsin) at two different concentrations (5ug/ml or 50ug/ml) for varying times:

Figure 13a: Pronase (5ug/ml), for (left to right) 0, 1, 3, 10 and 30mins;

Figure 13b: Pronase (50ug/ml), for (left to right) 0, 1, 3, 10 and 30mins;

Figure 13c: Papain (5ug/ml), for (left to right) 0, 1, 3, 10 and 30mins;

Figure 13d: Papain (50ug/ml), for (left to right) 0, 1, 3, 10 and 30mins;

Figure 13e: Trypsin (5ug/ml), for (left to right) 0, 1, 3, 10 and 30mins;

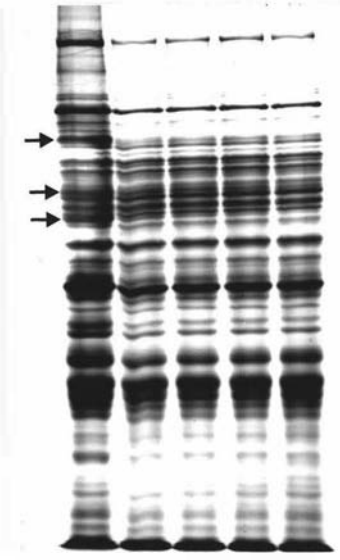
Figure 13f: Trypsin (50ug/ml), for (left to right) 0, 1, 3, 10 and 30mins.

Note:

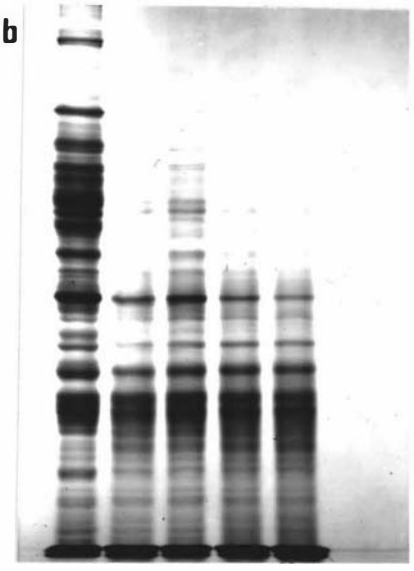
1. Arrows (Figure 13f) indicate bands which diminished in intensity following exposure of intact organisms to proteolytic enzymes in conditions which left most bands unaltered.

2. Pronase and Papain at high concentrations (13b and 13d), lysed the cells.

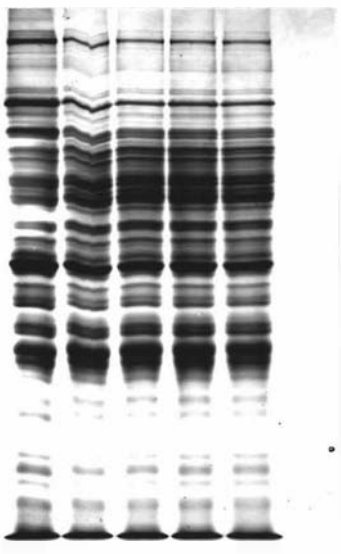
13a



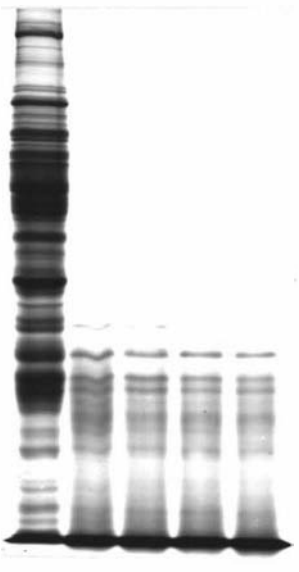
13b



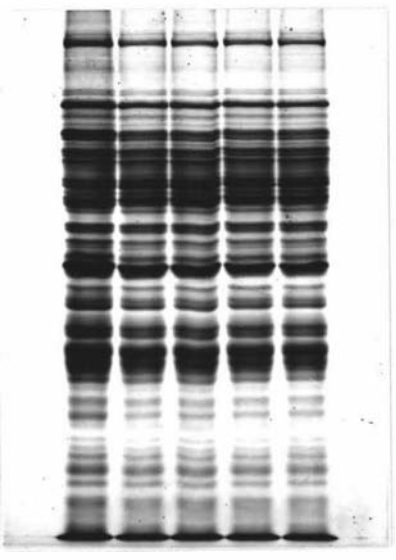
13c



13d



13e



13f

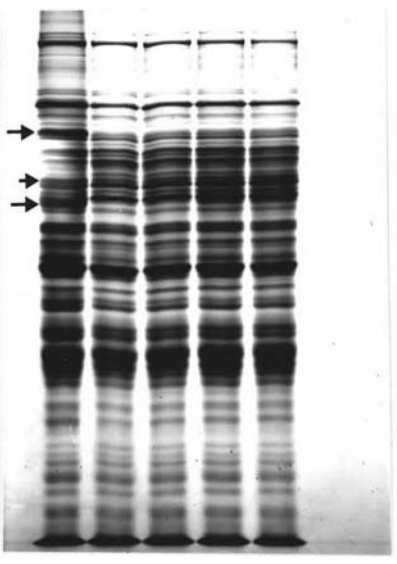


Figure 14a

A comparison of the total proteins of eight *M. ovipneumoniae* strains before and after exposure to 50ug/ml of trypsin for 10mins at 37^{o}C.

Lanes 1 and 2: strain 1 untreated and treated with trypsin;
Lanes 3 and 4: strain 2 untreated and treated with trypsin;
Lanes 5 and 6: strain 4 untreated and treated with trypsin;
Lanes 7 and 8: strain 5 untreated and treated with trypsin;
Lanes 9 and 10: strain 10 untreated and treated with trypsin;
Lanes 11 and 12: strain 37E untreated and treated with trypsin;
Lanes 13 and 14: strain MPP74 untreated and treated with trypsin;
Lanes 15 and 16: strain L3/C3 untreated and treated with trypsin.

Note:

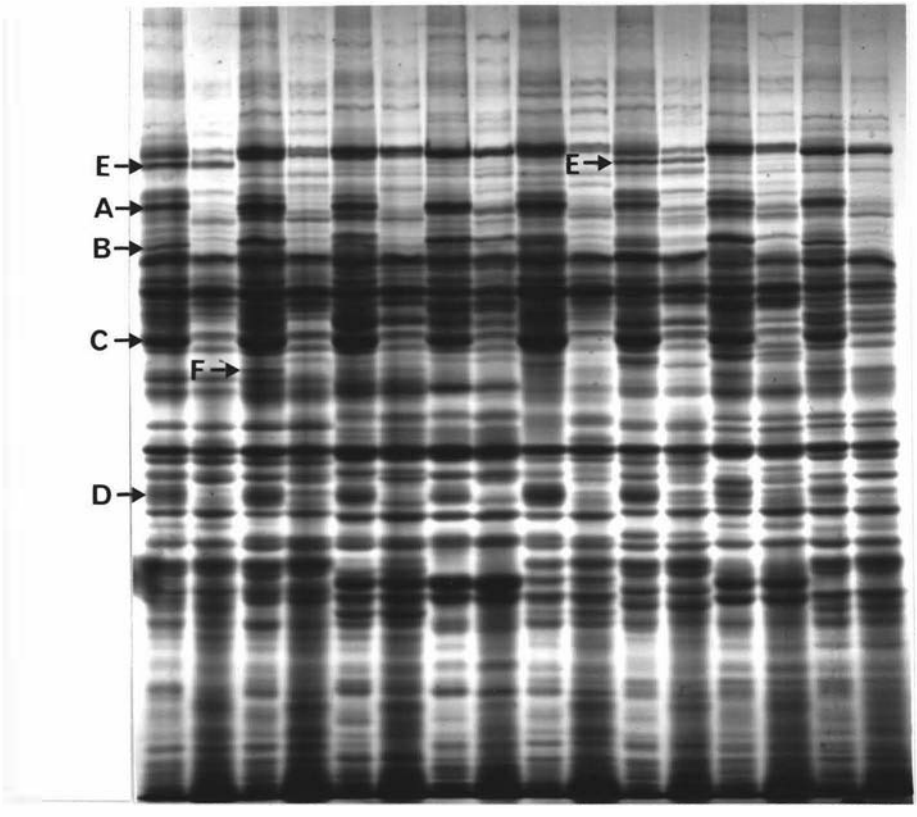
Four common protein bands (A, B, C and D) are diminished in intensity by enzymatic treatment. One protein band (E) shared between two strains and a small number of bands unique to individual isolates (e.g. band F arrow) are also removed or diminished in intensity by exposure to trypsin.

Technical Note:

The acrylamide gel concentration in this figure (in contrast to Figures 13) was reduced from 10% to 7.5% to increase the resolution of the middle to high molecular weight protein bands.

Figure 14b

This figure is an enlargement of Figure 14a and shows the region bounded by bands B and E. It illustrates that faint bands adjacent to band E are also removed by trypsin (see arrows).


E
A
B
C
F
D
E

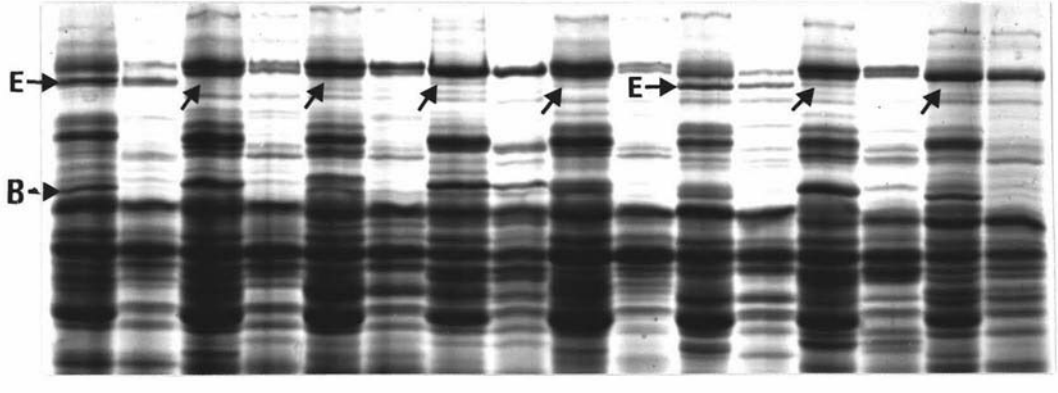

E
B
E

Figure 15

A comparison of eight *M. ovipneumoniae* strains before and after exposure to 50ug/ml of trypsin for 10mins at 37^{o}C. Stained with periodic acid-silver.

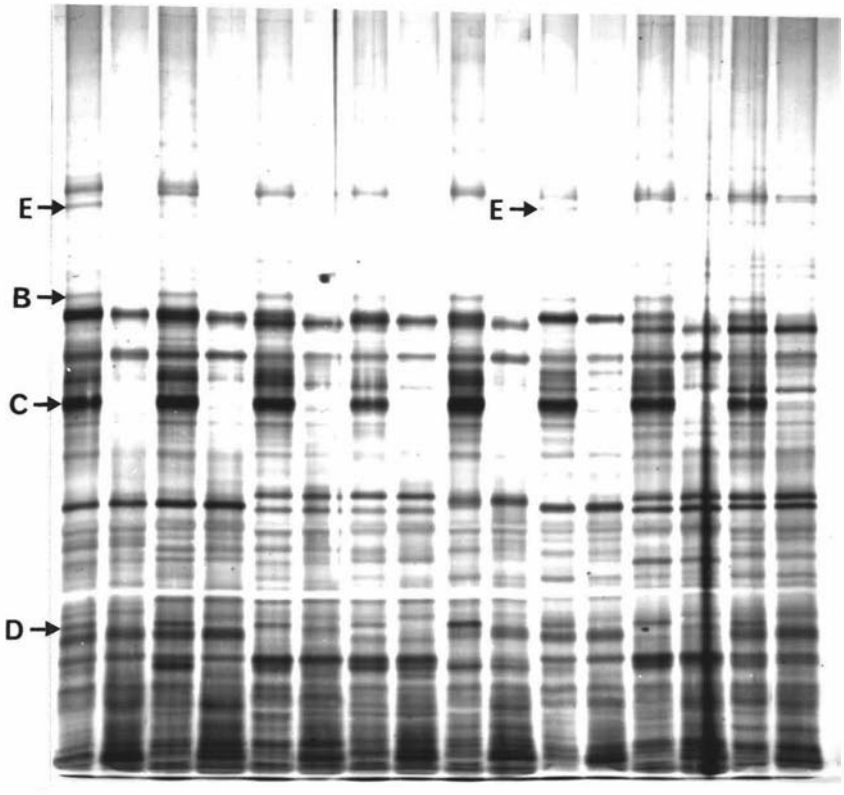
Lanes 1 and 2: strain 1 untreated and treated with trypsin;
Lanes 3 and 4: strain 2 untreated and treated with trypsin;
Lanes 5 and 6: strain 4 untreated and treated with trypsin;
Lanes 7 and 8: strain 5 untreated and treated with trypsin;
Lanes 9 and 10: strain 10 untreated and treated with trypsin;
Lanes 11 and 12: strain 37E untreated and treated with trypsin;
Lanes 13 and 14: strain MPP74 untreated and treated with trypsin;
Lanes 15 and 16: strain L3/C3 untreated and treated with trypsin.

Note:

Three bands (B, C and D) common to all isolates are seen. One band (E) is present in strains 1 and 37E. The letters correspond with the bands in Figure 14. However, Band A is not stained in this gel.

Technical Note:

Each lane was loaded with 3ug of protein.


E
E
B
C
D

A3.4 Discussion.

Pronase (5ug/ml) and trypsin (50ug/ml) were equally able to cause a diminution in the staining intensity of a limited number of bands (Figures 13a and 13f). Both of these proteolytic enzymes could therefore be regarded as suitable for the identification of surface proteins by selectively removing them. Trypsin treatment at a concentration of 50ug/ml for 10mins was chosen as a standard method of detecting surface proteins of a number of independent *M. ovipneumoniae* strains. It had the advantage that, unlike pronase, high concentrations for long periods, did not lyse the cells. Thus, even at concentration levels up to 350ug/ml for 10mins (data not shown), or up to 50ug/ml for 30mins (Figure 13f), it did not substantially alter the number of proteins digested.

Proteolytic enzyme digestion of eight intact *M. ovipneumoniae* strains by 50ug/ml of trypsin for 10mins, showed that four protein bands (A, B, C and D) were either removed or diminished in band intensity relative to adjacent bands (Figure 14). It was concluded that *M. ovipneumoniae* has at least four surface proteins which are common to all strains. Another surface protein (Figure 14, band E) was shown by only two *M. ovipneumoniae* strains. Finally, individual unique protein bands, were removed from some *M. ovipneumoniae* strains (Figure 14, unlabelled arrows).

Dubray and Bezard (1982), recommended the use of periodic acid-silver stain as a method of detecting glycoproteins. Since surface proteins of many micro-organisms including mycoplasmas are glycoproteins (Razin, 1978), we used a lightly loaded gel; 3ug of protein per lane, stained by this method to complement the above results. One band (Figure 15, band C) already identified as a surface protein stained strongly by periodic acid-silver stain and two bands were lightly stained (Figure 15, bands B and D). One band (Figure 15, band A) was not detected on the periodic acid-silver stained gel. It was therefore concluded that three of the four common protein bands are glycoproteins, but at least one common band (Figure 14, band A) is not glycosylated. Interestingly, a number of unique bands removed by proteolytic enzyme digestion in the coomassie blue stained gel (Figure 14, unlabelled arrows), were not detected in the periodic acid-silver stained gel (Figure 15). This suggests that these bands are not glycoproteins.

The possibility arose, that some of the trypsin-sensitive common protein bands could be absorbed media constituents. However, in a previous study (Ionas, 1983), to determine the

presence of absorbed media constituents, an *M. ovipneumoniae* strain was propagated in the presence of ^{14}C-amino acids and the electrophoresed protein bands were recorded by autoradiography. The protein bands now found to be trypsin-sensitive, were identical with the labelled bands of earlier experiments i.e. are not media constituents.

It is therefore concluded that *M. ovipneumoniae* has at least four surface proteins which (at least in the molecular weight sense) are common to all strains. Three of these are probably glycoproteins. Other surface proteins unique to individual strains (or shared by only a few strains) also exist. These do not appear to be glycoproteins.

The existance of common bands as shown by SDS-PAGE, does not necessarily imply that they are the same proteins i.e. the commonality could be limited to the molecular weight and relative amount of the protein present, rather than implying identical immunogenicity. Conversly, the appearance of a unique band does not prove unique antigenicity, since proteins of slightly different molecular weight, could be antigenically similar. These considerations are investigated in the next section using an "Immuno-Blot" technique.

B) Investigations of Some *M. ovipneumoniae* Proteins by the "Immuno-Blot" Technique.

B3.1 Introduction.

Examination of *M. ovipneumoniae* cells with and without trypsin treatment, allowed us to identify four proteins which were common to the surface of all isolates. We also identified a protein shared by two isolates, but absent from the other six tested, this protein was apparently a glycoprotein. Several other proteins, apparently not glycoproteins, which were unique to individual isolates were also identified.

SDS-PAGE gels separate proteins on the basis of size but proteins of the same molecular weight are not necessarily the same immunologically and proteins of slightly different molecular weights are not necessarily serologically distinct.

To investigate the serological relatedness we selected five proteins; three common, one shared between two isolates and one unique protein. These proteins were separated by SDS-PAGE, excised from the gel, used to immunise rabbits and the antisera was used to investigate the relatedness of the proteins by the use of the "Immuno-Blot" technique.

B3.2 Materials and Methods.

B3.2.1 Materials

1)**0.15M Saline**: see appendix

2)**Transfer Buffer** (pH 8.3)

Tris (Hydroxymethyl) Aminomethane	12.12g
Glycine	57.60g
Methanol (Analytical Grade)	800.0ml
Distilled Water to	4000.0ml

Note:

This solution does not require adjustment to pH 8.3 and the addition of HCl for minor pH corrections should be avoided as it will cause an ion imbalance.

3)**Tris Buffered Saline** (1xTBS)

Tris (Hydroxymethyl) Aminomethane	4.84g
NaCl	58.48g
Adjust to pH 7.5 with 12N HCl	
Distilled Water to	2000.0ml

Stored at room temperature.

4)**Tween-20 Tris Buffered Saline** (1xTTBS)

Tris (Hydroxymethyl) Aminomethane	4.84g
NaCl	58.48g
Adjust to pH 7.5 with 12N HCl	
Tween-20 (polyoxethylene sorbitan monolaurate)	1.0ml
Distilled Water to	2000.0ml

Stored at room temperature.

5)**Blocking Solution** (1xTTBS-3% Gelatin)

Tris (Hydroxymethyl) Aminomethane	0.24g
NaCl	2.92g
Adjust to pH 7.5 with 12N HCl	
Tween-20	0.05ml
Gelatin (EIA purity reagent, Bio-Rad™)	3.00g
Distilled Water to	100.0ml

This solution was made immediately prior to use. The gelatin was dissolved in pre-warmed (37°C) 1xTTBS and cooled to 28°C before use.

6)**Antibody Buffer** (1xTTBS-1% Gelatin)

Tris (Hydroxymethyl) Aminomethane	0.48g
NaCl	5.84g
Adjust to pH 7.5 with 12N HCl	
Tween-20	0.10ml
Gelatin	2.00g
Distilled Water to	200.0ml

This solution was made immediately prior to use. The gelatin was dissolved in pre-warmed (37^{o}C) 1xTTBS and cooled to 28^{o}C before use.

Note:

100ml of this solution was used for the first antibody reaction and the remainder was used for the second antibody reaction.

7)**Horseradish Peroxidase (HRP) Substrate Solution**

This solution is composed of two parts and was used immediately after mixing.

Part A:

60mg of 4-chloro-1-naphthol (stored at -20^{o}C) was added to 20ml of cold methanol (AR grade). This solution was dispensed into a dark bottle and stored at -20^{o}C for a few minutes until required.

Part B:

60ul of hydrogen peroxide (30% w/v) was added to 100ml of 1x TBS at room temperature*.

Parts A and B were then mixed immediately to produce the working solution and this was used immediately.

* Note:

If the working solution (part A plus part B) is stored at 0-4^{o}C, the substrate will precipitate out of the solution and detection sensitivity may be substantially decreased. The substrate (Part A) was placed at -20^{o}C and the 1x TBS-hydrogen peroxide solution was at room temperature prior to mixing, thus maximizing their activity and prolonging their useful life.

B3.2.2 Methods

B3.2.2.i The Excision of *M. ovipneumoniae* Surface Proteins from SDS-PAGE Gels for the Production of Monospecific Antisera.

M. ovipneumoniae lysates were electrophoresed in a 7.5% acrylamide gel as previously described in section C2.2.2.vi. However, a small modification to this method was included so as to allow large amounts of surface proteins to be excised from the SDS-PAGE gel. The comb used to cast ten wells in the stacking gel was substituted for a comb that could cast only two wells. The dimensions of the wells also differed. One well cast was approximately 0.8cm in width while the second was about 24.0cm.

The lysate of an intact *M. ovipneumoniae* suspension treated with trypsin (see section A3.2.2.i) with a protein concentration of 80ug/well, was dispensed into the smaller of the two wells. On the adjacent larger well, an aliquot containing approximately 2.5mg of protein from the same *M. ovipneumoniae* strain, but not treated with trypsin was dispensed. The lysates were electrophoresed through the acrylamide gel. This gel was stained and then destained as previously described in section C2.2.2.vi. Following the destaining procedure the gel was repeatedly washed with large volumes of distilled water to remove all trace of destaining solution (i.e. 10% acetic acid).

Surface proteins that were previously identified (sections A3.3) and were again identified by comparing the trypsin treated (0.8cm) lane with the non-treated (24.0cm) protein lane. Individual surface proteins were excised from the non-treated lane using a scalpel blade, diced into approximately 0.15cmx0.15cmx0.15cm cubes and placed into a 5ml Cornwall^{TM} luer-lock syringe containing 1.5ml of sterile 0.15M saline. This gave a total volume of about 3ml. The protein/acrylamide mixture was then connected to another 5ml syringe via a 12 gauge needle with luer-locks at either end and the mixture was squirted from one syringe to the other. This process was repeated until the mixture became pastelike.

B3.2.2.ii Antisera Production to Excised Surface Proteins from SDS-PAGE Gels.

3ml aliquots of homogenized protein/acrylamide mix was injected intraperitoneally into pre-bled New Zealand White rabbits at two weekly intervals for twelve weeks. The rabbits were bled seven days after the last inoculation and the sera were processed as described in section C2.2.2.ii.

B2.2.2.iil Electrophoretic Transfer of Proteins from a SDS-PAGE Gel to a Nitrocellulose Sheet ("Western-Blot").

a. Preparation of the SDS-PAGE Gel for Electrophoretic Transfer

Lysates of *M. ovipneumoniae* strains containing 80ug/well were electrophoresed in a 7.5% acrylamide gel. Following electrophoresis the acrylamide gel was removed from the gel apparatus and equilibrated with 500ml of transfer buffer for 30mins.

b. Assembly of the Blotting Apparatus

3500ml of transfer buffer was placed in a 30cm x 40cm photographic developing tray. The perspex gel holder (see Figure 16) was opened and one of the two panels (tinted smoky grey-the cathode end) was submerged in the transfer buffer. The other perspex panel which is transparent (the anode end) was allowed to rest an angle against the wall of the vessel. A sponge (21cm x 16cm x 1cm) was saturated in the transfer buffer and positioned over the submerged panel. Three layers of Whatman^{TM} 3mm filter paper were placed on top of the sponge, followed by a single sheet of Whatman^{TM} N^{o}1 filter. Care was taken to remove trapped air bubbles between each filter paper layer as this inhibits the transfer of the protein from the gel to the nitrocellulose sheet. The equilibrated acrylamide gel was then placed on top of the filter paper and trapped air bubbles were expelled by pressing down on the gel and working the bubbles to the edge. A square of nitrocellulose sheet (Schleicher and Schuell, West Germany) with a pore size of 0.45u was cut to the approximate dimensions of 11cm x 13cm. Care was taken to handle the nitrocellulose sheet with disposable plastic gloves, so as not to transfer proteins from the skin by contact. The nitrocellulose sheet was labelled with a marker pen on one corner so that the orientation of the sheet was clear and then soaked in transfer buffer by capillary action. This method of soaking the nitrocellulose sheet ensures that the entire sheet is soaked evenly. Sheets placed directly into transfer buffer in most cases, became blotchy and could not be used. The saturated sheet was then carefully laid on top of the acrylamide gel. Air bubbles trapped between the gel and the nitrocellulose sheet were expelled by rubbing the surface of the nitrocellulose sheet and working the air bubble to the edge. The rubbing and pressing process (to expell trapped air

Figure 16

Assembly of the apparatus to transfer proteins from an acrylamide gel to a nitrocellulose sheet. The gel "sandwich" was (from bottom up) in the following order.

a) The perspex gel holder
b) A sponge pad
c) Three layers of Whatman™ 3mm filter paper
d) One layer of Whatman™ N°1 filter paper
e) The acrylamide gel
f) A single sheet of nitrocellulose filter (Schleicher and Schuell, West Germany) with a pore size of 0.45u
g) One layer of Whatman™ N°1 filter paper
h) Three layers of Whatman™ 3mm filter paper
i) A sponge pad

The gel holder was closed and placed into the Trans-blot™ tank.

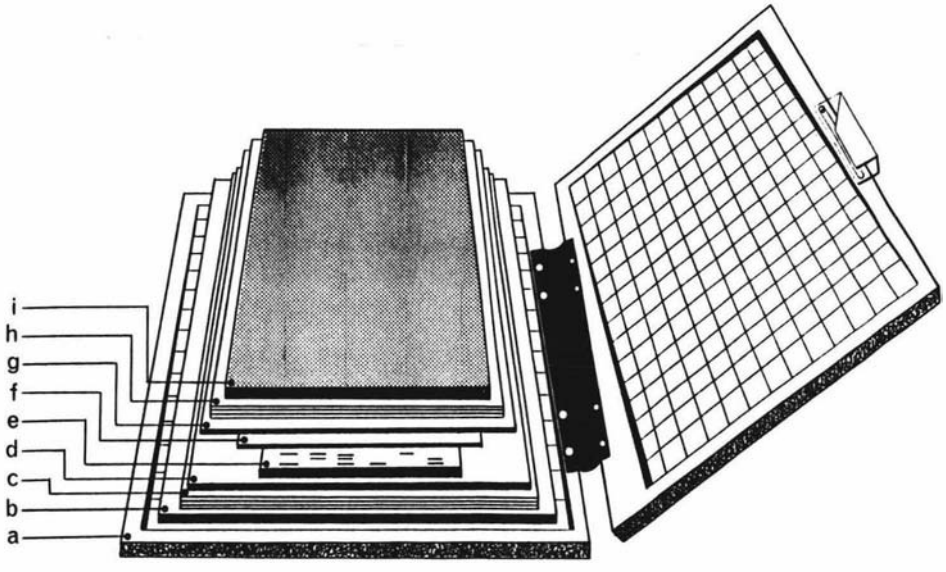
The "sandwich" was assembled while already submerged in 3500ml of transfer buffer. Bubbles between layers were avoided.

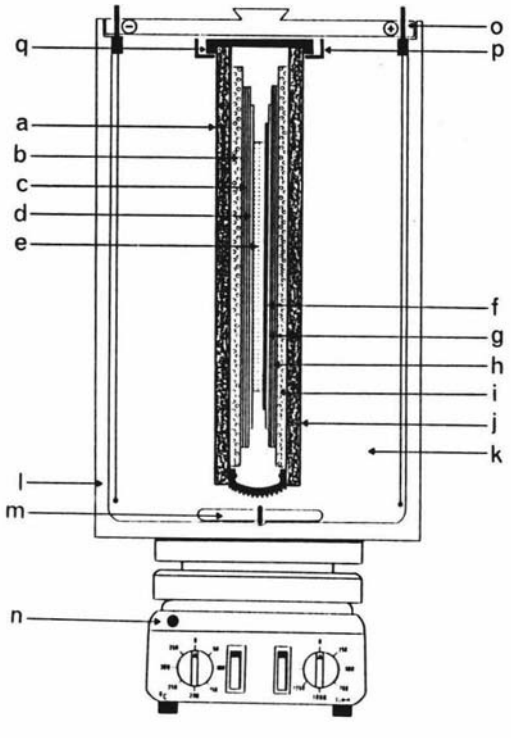
Figure 17

Transfer of the protein to the nitrocellulose sheet.

The gel "sandwich" was placed into the Trans-blot™ tank containing 3000ml of transfer buffer. This was stirred with a magnet.

a) The cathode side of the perspex gel holder
b) A sponge pad
c) Three layers of Whatman™ 3mm filter paper
d) One layer of Whatman™ N°1 filter paper
e) The acrylamide gel
f) A single sheet of nitrocellulose filter (Schleicher and Schuell, West Germany) with a pore size of 0.45u
g) One layer of Whatman™ N°1 filter paper
h) Three layers of Whatman™ 3mm filter paper
i) A sponge pad
j) The anode side of the perspex gel holder
k) 3000ml transfer buffer
l) Trans-blot™ tank
m) Magnet
n) Magnetic stirrer
o) Lid
p) Gel holder
q) Gel holder latch


i
h
g
f
e
d
c
b
a


q
o
p
a
b
c
d
e
f
g
h
i
j
k
l
m
n

bubbles) also ensured good contact between the two layers. The gel "sandwich" (sponge, three layers of 3mm filter paper, one layer of Whatman $N^{o}1$ filter paper, acrylamide gel and the nitrocellulose sheet) was completed by placing one layer of Whatman^{TM} $N^{o}1$, followed by three layers of Whatman^{TM} 3mm filter paper and a sponge pad, all of which were saturated with transfer buffer. The gel holder was then closed and placed into the Trans-blot^{TM} tank (see Figure 17) with the gel on the cathode side with respect to the nitrocellulose sheet. A magnetic "flea" was placed at the bottom of the tank, 3000ml of transfer buffer was added and the entire Trans-blot^{TM} apparatus was positioned on a magnetic stirrer. The mixing of the transfer buffer served two purposes: it ensured that the temperature of the transfer buffer was even throughout the tank and it also ensured an even distribution of ions throughout the solution while electrophoresis was occurring.

c. Running Conditions for Protein Transfer

The *M. ovipneumoniae* proteins were transferred from the acrylamide gel to the nitrocellulose sheet at room temperature by using a Bio-Rad^{TM} 250/2.5 power supply, set at a constant 30 volts (this corresponded to about 0.1 amp) for 18hrs. The next day the voltage was increased to 60 volts for 1-2hrs.

B3.2.2.iv Goat Anti-Rabbit Horseradish Peroxidase Conjugate Enzyme Immunoassay.

Following the transfer of the protein to the nitrocellulose sheet, the gel holder was removed from the trans-blot tank and opened. The nitrocellulose sheet was then immersed in 200ml of Tris buffered saline (1xTBS) for 10mins on a shaking platform set at 60rpm. The acrylamide gel was also removed from the holder and stained with coomassie blue as described in section C2.2.2.vi and examined for the presence of bands. No protein bands were seen following transfer indicating that all of the protein bands had migrated out of the gel.

The nitrocellulose sheet was removed from the Tris Buffered Saline and placed in 200ml of blocking solution consisting of TBS + 3% gelatin and incubated on a shaking platform for 4hrs at 28^{o}C. The sheet was then transferred to 200ml TBS + Tween 20 (TTBS) and washed twice for 5mins with gentle agitation at room temperature. Following this step the nitrocellulose sheet was placed in a 100ml solution of TTBS + 1% gelatin + 0.25ml of rabbit antibody to the excised surface proteins, giving a final antibody dilution of 1:400 (this was

excess antibody determined in experiments not recorded here) and incubated for 1-2hrs at 28^{o}C on a shaking platform set at 60rpm. At this point the nitrocellulose sheet could be left to incubate overnight for convenience. Prolonged incubation will increase the sensitivity but, this was not necessary in the present experiments. Following the exposure to the first antibody solution, the nitrocellulose sheet was washed three times for 5min intervals in 200ml of TTBS to remove unbound antibody. The nitrocellulose sheet was then transferred to 100ml of TTBS + 1% gelatin containing 33ul of goat anti-rabbit horseradish peroxidase conjugate (i.e. about a 1/3000 final dilution) and incubated for 1hr at 28^{o}C on a shaking platform. The nitrocellulose sheet was then washed twice for 5min intervals with 200ml of TTBS and once for 5min with TBS. During these washings of the nitrocellulose sheet, the horseradish peroxidase colour development solution was prepared as described in the materials of this section. Parts A and B of the colour development solution were mixed to give a final volume of 100ml. The nitrocellulose sheet was immediately immersed in the substrate solution and gently agitated. Bands started to appear almost immediately but the reaction was allowed to continue for up to 30mins to produce a strong purple colour. Further incubation did not increase the colour. The reaction was terminated by immersing the nitrocellulose sheet in 500ml of distilled water for 10mins. This final washing was repeated once.

B3.2.2.v Photographing Visualized Bands.

The reacted proteins to the goat anti-rabbit horseradish peroxidase conjugate was permanently recorded on Tech Pan (Kodak^{TM}) film. The nitrocellulose sheet was photographed submerged in distilled water. This procedure had the advantage of not allowing the sheet to dry under the heat of the photographic lights and prevented fading of the purple coloured bands which occurs on drying.

The film was exposed for 1/30 sec at f/5.6 and the negative was developed as described in section 4.2.2A.vi.

B3.3 Results.

Four protein bands (A, B, C and D) on SDS-PAGE gels had been identified as surface proteins. These bands are common to all eight isolates at least in the "molecular weight" sense.

Antisera to three bands (Figure 14, bands B, C and D) were used to probe the eight isolates by "immuno-blotting" (band A was not used as a probe due to the untimely death of the immunized animal).

The results for band C (Figure 19) indicates that this band is common, in the immunological sense, to all eight isolates. This also applies to band D (Figure 20). Band B reacted with five (including itself) of the eight isolates. However, interestingly it did not react with strains 4, 5 and 10 (Figure 18, lanes 3, 4 and 5).

To confirm the above results, a gel which included *M. arginini* and *A. laidlawii* as well as the eight *M. ovipneumoniae* isolates, was probed with antisera raised to the three common surface protein bands. All three bands reacted as described above and no antiserum reacted with *M. arginini* or *A. laidlawii* (Figure 21).

Another band (i.e. band E) was present in only two of the eight isolates (Figure 14). Antiserum to this band was used to probe the eight *M. ovipneumoniae* isolates. It reacted with all eight isolates (Figure 22). However, in some instances the reacting band was displaced upwards and in others it is clear that two bands are involved i.e. the apparently single band E represents at least two proteins, one or (more probably) both of which are present in all isolates albeit in slightly modified form as compared to strains 1 and 37E.

Since band E was resolved into two bands (see Figure 22, lanes 4, 5 and 7). This might imply that the original band E represents a surface protein and another protein. However, on re-examining an enlargement of the region bound by bands B and E (Figure 14b) it was seen that a faint band in adjacent tracts (see unlabelled arrows) was indeed removed by trypsin i.e. band E represents two proteins both of which are apparently surface proteins.

Antiserum to only one band (i.e. band F, Figure 14), identified as a surface protein unique to one isolate was used to probe eight *M. ovipneumoniae* isolates. The results (Figure 23) show that it is indeed a serologically unique protein i.e. it reacts with "itself" (lane 2), but did not react with any other isolate.

Figure 18

Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band B.

Antiserum raised to a common surface protein (Figure 14 - strain 2, band B) was used to probe eight *M. ovipneumoniae* isolates by "immunoblotting". Lane 1: strain 1; Lane 2: strain 2; Lane 3: strain 4; Lane 4: strain 5; Lane 5: strain 10; Lane 6: strain 37E; Lane 7: strain MPP74; Lane 8: strain L3/C3.

Note that although this protein appeared to be common to all isolates when examined by SDS-PAGE (Figure 14), the antiserum reacted with a single protein band in five (including the homologous strain-lane 2) of the eight isolates i.e. lanes 1, 2, 6, 7 and 8. It did not react with the isolates in lanes 3, 4 and 5.

Figure 19

Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band C.

Antiserum raised to a common surface protein (Figure 14 - strain 5, band C) was used to probe eight *M. ovipneumoniae* isolates by "immunoblotting". Lane 1: strain 1; Lane 2: strain 2; Lane 3: strain 4; Lane 4: strain 5; Lane 5: strain 10; Lane 6: strain 37E; Lane 7: strain MPP74; Lane 8: strain L3/C3.

Note that the antiserum raised to band C reacted with all eight *M. ovipneumoniae* isolates.


B

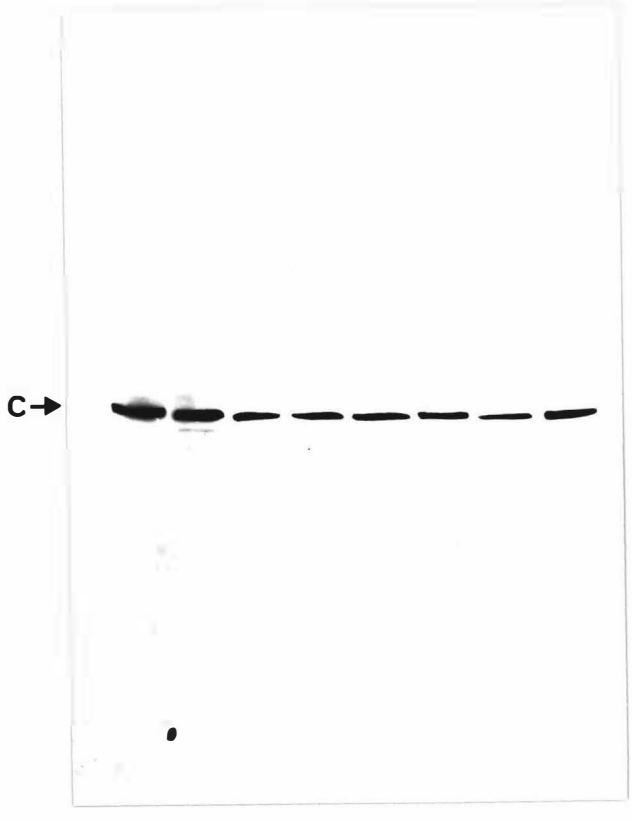

C

Figure 20

Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band D.

Antiserum raised to a common surface protein (Figure 14 - strain 5, band D) was used to probe eight *M. ovipneumoniae* isolates by "immunoblotting". Lane 1: strain 1; Lane 2: strain 2; Lane 3: strain 4; Lane 4: strain 5; Lane 5: strain 10; Lane 6: strain 37E; Lane 7: strain MPP74; Lane 8: strain L3/C3.

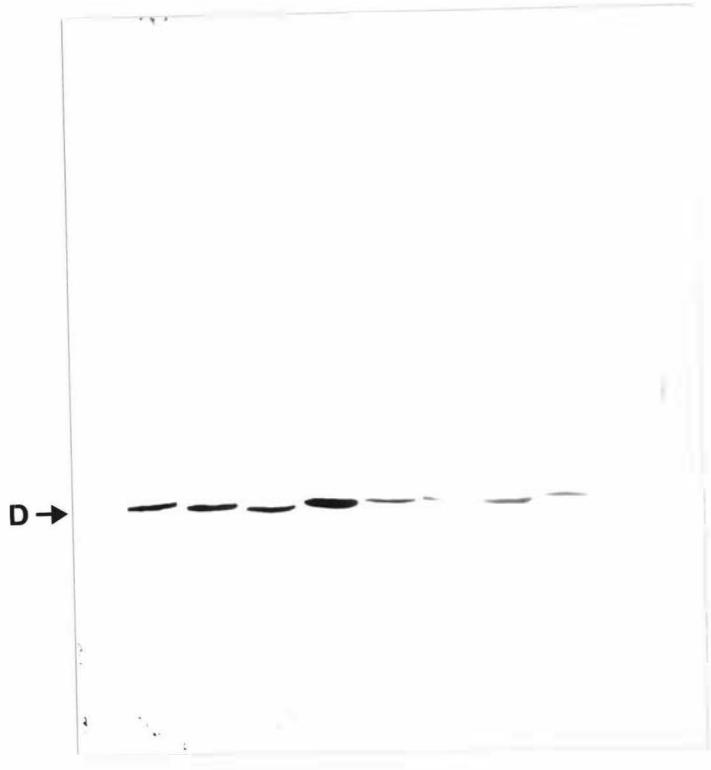
Note that the antiserum raised to band D reacted with all eight *M. ovipneumoniae* isolates.

Figure 21

Eight *M. ovipneumoniae* isolates "blotted" with antisera raised to bands B, C and D.

Antisera raised to three common surface proteins (Figure 14-strain 2, band B; strain 5, bands C and D) were used to simultaneously probe eight *M. ovipneumoniae* strains, one *M. arginini* strain and one *A. laidlawii* strain. Lanes 1 to 8: *M. ovipneumoniae* strains 1, 2, 4, 5, 10, 37E, MPP74 and L3/C3; Lane 9: *M. arginini* strain M30/C4; Lane 10: *A. laidlawii* (ATCC 23206).

Note that antisera raised to bands C and D are species specific in the sense that they reacted with all eight *M. ovipneumoniae* isolates but did not react with either of the other two organisms. The antisera raised to band B did not react with *M. arginini* or with *A. laidlawii*. However, band B is specific for a "sub-group" of *M. ovipneumoniae* i.e. it reacted with five of the eight *M. ovipneumoniae* isolates.


D

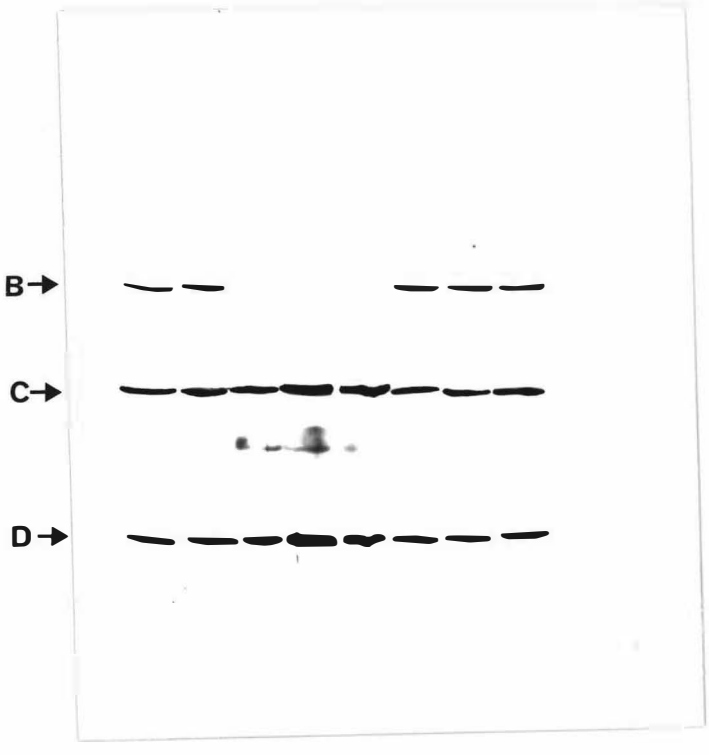

B
C
D

Figure 22

Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band E.

Antiserum raised to a surface protein apparently present (SDS-PAGE) in only two of eight strains (Figure 14 - strains 1 and 37E, band E) was used to probe the eight *M. ovipneumoniae* isolates by "immunoblotting". Lane 1: strain 1; Lane 2: strain 2; Lane 3: strain 4; Lane 4: strain 5; Lane 5: strain 10; Lane 6: strain 37E; Lane 7: strain MPP74; Lane 8: strain L3/C3.

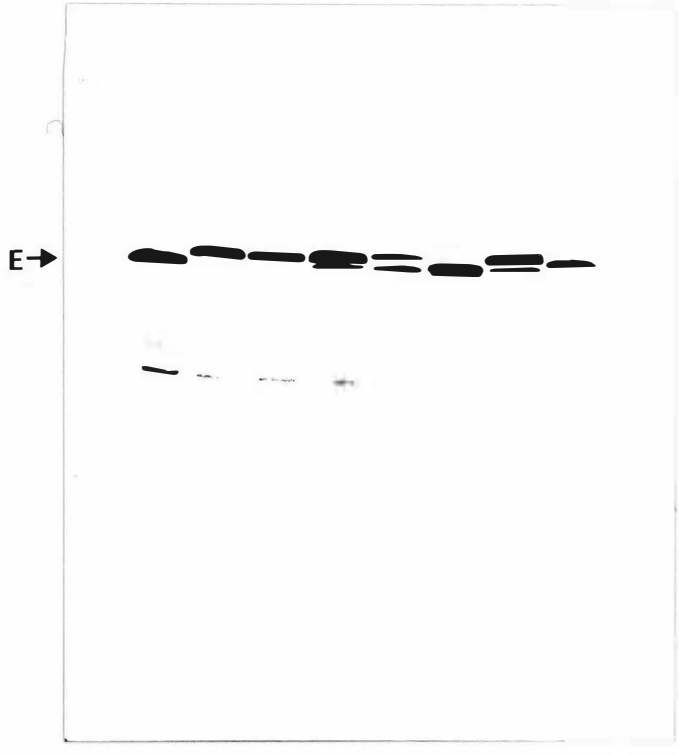
Note that, as expected, this antiserum reacted with strains 1 and 37E (lanes 1 and 6), but it also reacted with one or two bands of the remaining strains. However, in these cases, one of the bands represented a protein of slightly higher molecular weight. Band E therefore represents at least two proteins, one or both of which are present in all isolates.

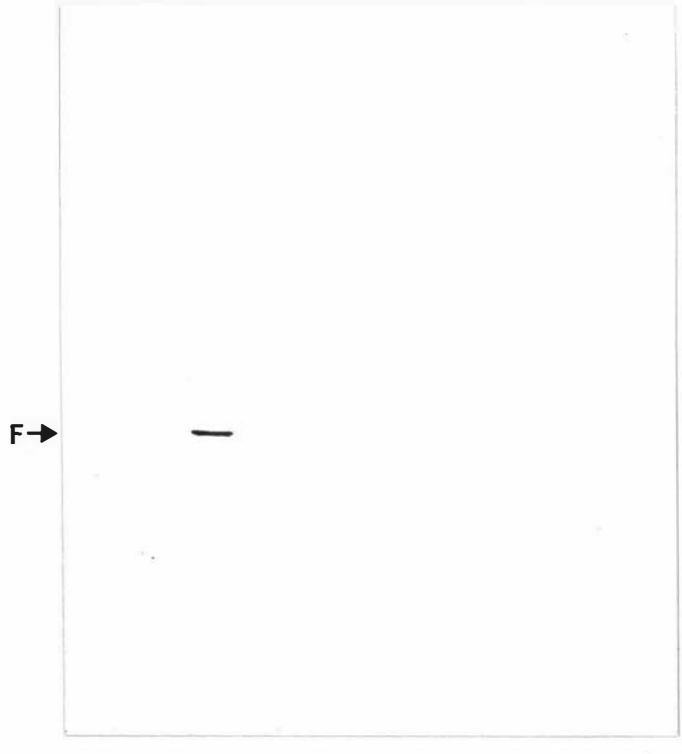
Figure 23

Eight *M. ovipneumoniae* isolates "blotted" with antiserum raised to band F.

Antiserum raised to an apparently unique surface protein (Figure 14 - strain 2, band F) was used to probe eight *M. ovipneumoniae* isolates by "immunoblotting". Lane 1: strain 1; Lane 2: strain 2; Lane 3: strain 4; Lane 4: strain 5; Lane 5: strain 10; Lane 6: strain 37E; Lane 7: strain MPP74; Lane 8: strain L3/C3.

Note that the antiserum raised to this band only reacted with the homologous strain (lane 2).


E


F

B3.4 Discussion.

SDS-PAGE allows the proteins of *M. ovipneumoniae* strains to be separated in an acrylamide gel according to their molecular weight. However, protein bands of equal molecular weight in different isolates are not necessarily the same proteins. So in this section of work we examined the serological relationship of several surface proteins and concluded that:

a) Antiserum to one surface protein (Figure 14, band B), apparently common to all *M. ovipneumoniae* isolates, was found to react with only five of the eight strains tested (Figure 18).

b) Two surface proteins (Figure 14, bands C and D) are common to all of the isolates tested. However, antisera to these bands did not react with other species (Figure 21) i.e. they are species-specific bands.

c) A surface protein band (Figure 14, band E) which was apparently shared by two strains was found to represent at least two different proteins (Figure 22). These proteins were present in the remaining six *M. ovipneumoniae* strains but, although serologically related, they differed slightly in molecular weight.

d) One apparently unique surface protein (Figure 14, band F) was indeed present in only one of the eight *M. ovipneumoniae* strains tested (Figure 23).

The possible application of these conclusions in distinguishing *M. ovipneumoniae* isolates is considered in the general discussion.

CHAPTER FOUR

Investigation of the Heterogeneity of *M. ovipneumoniae* Isolates using Various Restriction Endonucleases Including a Comparison of Multiple Isolates from Individual Pneumonic Lungs.

4.1 Introduction.

In a preliminary study (Mew *et al*, 1985) we showed that the BRENDA patterns of eight *M. ovipneumoniae* isolates were totally different (no common bands) when *Eco*R1 was used as the restriction enzyme. This contrasts with the findings for some other mycoplasma species. Razin *et al*, (1983) reported that *M. pneumoniae* isolates were homogeneous. The possibility (perhaps a small one) exists that the heterogeneity seen in *M. ovipneumoniae* could be demonstrated only by *Eco*R1. To investigate this possibility we examined the BRENDA patterns of *M. ovipneumoniae* using several restriction endonucleases.

The result (see below) of the above investigation confirmed the heterogeneity of *M. ovipneumoniae* DNA and in a parallel study, a colleague (Norman, 1985) examined the DNA from sixty isolates, which I recovered from sheep on twenty farms. Of these sixty isolates, fifty-eight were totally different in the BRENDA pattern and also had different protein banding patterns as shown by SDS-PAGE. Only three isolates (from sheep on one farm) were identical. The heterogeneity seemed to be remarkably high and implies that many different strains of *M. ovipneumoniae* must be maintained within all flocks of sheep. The sixty isolates came from pneumonic lungs and thus they did not represent differences between "nasal" and "lung" isolates. It occurred to us that with such heterogeneity it might be possible that lungs are colonized by more than one strain of *M. ovipneumoniae*. To investigate this, we examined multiple isolates of *M. ovipneumoniae* from each of six pneumonic sheep lungs. The opportunity to extend this to one pneumonic goat lung presented itself and is also included here.

4.2 Materials and Methods.

4.2.1 Materials

i. For Propagation and Preparation of *M. ovipneumoniae* Isolates for DNA Extraction.

1)**FM4 Medium**: see appendix

2)**FM4 Agar**: see appendix

3)**Phosphate Buffered Saline** (PBS): see appendix

4)**Tris-EDTA Solution**: see appendix

ii. For DNA Extraction.

1)**Sodium Dodecyl Sulphate** (SDS) 10%

SDS (Sigma)	10.0g
Distilled Water to	100.0ml

Stored at room temperature without autoclaving.

2)**Pronase Type XIV** 10mg/ml

Pronase (Sigma)	0.2g
Distilled WAter to	20.0ml

This solution was preincubated at 37^{o}C for three hours to self-digest contaminants, especially DNase activity. This was then stored at -20^{o}C.

3)**RNase** (Ribonuclease I) 2mg/ml

RNase-from bovine pancreas (Sigma)	0.02g
Distilled Water to	10.0ml

This solution was preincubated at 90^{o}C for 10mins to destroy DNase activity. It was then stored at -20^{o}C.

4)**5.0M NaCl**

NaCl	29.22g
Distilled Water to	100.0ml

5)**Saline Tris-EDTA (STE) Buffer**: see appendix

6)**Phenol/Chloroform/Iso-amyl Alcohol**

A 25:24:1 solution, respectively, was prepared.

7)**0.001M EDTA**

EDTA	0.37g
Distilled Water to	1000.0ml

8)**Dialysis Tubing**

10mm flat width dialysis tubing was cut to approximately 35cm lengths and boiled in 5% sodium carbonate solution. This process was repeated until no colour or odour could be detected. The tubing was then boiled in distilled water once; in 0.001M EDTA once; washed in distilled water and boiled in distilled water. The dialysis tubing in the distilled water was allowed to cool before being stored at $4^{o}C$.

9)**5M Sodium Perchlorate**

Sodium Perchlorate	61.22g
Distilled Water to	100.0ml

iii. For Spectrophotometric Determination of the Purity and Concentration of the DNA.

1)**TE Buffer**: see appendix

Iv. For the Digestion of DNA with *Eco*R1.

1)***Eco*R1** (BRL^{TM}) 10 units/ul

Stored at $-20^{o}C$

2)***Eco*R1 Buffer** (5x Concentrated)

1M Tris-HCL (pH 7.5)	50.0ml
5M NaCl	5.0ml
1M $MgCl_2$	2.5ml
Bovine Serum Albumin (Fraction V)	50.0mg
Distilled Water to	100.0ml

Stored at $4^{o}C$.

Note:

A total of sixteen different restriction endonucleases were used in this section. Buffers used with these different restriction enzymes to digest mycoplasma DNA were supplied by the manufacturer.

3)**Tris-EDTA** (TE Buffer) + 0.05% SDS + 20% Glycerol

1.0M Tris-HCl (pH 7.5)	0.1ml
0.2M EDTA (disodium salt) (pH 7.2)	0.05ml
Glycerol	2.0ml
10% Sodium Dodecyl Sulphate	0.05ml
Distilled Water to	10.0ml

Stored at room temperature.

4)**Bromophenol Blue Dye** (10x concentration)

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

Stored at room temperature.

v. For Gel Electrophoresis.

1)**Electrophoresis (E) Buffer** (10x concentrated)

Trizma Base (Sigma)	96.88g
EDTA (disodium salt)	7.44g
Sodium Acetate	8.20g
Glacial Acetic Acid to pH 7.8	
Distilled Water to	2000.0ml

Stored at 4^{o}C and diluted with distilled water when required.

2)**Ethidium Bromide Solution** (10mg/ml)

Ethidium Bromide	100.0mg
Distilled Water to	10.0ml

Stored in a dark bottle at 4^{o}C.

3)**Ultrapure Electrophoresis Grade Agarose** (Bio-Rad^{TM})

4.2.2 Methods

A) Examining the Heterogeneity of *M. ovipneumoniae* Isolates from Sheep on Different Farms by using Restriction Endonucleases.

4.2.2A.i Propagation and Preparation of *M. ovipneumoniae* Isolates for DNA Extraction.

A 1.5ml aliquot of thrice cloned *M. ovipneumoniae* culture stored at -70^{o}C was thawed and inoculated into 300ml of FM4 Medium. The culture was placed on a rotary shaker* set at 125rpm and incubated at 37^{o}C until the reaction of the medium reached approximately pH 6.8 (at this pH *M. ovipneumoniae* cells are still in the exponential growth phase).

The 300ml culture of *M. ovipneumoniae* cells was centrifuged at 15 500g for 20mins, the supernatant was discarded and the pellet was resuspended in 30ml of sterile PBS. The cells were recentrifuged and the deposit was resuspended in 1.5ml of Tris-EDTA Solution. 0.5ml

of this suspension was used in a gel precipitin test (to ensure that the culture was *M. ovipneumoniae*). The remaining 1.0ml cell suspension was placed in a 12ml Nalgene^{TM} centrifuge tube and then stored at -70^{o}C or used immediately for DNA extraction.

*Note:

In an earlier study Mew(1982) showed that *M. ovipneumoniae* cultures grew more rapidly and gave a better yield when the cultures were incubated on a rotary shaker than incubated stationary.

4.2.2A.ii DNA Extraction.

Pelleted preparations (see above) of *M. ovipneumoniae* isolates suspended in 1ml of Tris-EDTA Solution and stored at -70^{o}C were thawed in a 37^{o}C waterbath. 0.1ml of SDS (10%) and 0.1ml of Pronase Type XIV (10mg/ml) were added to each isolate and incubated at 50^{o}C overnight to ensure that all the cells were lysed and that their proteins were digested. 0.1ml of RNase was added to the lysate and incubated for 60mins to digest the RNA. An aliquot of Sodium Perchlorate was then added to give a final concentration of 1M and the lysate was incubated at 50^{o}C for a further 60mins.

Phenol was melted in a 70^{o}C waterbath and added to chloroform and iso-amyl alcohol to give a ratio of 25:24:1. This solution was mixed with one tenth its volume of STE Buffer. Air was bubbled through the preparation to facilitate mixing. The solution was cooled to room temperature and used to deproteinate the lysate.

An equal volume of Phenol/Chloroform/Iso-amyl alcohol solution was added to the lysate, mixed by repetitively inverting the centrifuge tube which was then left for 5mins until the interface reformed and the mixture was then centrifuged at 5 000g for 10mins at room temperature. The upper aqueous layer, containing the DNA, was removed using a 5ml serological pipette, placed into another centrifuge tube and the DNA was re-extracted twice more with the phenol/chloroform/iso-amyl alcohol solution. The DNA was then added to dialysis tube and dialysed at 4^{o}C against 8 litres (total 32 litres) of TE Buffer with three changes over a 48hr period. The DNA was then removed from the dialysis tubing and placed into a sterile bijou bottle and stores at 4^{o}C.

4.2.2A.iii Spectrophotometric Determination of the Purity and Concentration of the DNA.

0.1ml of DNA was diluted with 1.9ml of TE Buffer, gently mixed so as not to shear the DNA and placed into quartz cuvette and the optical density was measured using a CE 599 Cecil™ automatic scanning spectrophotometer which was zeroed against the dialysis buffer (*viz* TE Buffer).

Readings were taken at 230nm, 258nm, 260nm, 270nm, 280nm and 300nm. Spectral ratios 260/230 = 1.8 to 2.5 and 260/280 = 1.8 to 2.0 were regarded as satisfactory. If the purity was unsatisfactory the DNA was re-extracted as previously described. If the absorbance at 270nm was greater than at 260nm this indicated phenol contamination of the DNA so the sample was re-dialysed and re-assayed.

The concentration of the DNA was determined as described by Brenner and Falkow (1971). i.e.

$$\text{Concentration of DNA (mg/ml)} = \frac{OD_{258nm} - OD_{300nm}}{20} \times \text{Dilution Factor}$$

A typical spectrophotometric profile is shown below:

Wavelength (nm)	230	258	260	270	280	300	$\frac{260}{280}$	$\frac{260}{230}$
M. ovipneumoniae L4l3 (OD units)	0.335	0.820	0.818	0.672	0.415	0.032	1.98	2.45

Therefore the DNA concentration of *M. ovipneumoniae* isolate L4l3 diluted 1/20 is:

$$\frac{0.820 - 0.032 \times 20}{20} = 0.788\text{mg/ml}$$

4.2.2A.iv Digestion of DNA with *Eco*R1.

An aliquot of DNA calculated to contain 7.5ug was added to a 1.5ml Eppendorf microfuge tube containing 20ul of 5x *Eco*RI Buffer. This mixture was diluted with distilled water to

100ul, then 5ul (50 units) of *Eco*RI restriction enzyme was added to the reaction mixture and mixed gently. The eppendorf tube was pulsed on the microfuge to assure that all of the reaction mixture was at the base of the tube and incubated at 37^{o}C for 60mins. The digestion was terminated by heating the mixture at 65^{o}C for 10mins. The digested DNA was left to cool to room temperature then a 1/20th of the reaction mixture volume of 5M NaCl was added, followed by two volumes of cold (-20^{o}C) absolute ethanol. The tubes were inverted to mix the contents and placed at -20^{o}C for 30mins (or overnight) to precipitate the DNA.

The DNA was sedimented in a microfuge at 15 500g for 25mins to ensure that the pellet was firmly attatched to the base of the tube. The supernatant was discarded and 1ml of cold (-20^{o}C) absolute ethanol was added to wash the surface of the pellet and quickly discarded. The pellet was dried under vacuum for 15-20mins in a dessicator.

The dried pellet was re-suspended in 35ul of Tris-EDTA Buffer + 0.05% SDS + 20% Glycerol and 5ul of Bromophenol Blue Dye. The mixture was microfuged at 15 500g for 5mins to sediment any debris and the supernatant was used for electrophoresis.

4.2.2A.v Gel Electrophoresis.

The gel apparatus seen in Figure 24 was used to form the agarose gel and for the electrophoresis of the DNA samples. 75ul of Ethidium Bromide Solution* was added to 150ml of E Buffer and diluted to 1500ml with distilled water (giving a final concentration of 0.5ug/ml of ethidium bromide). 100ml was added to 0.7g of agarose** in a 250ml round bottomed Quick Fit™ flask fitted with a reflux condenser and boiled for 5mins to solubilize the agarose.

The agarose was cooled to approximately 40^{o}C and poured onto a levelled glass plate (20cm x 15cm) which was edged with adhesive tape to form a wall. A perspex "comb" was placed approximately 1cm from one end of the glass plate to form wells in the agarose and the gel was allowed to solidify for 20-30mins. The "comb" was extracted (forming the wells), the glass plate was taken from the electrophoresis apparatus and the adhesive tape was removed. This left a gel slab of 20cm x 15cm x 0.5cm adhering to the glass plate. This was then placed on the electrophoresis apparatus and submerged by filling the tank compartments with E Buffer (used in the preparation of the agarose gel) to a height approximately 2-3mm above the gel.

Figure 24

Preparation of agarose gels for bacterial restriction endonuclease DNA analysis.

a) A glass plate (20cm x 15cm) was edged with adhesive tape to form a wall.

b) The glass plate was placed into the electrophoresis tank.

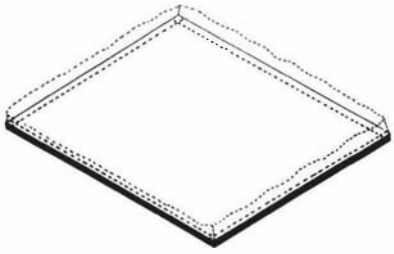
c) Agarose at the desired concentration (0.7% to 1.6%) was added to TE buffer and boiled for 5mins to dissolve. The agarose was allowed to cool to about 50^{o}C before it was poured onto the glass plate edged with adhesive tape. A "comb" used to cast wells was also inserted into the agarose solution which was then left to solidify.

d) The "comb" was extracted leaving the wells.

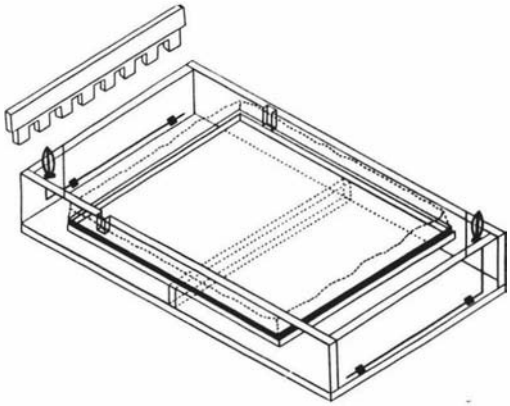
e) The glass plate with the agarose gel was removed from the apparatus and the adhesive tape was removed.

f) The glass plate with the gel was returned to the electrophoresis apparatus and TE buffer was added to submerge the gel. The DNA samples were added to the wells and electrophoresed through the gel for approximately 6hrs at 80 volts.

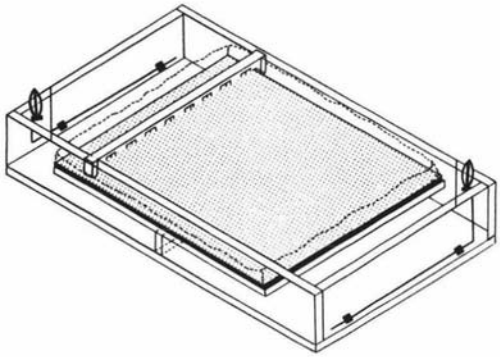
a



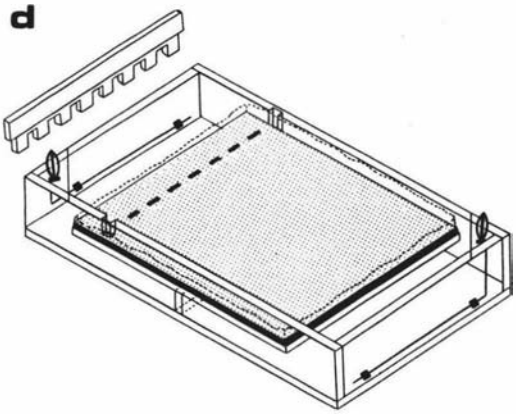
b



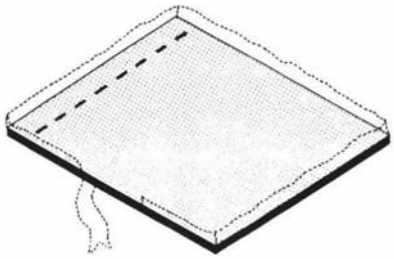
c



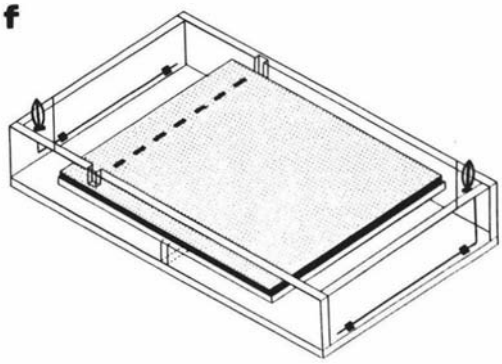
d



e



f



The DNA samples were added to the wells beneath the E Buffer and settled to the base of the wells due to the presence of glycerol in the DNA sample. Electrophoresis was carried out at 4 volts/cm until the Bromophenol Blue Dye had travelled about 16cm from the wells, this took approximately 5 to 6 hours.

* Note:

Because the gel bed was large it could be easily broken in a standard staining process. To avoid this ethidium bromide was added to the E Buffer before casting the gel. For safty reasons this procedure was carried out in a fume hood.

** Note:

0.7% agarose gel concentrations was used for all restriction endonucleases which recognized six-base sequences. However, restriction endonucleases which recognize four-base sequences, agarose gel concentrations were varied from 1.2% to 1.6% to give the best resolution and hence the maximum number of cleavage patterns.

4.2.2A.vI Photographing the Gel.

When the electrophoresis was completed the agarose gel was placed in a tray of distilled water to wash off excess ethidium bromide. The gel was then removed by tilting one end of the glass plate which caused it to slide onto a UV transilluminator filter.

The gel was photographed through a Wratten^{TM} 23A gelatin filter on Kodak^{TM} Tri-X 12.5cm x 10.0cm size film.

The film was exposed for 30secs at f/11* and developed for 5mins in D-19 Kodak^{TM} developer; 60secs in a stop bath solution (3% Acetic Acid); 5mins in Ilford^{TM} Rapid Fixer with hardener added; washed for 10mins in excess water and hung to drip dry.

* Note:

A focal ratio of f/11 was used because it has a greater depth of focus and thus DNA cleavage bands through the thickness of the gel will remain in focus.

B. Isolation of Multiple *M. ovipneumoniae* Isolates from Six Pneumonic Sheep and One Pneumonic Goat Lung.

Three different methods were used for the isolation of multiple *M. ovipneumoniae* isolates from each of six pneumonic sheep lungs and from one pneumonic goat lung. One of the three isolation methods was described in section C2.2.2.i, however it is briefly repeated here:

4.2.2B.I "Normal" Method of *M. ovipneumoniae* Isolation.

Infected lung tissue was cut with scissors into small pieces i.e. about 3-4mm^3, placed in 3ml of FM4 medium and mixed thoroughly for 60secs using a vortex mixer. A 0.3ml aliquot was removed and added to another bottle containing 2.7ml of FM4 medium. This was incubated at 37^{o}C until a pH (colour) change was detected. This isolation procedure was repeated ten times with fresh lung material. This gave ten independant isolates.

4.2.2B.ii Isolation of *M. ovipneumoniae* Isolates Directly from FM4 Agar Plates and by Limit Dilution.

Approximately 1g of pneumonic lung tissue exhibiting CNP lesions was placed in a sterile mortar. About 2g of sterile sand was added and the lung tissue was ground with the slow addition of 9ml FM4 Medium to give a homogenized suspension, regarded as a 10^{-1} dilution of the original lung. The sand settled rapidly and some of the supernatant was removed with a pipette.

Ten 0.3ml aliquots were used to prepare ten-fold serial dilutions in FM4 Medium from 10^{-2} to 10^{-10}. 100ul from each of (ten) the 10^{-2}, 10^{-3} and 10^{-4} dilutions were spotted onto FM4 agar plates. The plates were incubated at 37^{o}C and examined for colonies from 3 to 7 days after inoculation. Well spaced colonies (usually from the 10^{-4} dilution) were picked of with a plug of agar using a pasteur pipette under 40x magnification. At least one colony was picked from each of the ten separate dilution series.

For the limit dilution method the original dilution series was incubated and examined daily for a colour change. The greatest dilution of each of the ten parallel titrations which showed a colour change was selected and the isolate from each was cloned.

4.2.2B.iii Cloning of Isolates.

All thirty isolates obtained from each lung i.e. ten isolates derived by "normal" isolation techniques, ten from FM4 agar and ten obtained by limit dilution were each cloned three times as described in section C2.2.2.i.

4.2.2B.Iv Identification of Multiple Isolates Obtained from Individual Lungs as *M. ovipneumoniae*.

All isolates were identified as *M. ovipneumoniae* in a gel precipitin test using antisera raised against *M. ovipneumoniae* (strain 5).

4.3 Results.

A) The Heterogeneity of *M. ovipneumoniae* Isolates from Sheep on Different Farms Examined by using Four Restriction Endonucleases.

The DNA from eight isolates of *M. ovipneumoniae* from sheep on different farms were digested with each of four different restriction endonucleases and electrophoresed in a 0.7% agarose gel. The results are shown as follows: Figure 25, *Eco*RI; Figure 26, *Dra*I; Figure 27, *Hpa*I; Figure 28, *Msp*I.

Note:

1) Each of the eight strains give a unique pattern with each of the four restriction endonucleases.

2) The pattern is unique, not just in the sense that each is different but in the sense that no bands common to all eight strains were detected with any restriction endonuclease.

3) In Figure 27 a high molecular weight band is shared by three of the eight strains (i.e. strains 1, 10 and 37E). This is further investigated and discussed in the next chapter.

B) The Heterogeneity of Isolates Recovered from Individual Lungs of Six Sheep and a Goat.

From each of six pneumonic sheep lungs, ten isolates of *M. ovipneumoniae* were obtained by the "normal" method, ten from material directly plated on FM4 agar and ten by simultaneous limit dilution titrations of the original lung (see materials and methods). The results from one of the six lungs are illustrated in Figures 29a, b and c. Figure 29a shows ten isolates derived by the normal isolation procedure. Note that nine isolates are identical or almost identical (pattern P) whereas one isolate (lane 8) is markedly different (pattern Q).

Figure 29b shows ten isolates derived from ten original colonies on FM4 agar. Note that lanes 3, 5, 7, 8 and 9 (lane 8 differs slightly from the others) show one general pattern Q. Lanes 1, 2, 4, 6 and 10 show a different pattern (i.e. pattern R).

The final ten isolates (Figure 29c) obtained by limit dilution also showed two general patterns: Lanes 3, 5 and 7 (i.e. pattern S) and lanes 1, 2, 4, 6, 8, 9 and 10 (i.e. pattern R). The latter patterns are similar to one of the patterns seen in Figure 29b, while the former pattern (S) is different. To facilitate the comparisons, this will be further illustrated later (see Figure 35). Thus, of the thirty isolates, we obtained four different DNA restriction patterns using *Eco*RI, however, small variations are detected within each of these four patterns. These results (from lung 6) are tabulated at the bottom of Table V using the following convention: Each of the four basic patterns was given a letter e.g. P, Q, R and S for lung 6. However, minor differences (one or a few bands only) occurred within each pattern. Thus, nine isolates had pattern P but, within this, four slight variations were seen. One variation was represented by four isolates, one by two isolates, another by two isolates and one by one isolate (total nine).

It is difficult to compare restriction patterns in different gels so it would not therefore be useful to show the digests of all 180 isolates. However, the results for all six lungs are tabulated in Table V.

Table V: The Number of *M. ovipneumoniae* Strains and Variants Found in Six Sheep Lungs.

Lung Nº	Pattern	Variant Types 1	2	3	4
1	A	27	1	1	
	B	1			
2	C	25			
	D	5			
3	E	14	9		
	F	3	1		
	G	2			
	H	1			
4	I	8	3	1	
	J	8	2		
	K	4	2		
	L	2			
5	M	18	4	3	2
	N	2			
	O	1			
6	P	4	2	2	1
	Q	3	2	1	
	R	11	1		
	S	3			

The major points in Table V (above) are that two different patterns (A and B) were seen in lung 1; two (C and D) in lung 2; four (E, F, G and H) in lung 3; four (I, J, K and L) in lung 4; three (M, N, O) in lung 5 and four (P, Q, R and S) in lung 6. To illustrate these differences, one example of each of the different restriction patterns from each lung, were re-examined within one gel (see Figures 30, 31, 32, 33, 34 and 35). These results confirm the above conclusions.

To return to Table V, the minor point is that patterns have minor variants. e.g. pattern A has three variants; twenty-seven isolates were of one variant and one each of the other two. Lung 2 had two patterns (C and D). These showed no variations etc.... (see Tabel V). These minor variations are illustrated later (see Figure 48).

C) Further Investigations of the Heterogeneity of *M. ovipneumoniae* Isolates Derived Simultaneously from One Pneumonic Lung.

Lung 6 was arbitrarily chosen and from it, four isolates which gave four markedly different patterns using *Eco*RI (Figure 35) were selected. These were further examined using a number of different restriction endonucleases. The results are shown in Figure 36 through to 47 and are summarized in Table VI below.

Table VI: The Examination of Four Simultaneous Isolates, from One Lung (Lung 6), Using a Range of Restriction Enzymes.

Figure[1]	Restriction Endonuclease	Recognition Sequence[2]	Different Patterns[3]	Digestion of DNA[4]
36	*Xba*I	T^{V}CTAGA	yes	++++[5]
37	*Bgl*II	A^{V}GATCT	yes	++++
38	*Hind*III	A^{V}AGCTT	yes	++++
39	*Msp*I	C^{V}CGG	yes	++++
40	*Dra*I	TTT^{V}AAA	yes	++++
41	*Hpa*II	C^{V}CGG	yes	+±+±
42	*Kpn*I	GGTAC^{V}C	yes	±±±±
43	*Sal*I	G^{V}TCGAC	yes	±±±±
44	*Bam*HI	G^{V}GATCC	yes	±±±±
45	*Sma*I	CCC^{V}GGG	yes	±±±±
46	*Xho*I	C^{V}TCGAG	yes	±±±-
47	*Hae*III	GG^{V}CC	yes	+±+-

1) Consolidated results of Figures 36 to 47.

2) Recognition sequence is read from 5' to 3' end of DNA.

3) If "yes", the four restriction patterns remain different using the indicated endonuclease.

4) + : Complete digestion of *M. ovipneumoniae* DNA.
± : Partial digestion of *M. ovipneumoniae* DNA.
- : No digestion of *M. ovipneumoniae* DNA.

5) The row of four signs (+) refers to Figure 36 and (left to right) indicates, with *Xba* I, that the DNA from all four isolates were totally digested. Taking the bottom row as a second example Figure 47 shows that the DNA from four isolates (left to right in Figure 47) were totally digested (+); partially digested (±) (note the single high molecular-weight band in the third row from the right); totally digested (+) and totally uncleaved (-).

Note:

a) In all cases the isolates showing different patterns with *Eco*RI remained different, when other restriction endonucleases were used.

b) Partial digests were seen with some enzymes and one isolate (marked -), see the last column, failed totally to cut with *Xho*I and *Hae*III.

c) The recognition sequence given does not include data on the effects of methylation.

While these results are considered in the discussion, it should be noted that partial digests and no digestion involved restriction endonucleases with a recognition sequence containing a high GC to AT ratio. This remark is not relevant to *Msp*I which can cut both methylated and non-methylated sites (see chapter five).

D) Further Investigation of Minor DNA Variations Detected by *Eco*R1:

The minor variations in isolates from within one pneumonic lung.

The DNA from isolates from lung 6 digested with *Eco*RI were found to give four markedly different patterns and within these patterns minor variations, which require careful inspection to detect, occurred (Figure 48, see arrows). These results were obtained with *Eco*RI, so to find if they also occurred with other restriction endonucleases, one pattern (Table V, pattern P) which had four variants were examined using twelve restriction endonucleases. The results are shown in Figures 49 through 60 and are summarized in Table VII below.

Table VII: The Examination of Four Simultaneous Isolates (From Lung 6) Which Showed Only Minor Restriction Pattern Differences with *Eco*RI, Using a Range of Restriction Endonucleases.

Figure[1]	Restriction Endonuclease	Recognition Sequence[2]	Different Patterns[3]	Digestion of DNA[4]
49	*Xba*I	T^{v}CTAGA	no	++++
50	*Bgl*I	A^{v}GATCT	no	++++
51	*Hin*dIII	A^{v}AGCTT	no	++++
52	*Msp*I	C^{v}CGG	no	++++
53	*Dra*I	TTT^{v}AAA	no	++++
54	*Hpa*II	C^{v}CGG	no	++++
55	*Kpn*I	GGTAC^{v}C	no	±±±±
56	*Sal*I	G^{v}TCGAC	no	±±±±
57	*Bam*HI	G^{v}GATCC	no	±±±±
58	*Sma*I	CCC^{v}GGG	no	±±±±
59	*Xho*I	C^{v}TCGAG	no	±±±±
60	*Hae*III	GG^{v}CC	no	++++

1) Consolidated results of Figures 49 to 60.

2) Recognition sequence is read from 5' to 3' end of DNA.

3) If "no", the four restriction patterns are indistinguishable.

4) + : Complete digestion of *M. ovipneumoniae* DNA.
± : Partial digestion of *M. ovipneumoniae* DNA.
- : No digestion of *M. ovipneumoniae* DNA.

Note:

a) The isolates showing minor restriction pattern differences (i.e. one to three bands) with *Eco*RI, showed identical patterns when any of the other restriction endonucleases were used.

b) Partial digests were seen with some restriction endonucleases. This is discussed in the next chapter (five).

c) The recognition sequence given does not include data on the effects of methylation.

E) A Comparison of the Total Proteins of *M. ovipneumoniae* Isolated from Six Pneumonic Lungs using SDS-PAGE.

180 isolates, i.e. thirty isolates from each of six lungs, were examined by SDS-PAGE. The results are summarized below but are illustrated in full for one lung only in Figures 61a, b and c.

1) Variation in SDS-PAGE patterns of multiple isolates from one lung were seen in five of the six cases. This is illustrated using all the isolates from one representative pneumonic lung i.e. lung 6 (Figures 61a, b and 61c) which shows four different patterns.

2) The differences referred to, although unequivocal, were nevertheless small and in general were smaller than the SDS-PAGE differences, when isolates from sheep on different farms are compared (see chapter two).

3) The differences in SDS-PAGE patterns correlated well, but not perfectly, with the BRENDA patterns e.g. the protein patterns in Figure 61b, lanes 1, 2, 4, 6 and 10 had one pattern while lanes 3, 5, 7, 8 and 9 had another slightly different protein pattern. These correlate with the major differences seen in the BRENDA patterns (Figure 29b) in which lanes 1, 2, 4, 6 and 10 had one pattern, while lanes 3, 5, 7, 8 and 9 had the second pattern. However, isolates which had identical protein patterns (Figure 61a, lanes 1 to 10, including lane 8), did not necessarily have identical BRENDA patterns. For example, compare Figure 61a (10 identical isolates by SDS-PAGE) with Figure 29a, which shows two patterns i.e. lanes 1 to 7 and 9 to 10 (one pattern) and lane 8 (a second pattern).

Since it is difficult to compare results between gels, the correlation of the SDS-PAGE and BRENDA results was confirmed for isolates from all six pneumonic lungs by taking just one of each of the major restriction endonuclease differences and comparing these isolates by SDS-PAGE. The different SDS-PAGE protein patterns of isolates from any one lung are shown for lungs 1 to 6 in Figures 62 to 67 respectively. Note that in lung 1 (Figure 62), both SDS-PAGE protein patterns are identical, while their DNA restriction endonuclease patterns were markedly different (c.f. Figure 30). Lung 2 shows minor variations in the SDS-PAGE protein pattern (Figure 63, see arrows), compared with the markedly different DNA patterns (Figure 31). Lung 3 showed one to two protein band differences in the first three isolates

(Figure 64), but the last isolate (far right lane) has an identical protein pattern with the third lane. Lung 4 showed the greatest differences in the protein patterns within one lung (Figure 65, bands arrowed), all four lanes showed protein band differences in the high, middle and low molecular weight range. Lung 5 (Figure 66) shows that three isolates which gave totally different DNA patterns (Figure 34) give only minor protein band differences (Figure 66). Lung 6 (Figure 67), which shows four isolates with different DNA patterns, gave three SDS-PAGE patterns. Lanes 1 and 2 are identical whereas the remaining lanes have a slightly different protein banding pattern.

The examination of the heterogeneity of multiple *M. ovipneumoniae* isolates recovered from six individual pneumonic sheep lungs by BRENDA and SDS-PAGE was extended to include the examination of a single pneumonic goat lung. The isolates of mycoplasmas from the pneumonic goat lung was as described for the recovery of multiple *M. ovipneumoniae* isolates from within one pneumonic sheep lung (see Methods, sections 4.2.2B.i and 4.2.2B.ii).

All goat isolates were identified as *M. ovipneumoniae* in a gel precipitin test using antisera raised to a standard *M. ovipneumoniae* strain (Figure 68). Also, one goat isolate (GT10) was arbitrarily chosen and electrophoresed in a 10% SDS-PAGE gel. Its protein banding pattern was compared with standard strains of *M. ovipneumoniae* (strains 1, 5 and 10) and is shown in Figure 69. Note that the goat isolate (GT10) had most of its protein bands in common with the other three *M. ovipneumoniae* strains and was no more different from sheep isolates than was one sheep isolate from another.

The thirty *M. ovipneumoniae* isolates were digested with *Eco*RI (Figures 70a, b and c). Figure 70a shows 10 isolates derived by the normal isolation procedure. Note that eight isolates have one pattern whereas two isolates (lanes 7 and 10) have a second pattern e.g. compare lanes 9 and 10 in which the higher molecular weight bands differ although common bands are seen in the lower molecular weight region.

Figure 70b shows ten isolates derived from colonies on agar. Nine show identical DNA restriction patterns, however lane 6 shows a different pattern. The nine lanes are similar to the DNA restriction patterns seen in Figure 70a, lanes 1 to 5, 7, 8, 9 and 10. Lane 6 (Figure 70b) is identical to lanes 7 and 10 in Figure 70a.

The final 10 isolates shown in Figure 70c, obtained by limit dilution, also shows two general patterns corresponding to lanes 1, 4, 7, 8 and 10 and lanes 2, 3, 5, 6 and 9. These two patterns are identical to the two patterns shown in Figures 70a and b. Thus, of the thirty pneumonic goat isolates, we obtianed two markedly different patterns (a comparison is perhaps best made by examining lanes 5 and 6, Figure 70b) and within one pattern, there were up to four different variations (one to two band differences) seen. These results from the pneumonic goat lung are tabulated in Table VIII.

Table VIII: Variations in BRENDA Patterns of Thirty *M. ovipneumoniae* Isolates Derived from One Pneumonic Goat Lung.

	Variant Types			
Pattern	1	2	3	4
A	16	4	1	1
B	8			

Note:

1) Two different patterns are seen (patterns A and B)
2) Pattern A consists of twenty-two isolates with four minor variations
3) Pattern B has eight isolates which are all identical.

SDS-PAGE examination of the thirty *M. ovipneumoniae* isolates showed only small variations which were restricted to minor bands in the high moleular weight region. The differences are at the limit of detection. However, since it was suggested that the two BRENDA patterns could be compared by examining lanes 5 and 6 in Figure 70b. The same isolates can be compared by SDS-PAGE by examining lanes 5 and 6 in Figure 71b. Small differences in the top 2 cm of the gel are seen.

Figure 25

Bacterial restriction endonuclease DNA analysis of eight *M. ovipneumoniae* strains obtained from sheep on different farms. The DNA was cleaved using *Eco*RI (5'-G^{V}AATTC-3'). Lane 1: Molecular-weight marker (i.e. lambda DNA cut with *Eco*RI); Lane 2: strain 1; Lane 3: strain 2; Lane 4: strain 4; Lane 5: strain 5; Lane 6: strain 10; Lane 7: strain 37E; Lane 8: strain MPP74; Lane 9: strain L3/C3.

Note that all *M. ovipneumoniae* strains gave totally different restriction banding patterns.

Technical Note:

The cleaved DNA was electrophoresed through a 0.8% agarose gel.

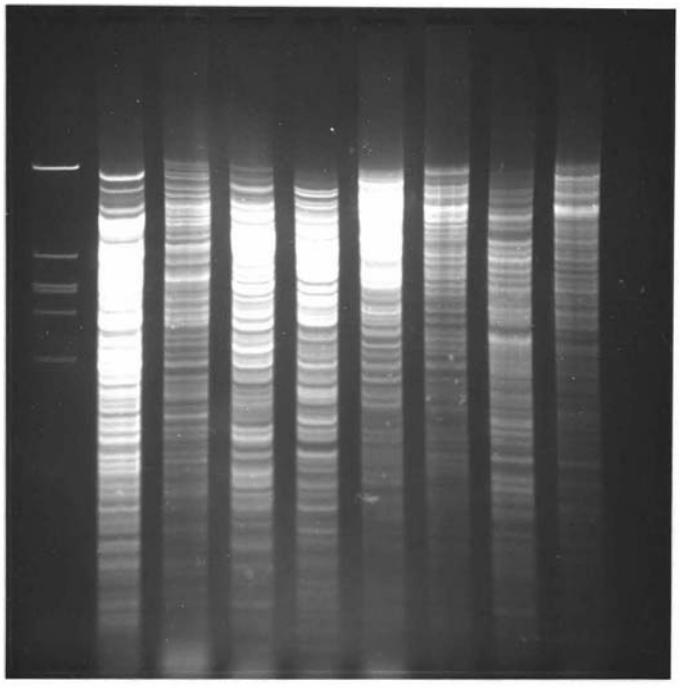
Figure 26

Bacterial restriction endonuclease DNA analysis of eight *M. ovipneumoniae* strains obtained from sheep on different farms. The DNA was cleaved using *Dra*I (5'-TTT^{V}AAA-3'). Lane 1: Molecular-weight marker (i.e. lambda DNA cut with *Dra*I); Lane 2: strain 1; Lane 3: strain 2; Lane 4: strain 4; Lane 5: strain 5; Lane 6: strain 10; Lane 7: strain 37E; Lane 8: strain MPP74; Lane 9: strain L3/C3.

Note that all *M. ovipneumoniae* strains gave totally different restriction banding patterns.

Technical Note:

The cleaved DNA was electrophoresed through a 1.6% agarose gel.



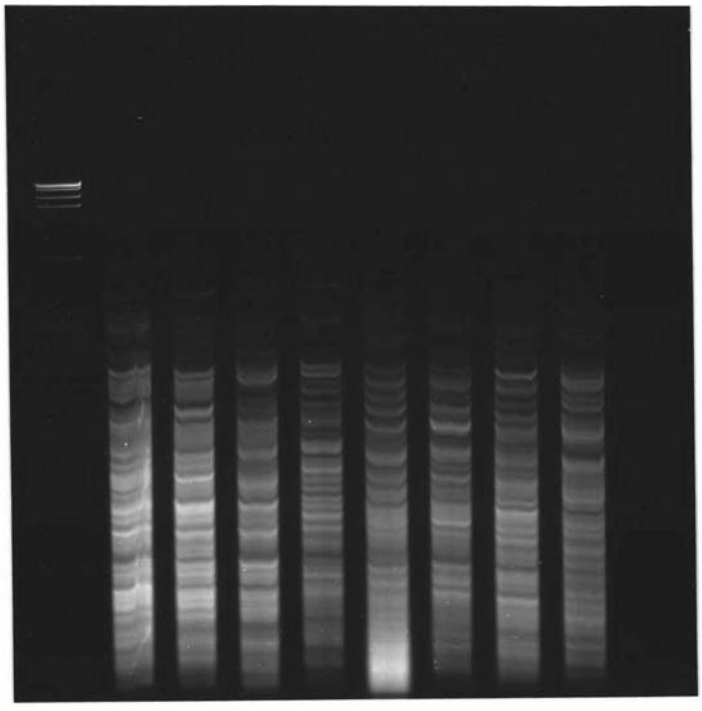


Figure 27

Bacterial restriction endonuclease DNA analysis of eight *M. ovipneumoniae* strains obtained from sheep on different farms. The DNA was cleaved using *Hpa*II (5'-C^{V}CGG-3'). Lane 1: Molecular-weight marker (i.e. lambda DNA cut with *Hpa*II); Lane 2: strain 1; Lane 3: strain 2; Lane 4: strain 4; Lane 5: strain 5; Lane 6: strain 10; Lane 7: strain 37E; Lane 8: strain MPP74; Lane 9: strain L3/C3.

Note that all *M. ovipneumoniae* strains gave totally different restriction banding patterns.

Technical Note:

The cleaved DNA was electrophoresed through a 1.6% agarose gel.

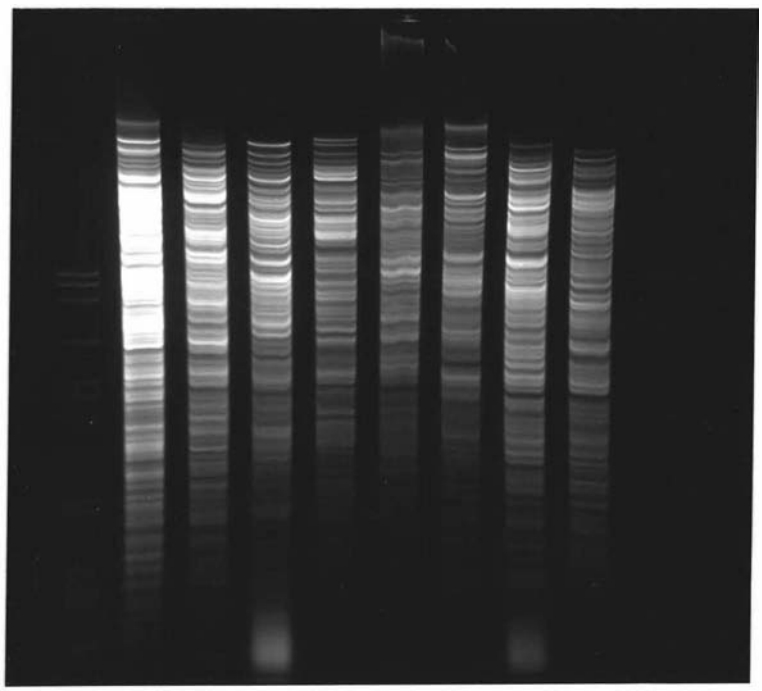
Figure 28

Bacterial restriction endonuclease DNA analysis of eight *M. ovipneumoniae* strains obtained from sheep on different farms. The DNA was cleaved using *Msp*I (5'-C^{V}CGG-3'). Lane 1: strain 1; Lane 2: strain 2; Lane 3: strain 4; Lane 4: strain 5; Lane 5: strain 10; Lane 6: strain 37E; Lane 7: strain MPP74; Lane 8: strain L3/C3.

Note that all *M. ovipneumoniae* strains gave totally different restriction banding patterns.

Technical Note:

The cleaved DNA was electrophoresed through a 1.6% agarose gel.



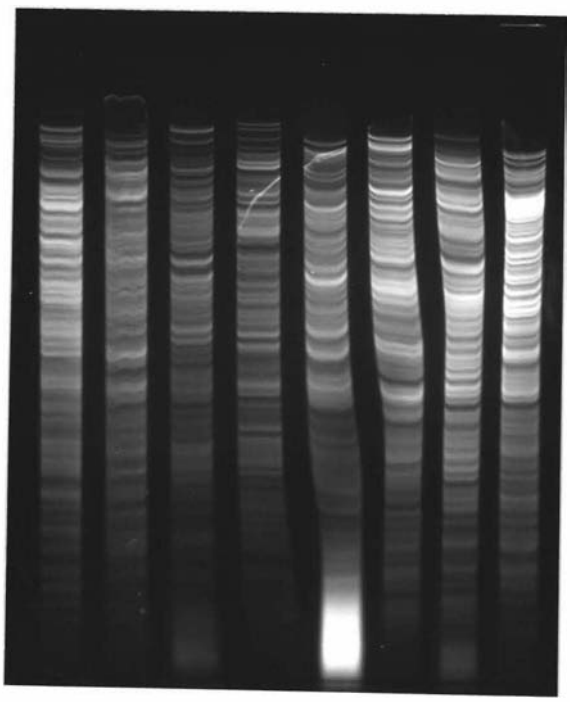


Figure 29a

Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates which were recovered simultaneously from a single pneumonic sheep lung (i.e. lung 6) by the "normal" isolation method. The DNA was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Lane 1: L6I1 (P); Lane 2: L6I2 (P); Lane 3: L6I3 (P); Lane 4: L6I4 (P); Lane 5: L6I5 (P); Lane 6: L6I6 (P); Lane 7: L6I7 (P); Lane 8: L6I8 (Q); Lane 9: L6I9 (P); Lane 10: L6I10 (P).

Note that nine isolates (designated as pattern P) are identical or almost identical, whereas one isolate (lane 8, pattern Q) has a totally different restriction banding pattern.

Figure 29b

Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates recovered from a single pneumonic sheep lung (i.e. lung 6) by selecting ten colonies derived from an inoculum of original lung material on FM4 agar. The DNA was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Lane 1: L6A1 (R); Lane 2: L6A2 (R); Lane 3: L6A3 (Q); Lane 4: L6A4 (R); Lane 5: L6A5 (Q); Lane 6: L6A6 (R); Lane 7: L6A7 (Q); Lane 8: L6A8 (Q); Lane 9: L6A9 (Q); Lane 10: L6A10 (R).

Note that two patterns are present *viz* lanes 3, 5, 7, 8 and 9 (pattern Q) which is similar to lane 8, Figure 29a and lanes 1, 2, 4, 6 and 10 (pattern R), which are different from both patterns in Figure 29a.

Figure 29c

Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates recovered from a single pneumonic sheep lung (i.e. lung 6) by limit dilution. The DNA was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Lane 1: L6T1 (R); Lane 2: L6T2 (R); Lane 3: L6T3 (S); Lane 4: L6T4 (R); Lane 5: L6T5 (S); Lane 6: L6T6 (R); Lane 7: L6T7 (S); Lane 8: L6T8 (S); Lane 9: L6T9 (S); Lane 10: L6T10 (S).

Note that two general patterns are seen i.e. lanes 1, 2, 4, 6, 8, 9 and 10 (pattern R) which are similar to one of the patterns in Figure 29b and lanes 3, 5 and 7 (pattern S), which are different to other patterns in Figure 29a and 29b. Thus, four patterns: P, Q, R and S were exhibited by the thirty isolates from lung 6.

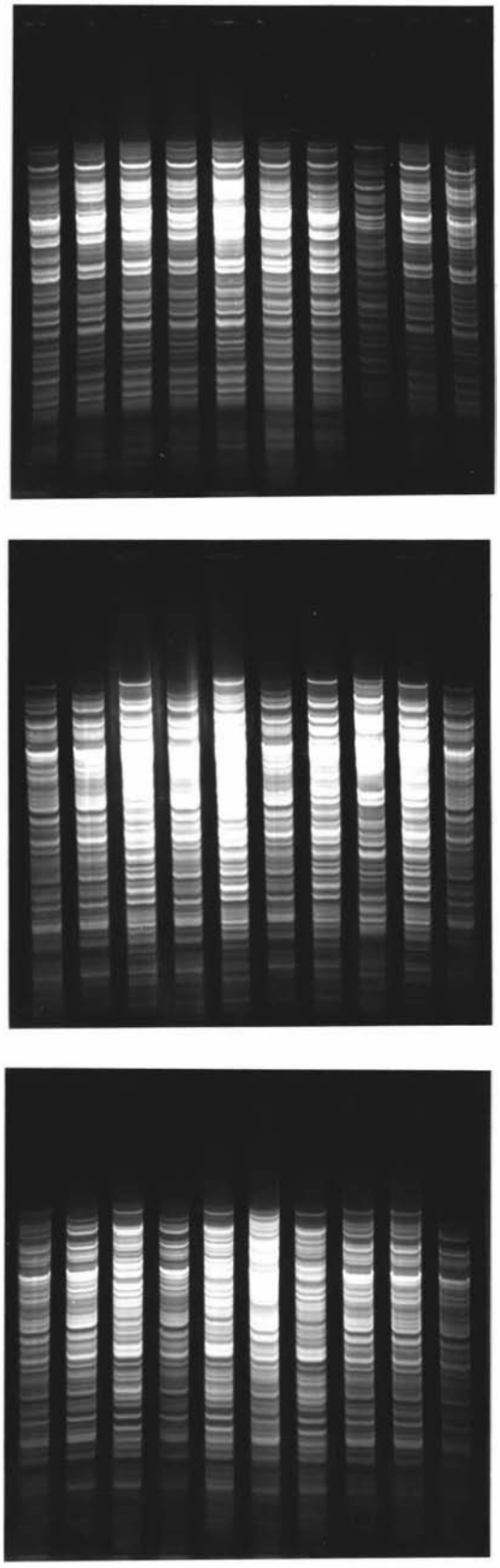
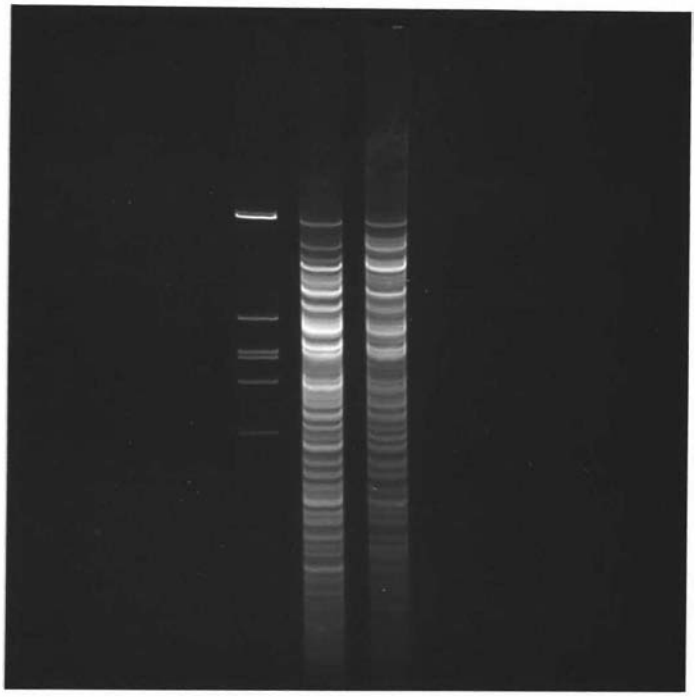


Figure 30

Bacterial restriction endonuclease DNA analysis of two *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 1). The DNA was digested with *Eco*RI (5'-G^{V}AATTC-3') and electrophoresed through a 0.7% agarose gel. Lane 1: Molecular weight marker (lambda DNA cut with *Eco*RI); Lane 2: L1A5; Lane 3: L1T2. The DNA showed marked differences in their cleavage patterns, although some common bands can be observed. The cleavage patterns for the other twenty-eight isolates were identical to one or other of the patterns shown.

Figure 31

Bacterial restriction endonuclease DNA analysis of two *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 2). The DNA was digested with *Eco*RI (5'-G^{V}AATTC-3') and electrophoresed through a 0.7% agarose gel. Lane 1: Molecular weight marker (lambda DNA cut with *Eco*RI); Lane 2: L2A5; Lane 3: L2I6. The DNA showed marked differences in their cleavage patterns, although some common bands can be observed. The cleavage patterns for the other twenty-eight isolates were identical to one or other of the patterns shown.



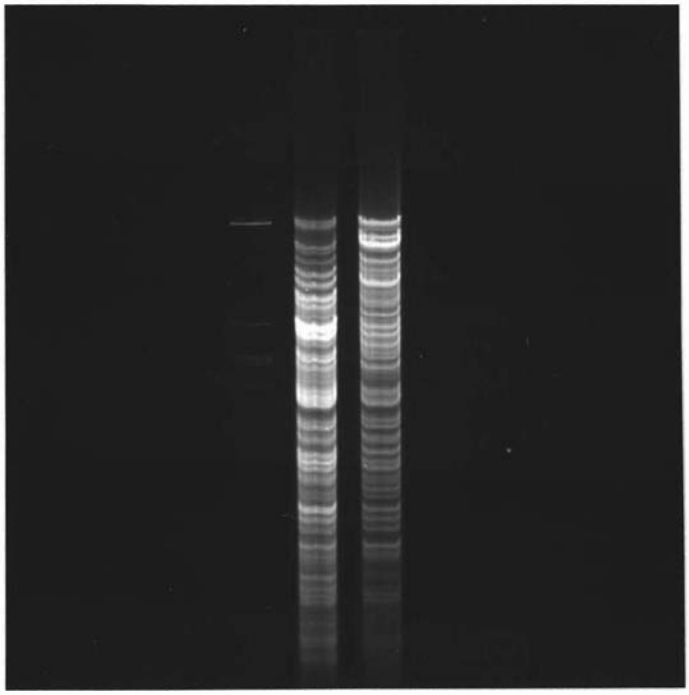
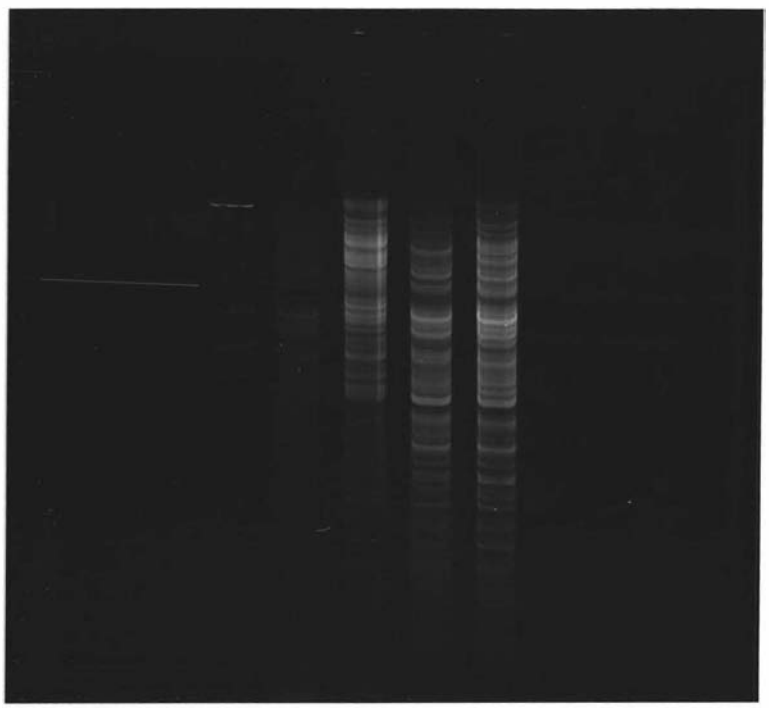


Figure 32

Bacterial restriction endonuclease DNA analysis of four *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 3). The DNA was digested with *Eco*RI (5'-G^{V}AATTC-3') and electrophoresed through a 0.7% agarose gel. Lane 1: Molecular weight marker (lambda DNA cut with *Eco*RI); Lane 2: L3T4; Lane 3: L3I6; Lane 4: L3I8; Lane 5: L3I9. The DNA showed marked differences in their cleavage patterns, although some common bands can be observed. The cleavage patterns for the other twenty-six isolates were identical to one or other of the patterns shown.

Figure 33

Bacterial restriction endonuclease DNA analysis of four *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 4). The DNA was digested with *Eco*RI (5'-G^{V}AATTC-3') and electrophoresed through a 0.7% agarose gel. Lane 1: Molecular weight marker (lambda DNA cut with *Eco*RI); Lane 2: L4I8; Lane 3: L4A5; Lane 4: L4A8; Lane 5: L4T9. The DNA showed marked differences in their cleavage patterns, including the small molecular size fragments. The cleavage patterns for the other twenty-six isolates were identical to one or other of the patterns shown.



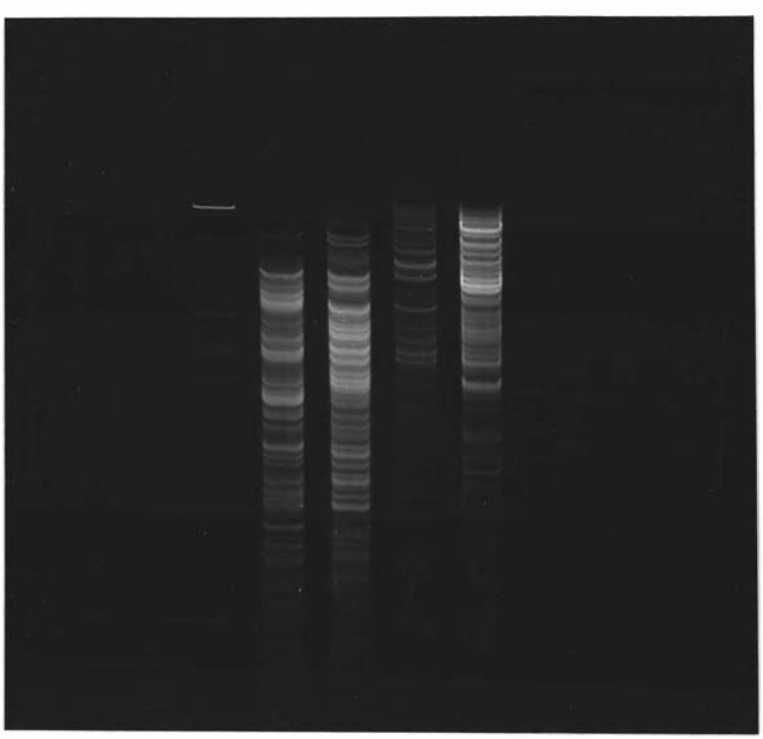
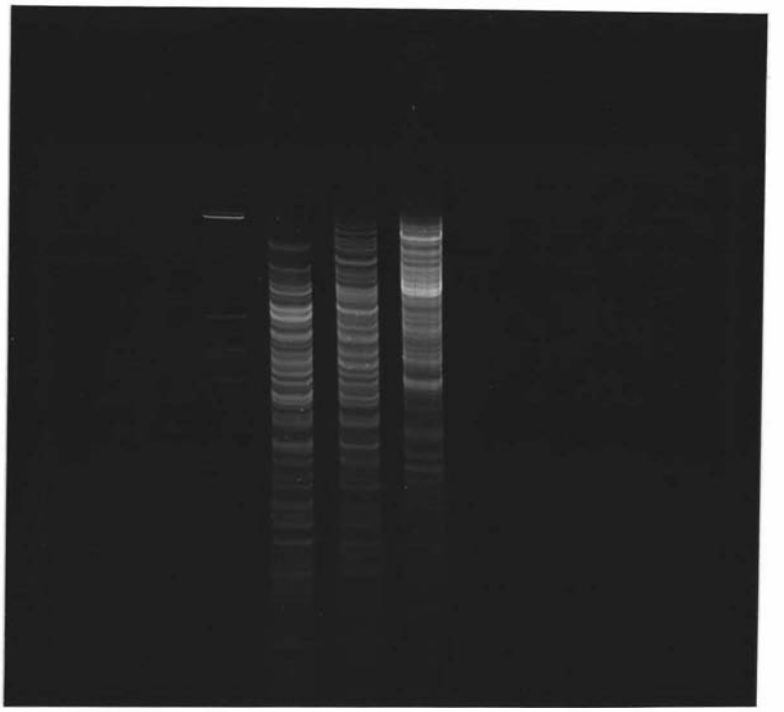


Figure 34

Bacterial restriction endonuclease DNA analysis of three *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 5). The DNA was digested with *Eco*RI (5'-G^{V}AATTC-3') and electrophoresed through a 0.7% agarose gel. Lane 1: Molecular weight marker (lambda DNA cut with *Eco*RI); Lane 2: L5T7; Lane 3: L5I8; Lane 4: L5T6. The DNA showed marked differences in their cleavage patterns, although some common bands can be observed. The cleavage patterns for the other twenty-seven isolates were identical to one or other of the patterns shown.

Figure 35

Bacterial restriction endonuclease DNA analysis of four *M. ovipneumoniae* isolates obtained from a single pneumonic sheep lung (i.e. lung 6). The DNA was digested with *Eco*RI (5'-G^{V}AATTC-3') and electrophoresed through a 0.7% agarose gel. Lane 1: Molecular weight marker (lambda DNA cut with *Eco*RI); Lane 2: L6I3; Lane 3: L6I8; Lane 4: L6A6; Lane 5: L6T3. The DNA showed marked differences in their cleavage patterns, including the small molecular size fragments. The cleavage patterns for the other twenty-six isolates were identical to one or other of the patterns shown.



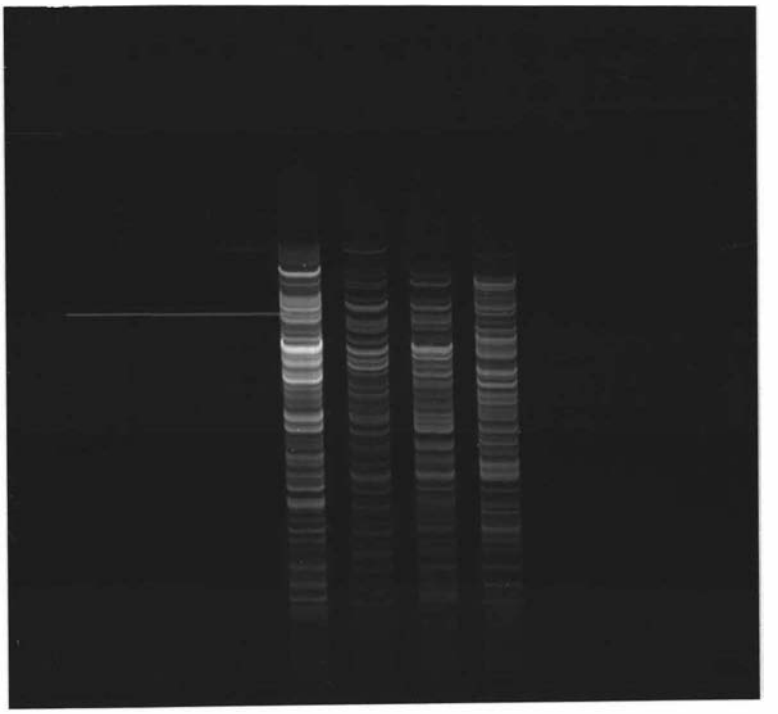


Figure 36

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Four isolates representing each of four patterns were digested with *Xba*I (5'-T^{V}CTAGA-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Xba*I); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

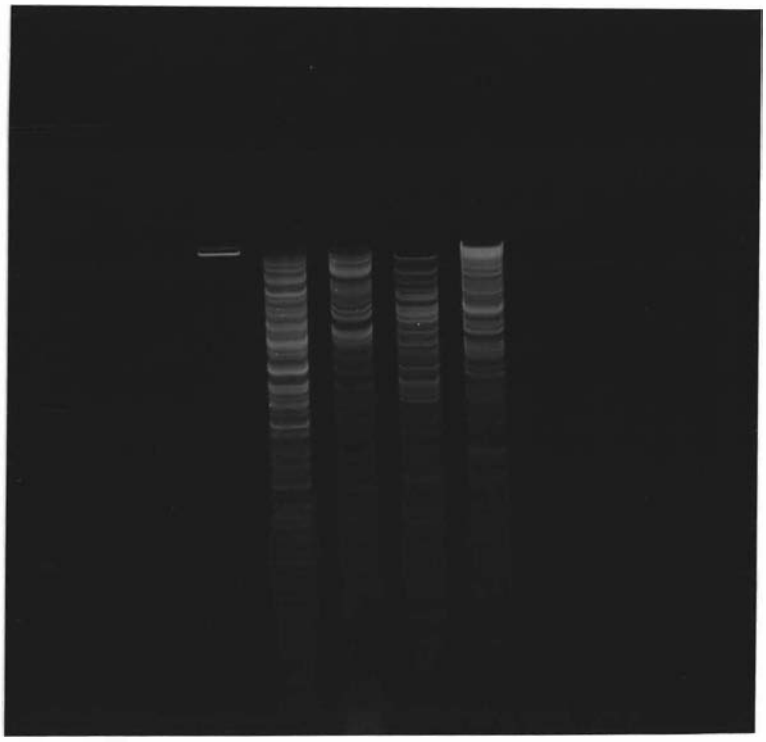
Note that all four patterns are markedly different with *Xba*I.

Figure 37

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Bgl*II (5'-A^{V}GATCT-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Bgl*II); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

Note that all four patterns are markedly different with *Bgl*II.



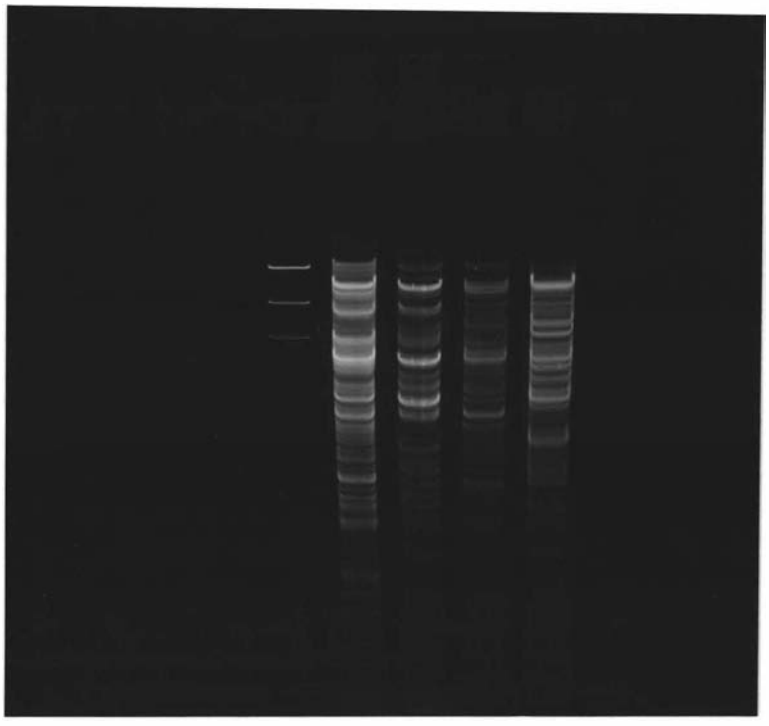


Figure 38

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Hin*dIII (5'-A^{V}AGCTT-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Hin*dIII); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

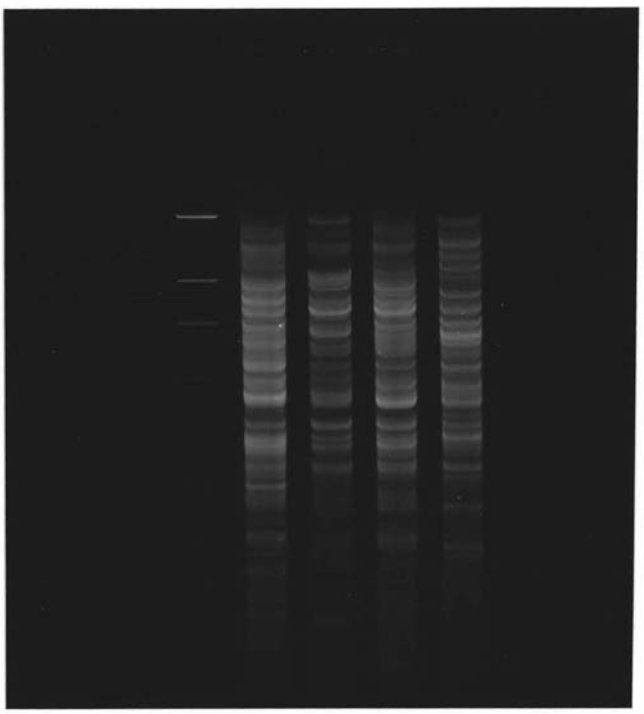
Note that all four patterns are markedly different with *Hin*dIII.

Figure 39

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Msp*I (5'-C^{V}CGG-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Msp*I); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

Note that all four patterns are markedly different with *Msp*I.



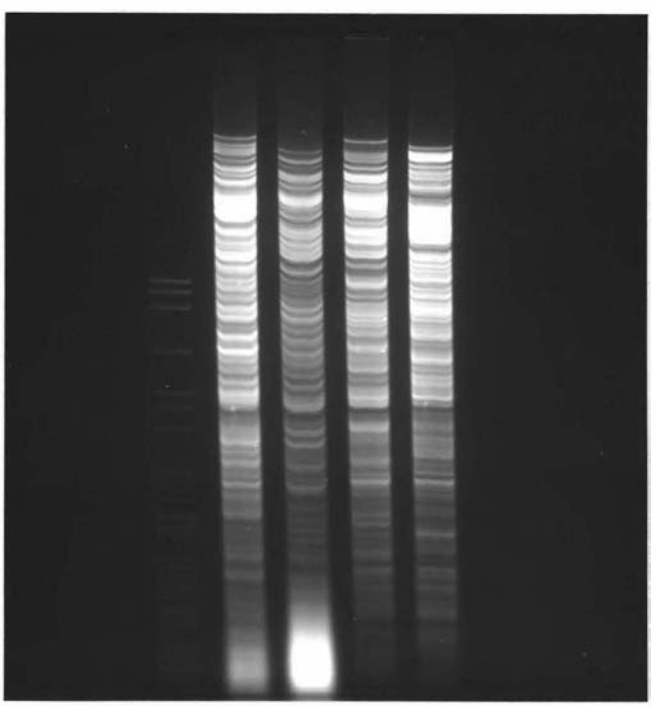


Figure 40

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Dra*I (5'-TTT^{V}AAA-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Dra*I); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

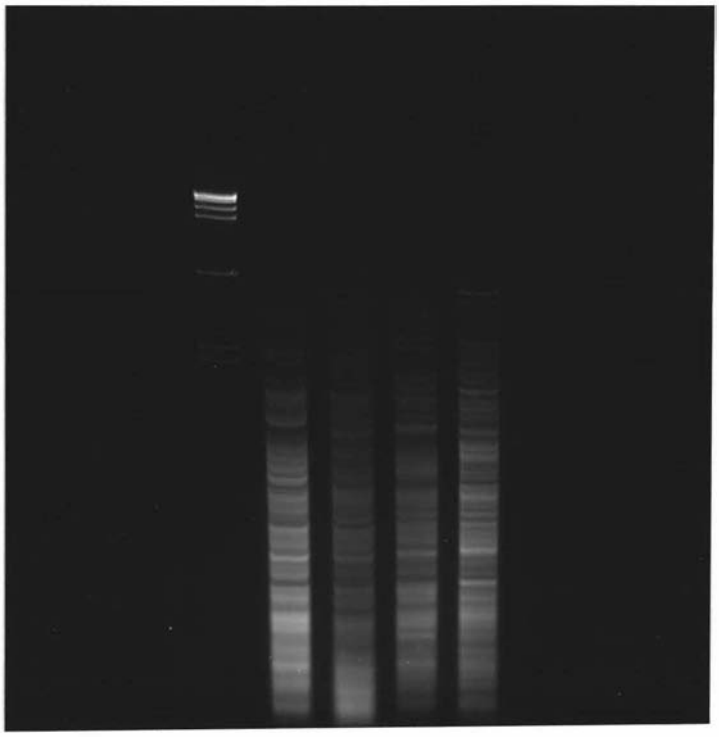
Note that all four patterns are markedly different with *Dra*I.

Figure 41

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Hpa*II (5'-C^{V}CGG-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Hpa*II); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

Note that all four patterns are markedly different with *Hpa*II.



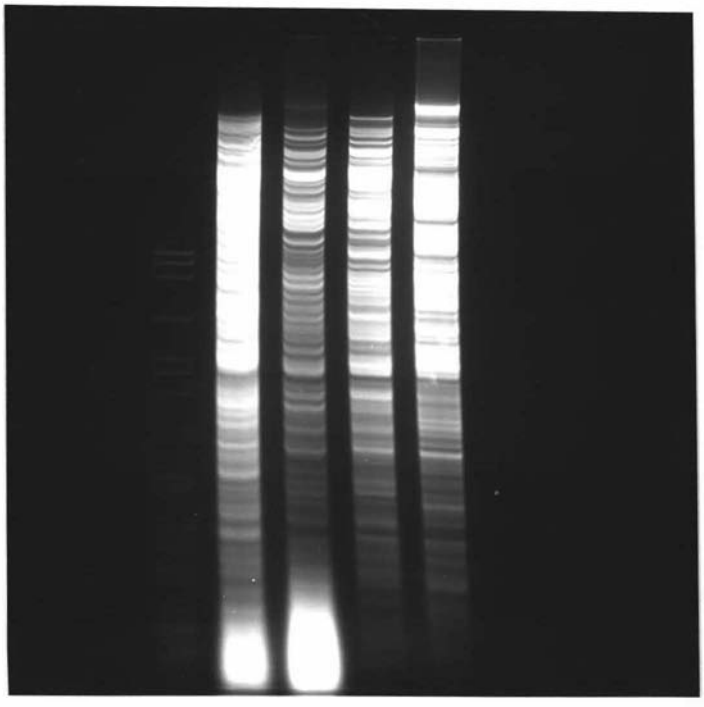


Figure 42

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Kpn*I (5'-GGTAC^{V}C-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Kpn*I); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

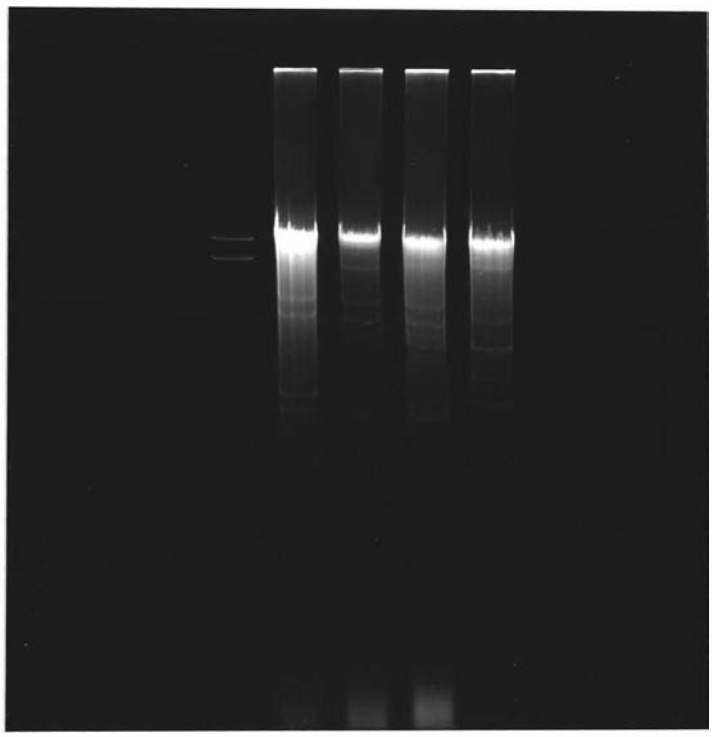
Note that all four patterns are markedly different with *Kpn*I.

Figure 43

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Sal*I (5'-G^{V}TCGAC-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Sal*I); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

Note that all four patterns are markedly different with *Sal*I.



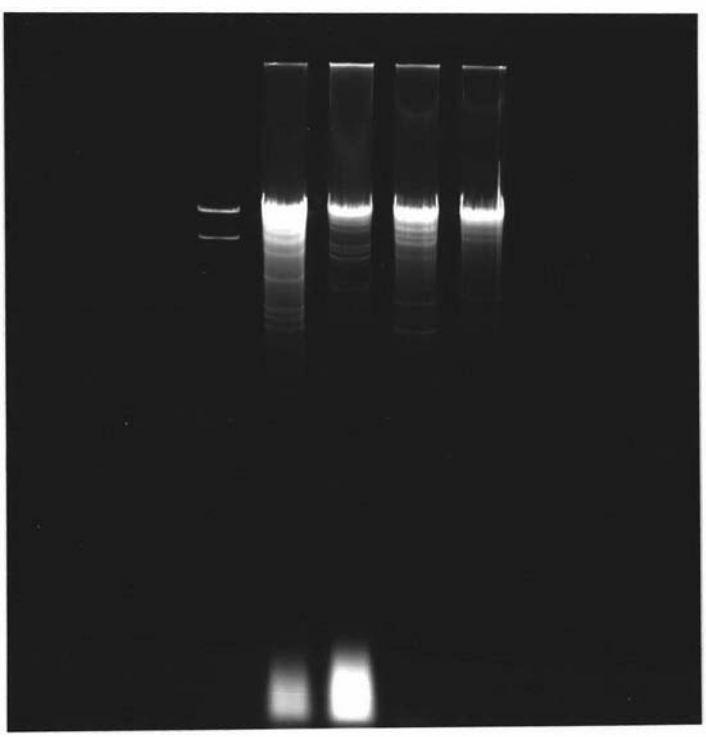


Figure 44

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Bam*HI (5'-G^{V}GATCC-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Bam*HI); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

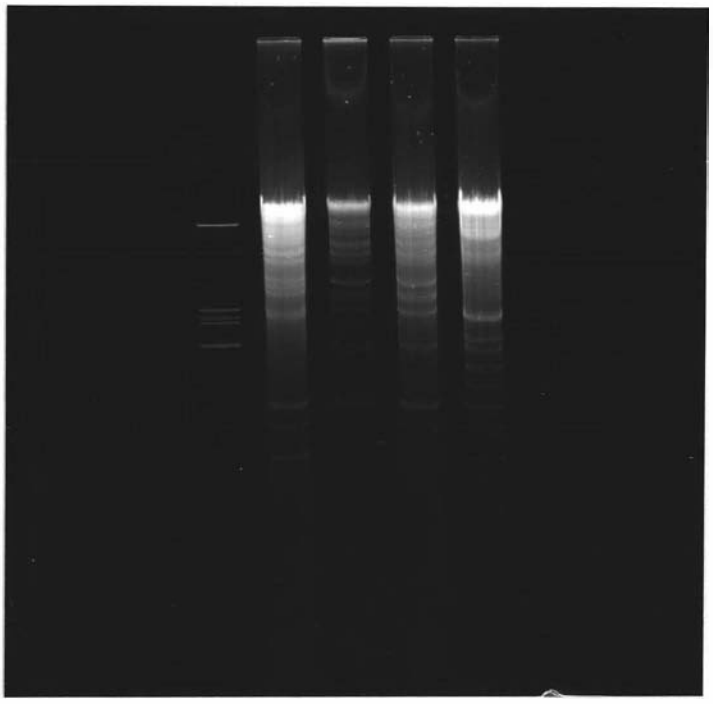
Note that all four patterns are markedly different with *Bam*HI.

Figure 45

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Sma*I (5'-CCC^{V}GGG-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Sma*I); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

Note that all four patterns are markedly different with *Sma*I.



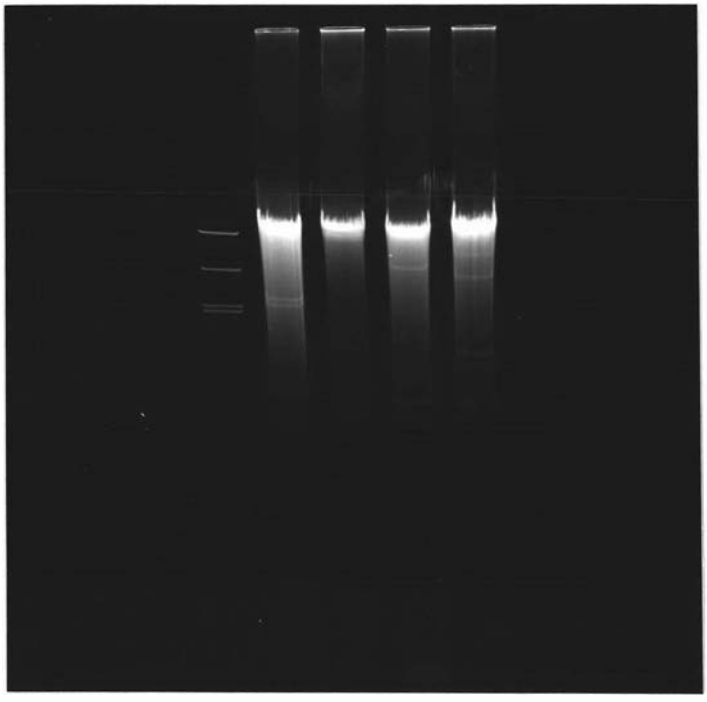


Figure 46

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Xho*I (5'-C^{V}TCGAC-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Xho*I); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

Note that all four patterns are markedly different with *Xho*I.

The uncleaved pattern in lane 5 is discussed in the text in relationship to methylation of cytosine.

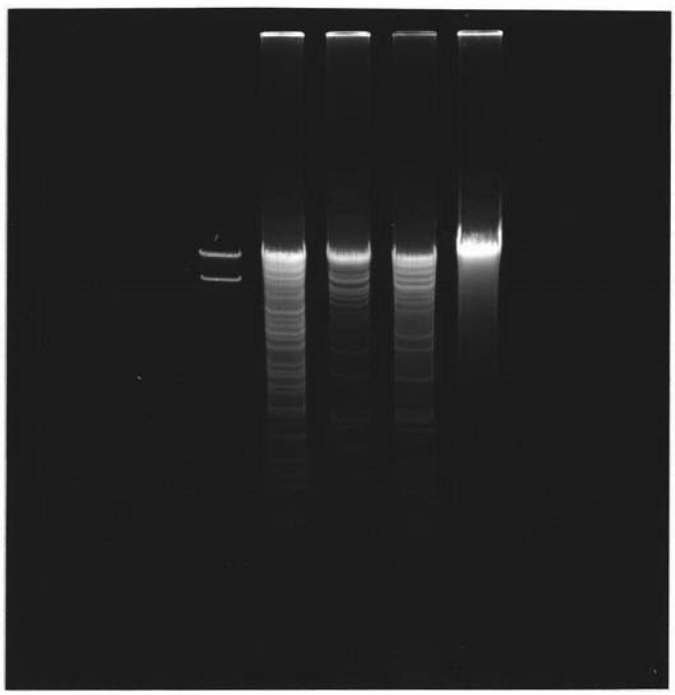
Figure 47

Thirty isolates of *M. ovipneumoniae* from one sheep lung (i.e. lung 6 which was chosen arbitrarily), showed four different DNA patterns (P, Q, R and S) when digested by *Eco*RI.

Isolates representing each pattern were examined using *Hae*III (5'-GG^{V}CC-3'). The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Hae*III); Lane 2: L6I3 (P); Lane 3: L6I8 (Q); Lane 4: L6A6 (R); Lane 5: L6T3 (S).

Note that all four patterns are markedly different with *Hae*III.

The uncleaved pattern in lane 5 is discussed in the text in relationship to methylation of cytosine.



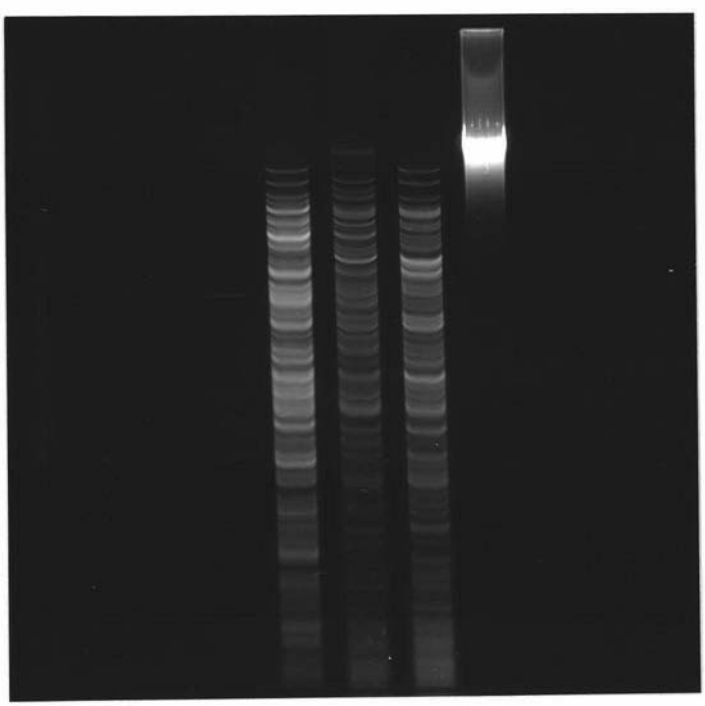


Figure 49

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Xba*I (5'-T^{V}CTAGA-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Xba*I); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

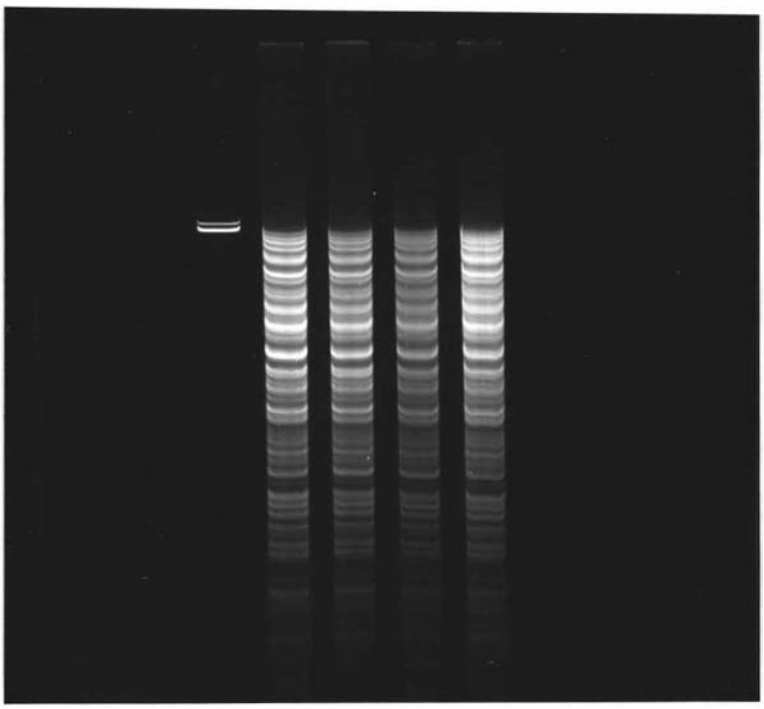
Note that all four patterns are identical.

Figure 50

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Bgl*II (5'-A^{V}GATCT-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Bgl*II); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

Note that all four patterns are identical.



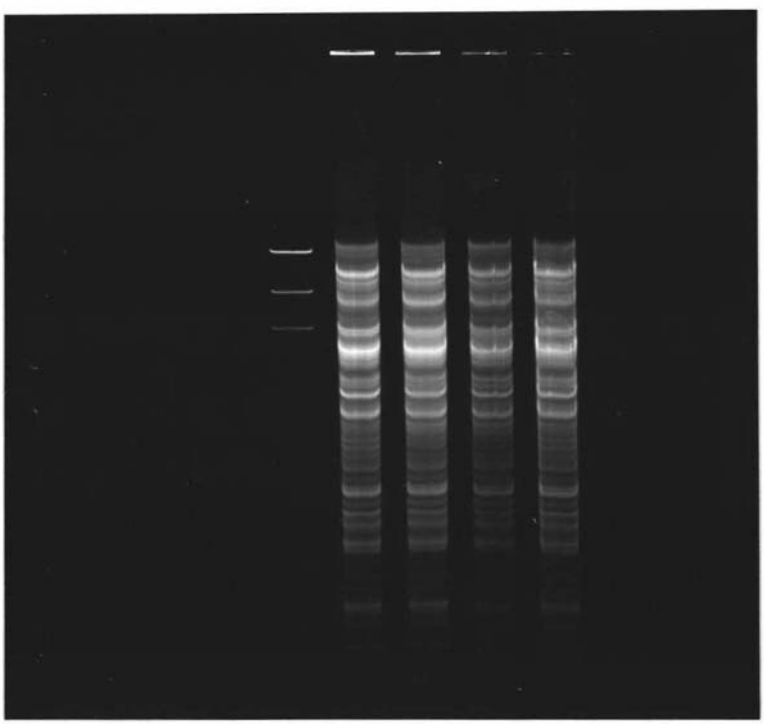


Figure 51

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Hin*dIII (5'-A^{V}AGCTT-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Hin*dIII); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

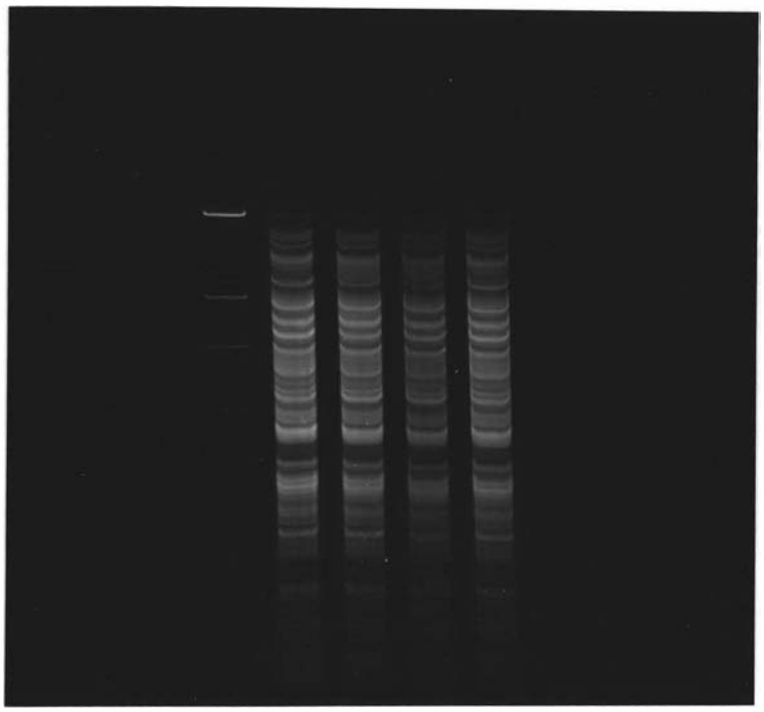
Note that all four patterns are identical.

Figure 52

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Msp*I (5'-C^{V}CGG-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Msp*I); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

Note that all four patterns are identical.



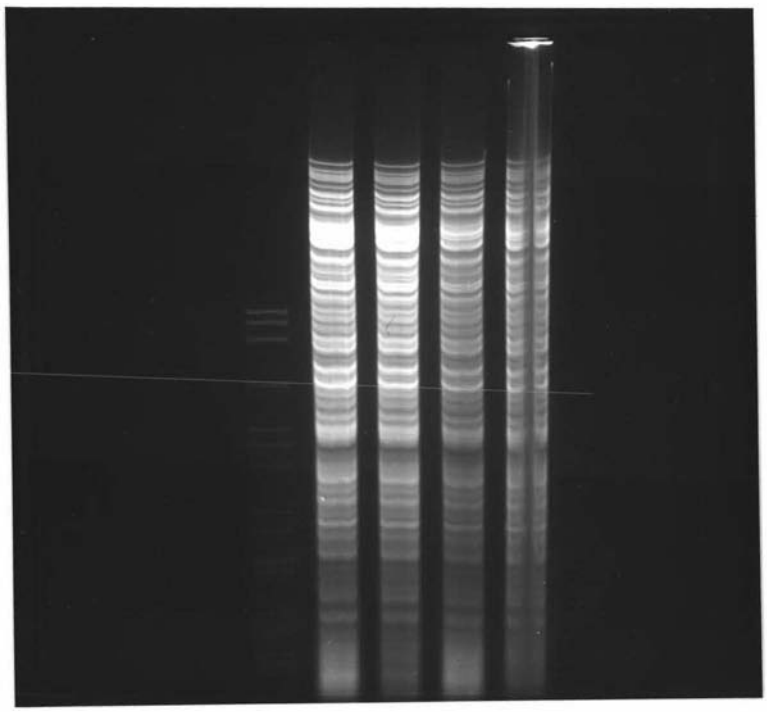


Figure 53

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Dra*I (5'-TTT^{V}AAA-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Dra*I); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

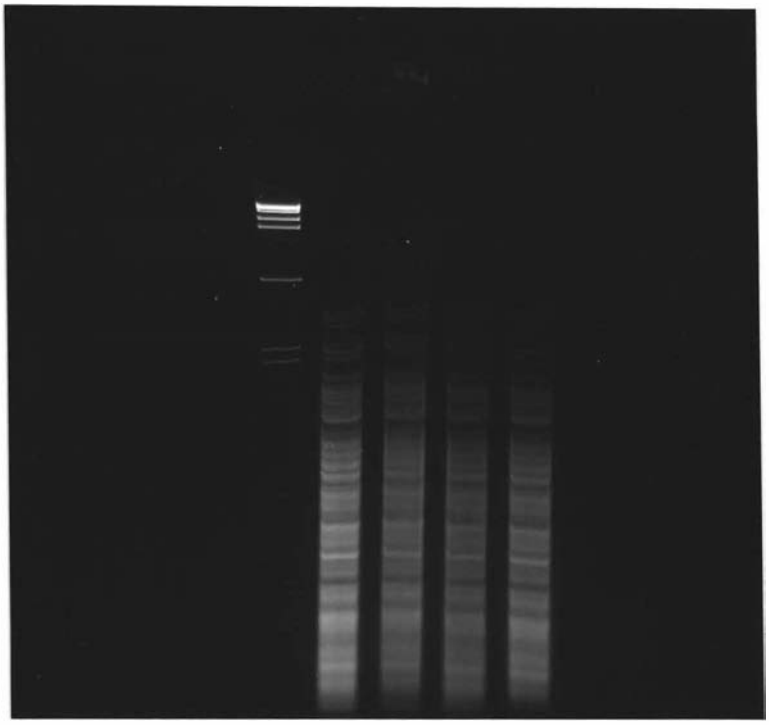
Note that all four patterns are identical.

Figure 54

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Hpa*II (5'-C^{V}CGG-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Hpa*II); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

Note that all four patterns are identical.



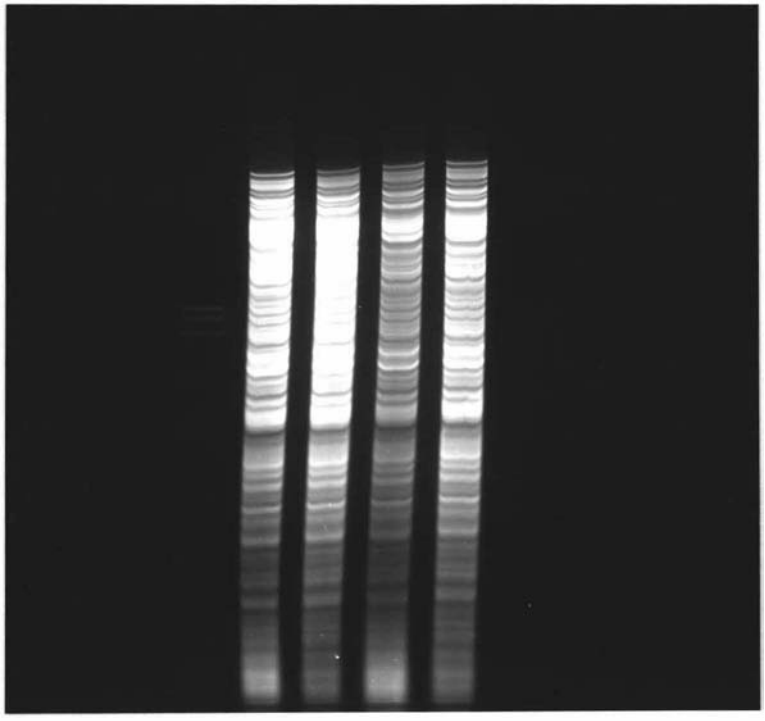


Figure 55

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Kpn*I (5'-GGTAC^{V}C-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Kpn*I); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

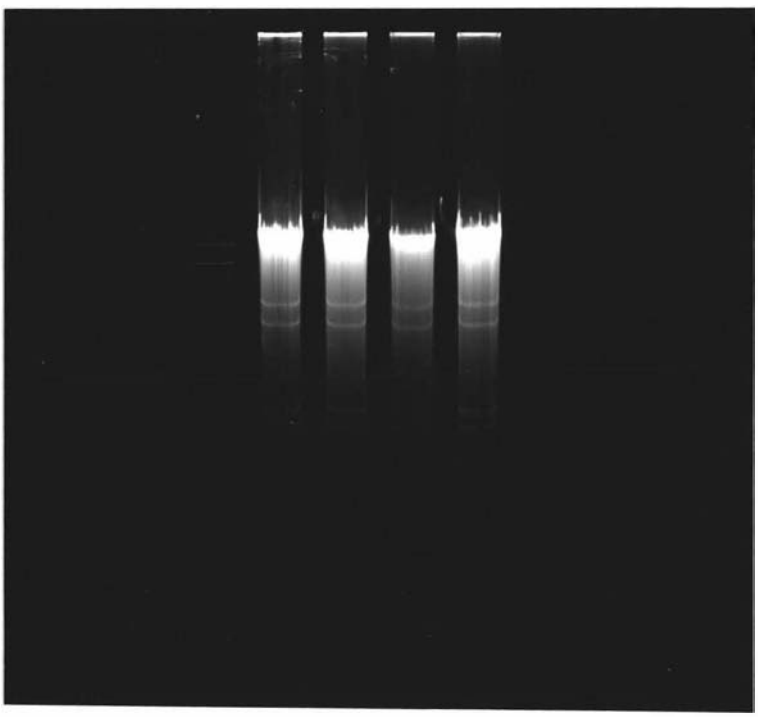
Note that all four patterns are similar.

Figure 56

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Sal*I (5'-G^{V}TCGAC-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Sal*I); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

Note that all four patterns are identical.



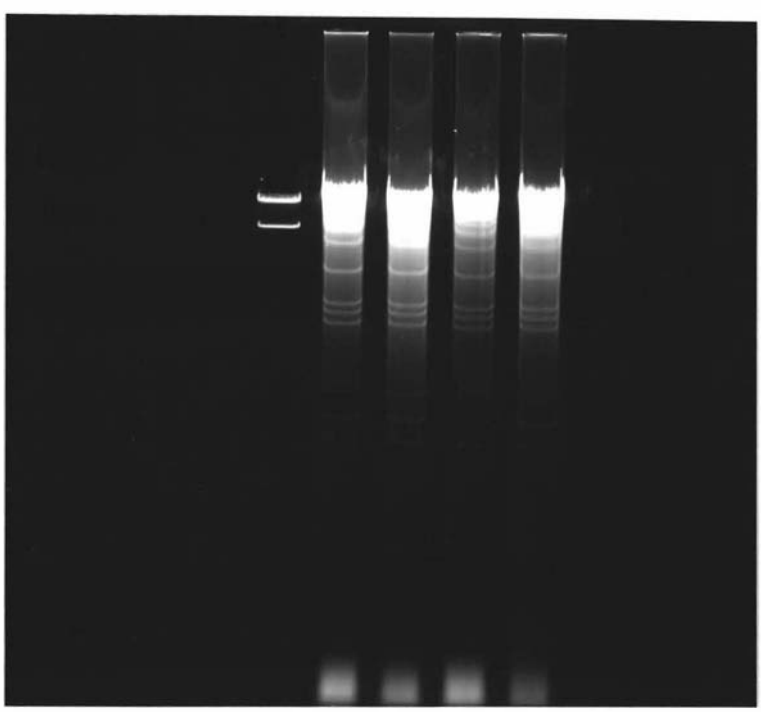


Figure 57

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Bam*HI (5'-G^{V}GATCC-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Bam*HI); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

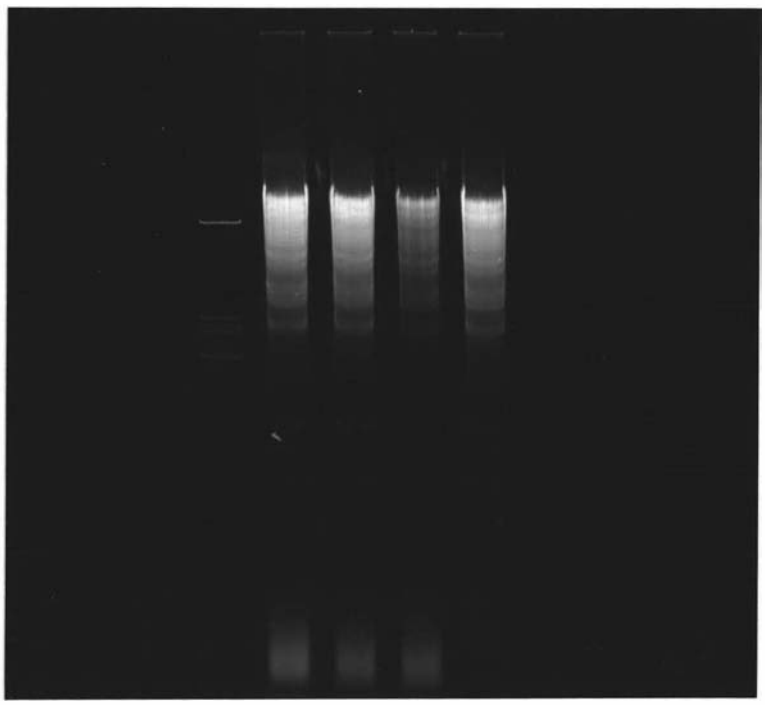
Note that all four patterns are identical.

Figure 58

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Sma*I (5'-CCC^{V}GGG-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Sma*I); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

Note that all four patterns are identical.



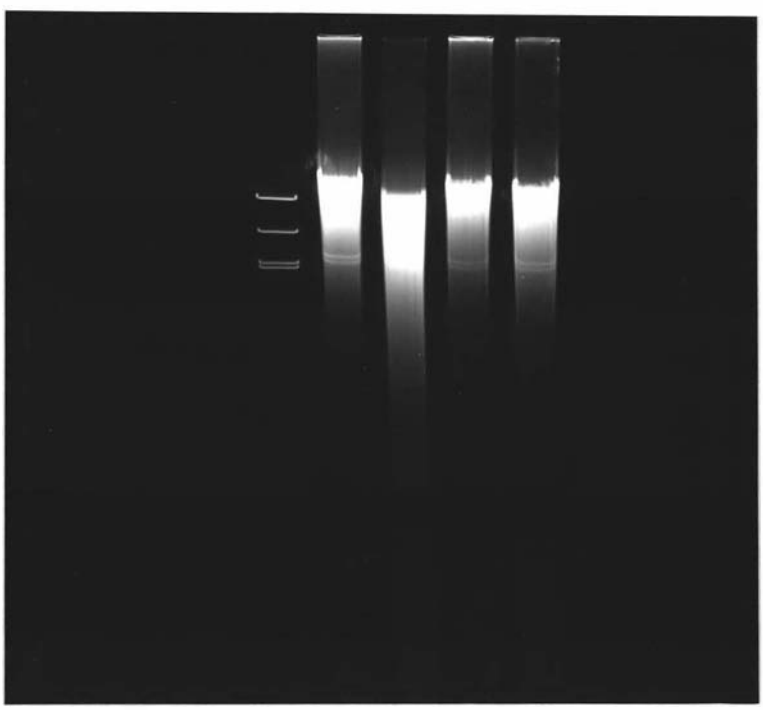


Figure 59

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Xho*I (5'-C^{V}TCGA-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Xho*I); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

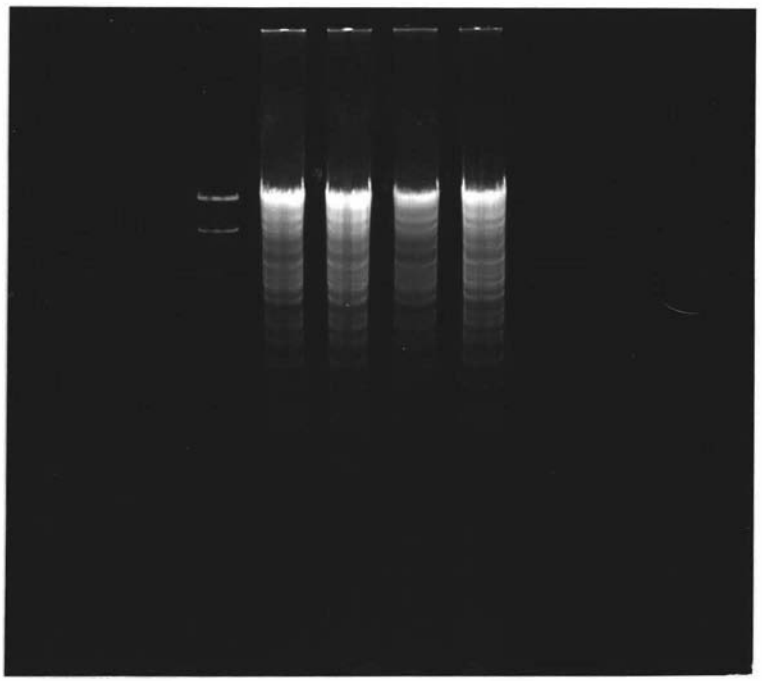
Note that all four patterns are identical.

Figure 60

Four isolates of *M. ovipneumoniae* derived from one pneumonic sheep (i.e. lung 6) and showing a similar, but not quite identical, pattern (pattern P, see Figure 48) were examined by *Hae*III (5'-GG^{V}CC-3').

The results are shown as follows: Lane 1: Molecular weight marker (lambda DNA cut with *Hae*III); Lane 2: L6I1; Lane 3: L6I2; Lane 4: L6I4; Lane 5: L6I6.

Note that all four patterns are identical.



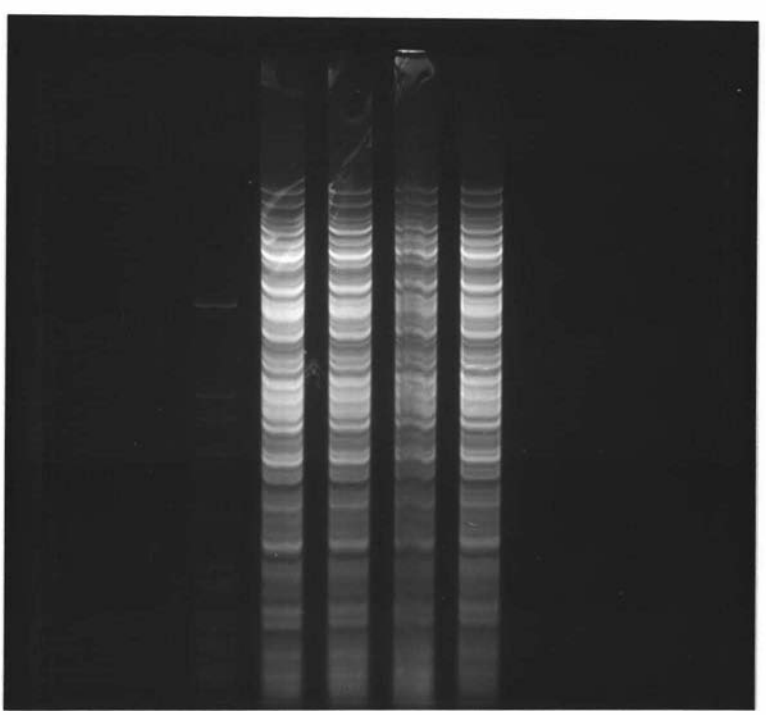


Figure 61a

SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered simultaneously from a single pneumonic sheep lung (i.e. lung 6) by the "normal" isolation method. The proteins were separated in a 10% acrylamide gel. Lane 1: L6I1; Lane 2: L6I2; Lane 3: L6I3; Lane 4: L6I4; Lane 5: L6I5; Lane 6: L6I6; Lane 7: L6I7; Lane 8: L6I8; Lane 9: L6I9; Lane 10: L6I10.

Note that all ten isolates are identical (pattern A).

Figure 61b

SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered from a single pneumonic sheep lung (i.e. lung 6) by selecting ten colonies derived from an inoculum of original lung material on FM4 agar. The proteins were separated in a 10% acrylamide gel. Lane 1: L6A1; Lane 2: L6A2; Lane 3: L6A3; Lane 4: L6A4; Lane 5: L6A5; Lane 6: L6A6; Lane 7: L6A7; Lane 8: L6A8; Lane 9: L6A9; Lane 10: L6A10.

Note that two patterns are present *viz*; lanes 3, 5, 7, 8 and 9 (pattern B) and lanes 1, 2, 4, 5, 6 and 8 (pattern C) differ in several bands at the limit of detection. These differences can best be noted by comparing lanes 3 and 4. The position of differences are marked by an asterisk (*).

The two patterns shown in this figure divide the isolates into the same two groups as the DNA restriction patterns (c.f. Figures 29a and 29b with 61a and 61b).

Figure 61c

SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered by ten parallel limit dilution titrations from a homogenate of single pneumonic lung lesion (i.e. lung 6). The proteins were separated in a 10% acrylamide gel. Lane 1: L6T1; Lane 2: L6T2; Lane 3: L6T3; Lane 4: L6T4; Lane 5: L6T5; Lane 6: L6T6; Lane 7: L6T7; Lane 8: L6T8; Lane 9: L6T9; Lane 10: L6T10.

Two patterns are present *viz*; lanes 1, 2, 4, 6, 8, 9 and 10 (pattern B) and lanes 3, 5 and 7 (pattern D). The former pattern is similar to one of the patterns seen in Figure 61b, while the latter is different from the patterns seen in Figures 61a and 61b, i.e. there are four patterns of isolates from one lung lesion. The two patterns seen in this figure can best be noted by comparing lanes 3 and 4. The position of differences are marked by an asterisk (*).

The two patterns shown in this figure divide the isolates into the same two groups as the DNA restriction patterns (c.f. Figures 29c and 61c).

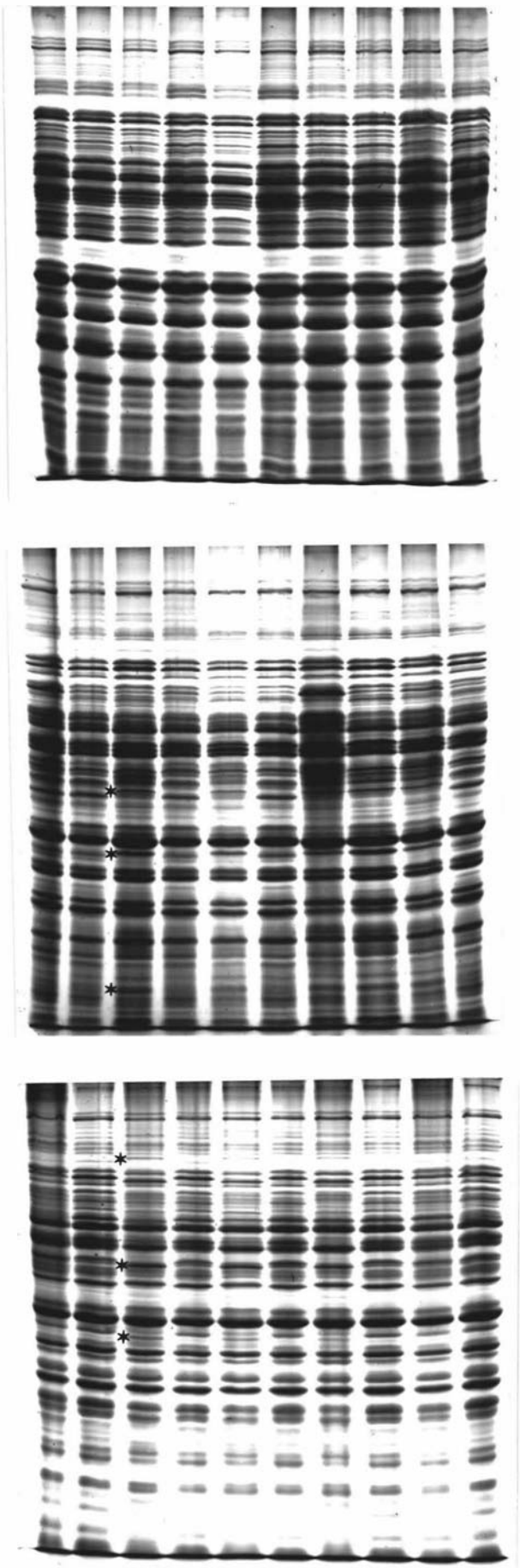


Figure 62

SDS-PAGE examination of two *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 1). The proteins were separated in a 10% acrylamide gel. Lane 1: LIA5; Lane 2: LIT2.

Note that both protein patterns are identical. These isolates differed in their restriction endonuclease pattern (see Figure 30).

Figure 63

SDS-PAGE examination of two *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 2). The proteins were separated in a 10% acrylamide gel. Lane 1: L2A5; Lane 2: L2I6.

The protein banding patterns differ slightly at two points (see arrows).

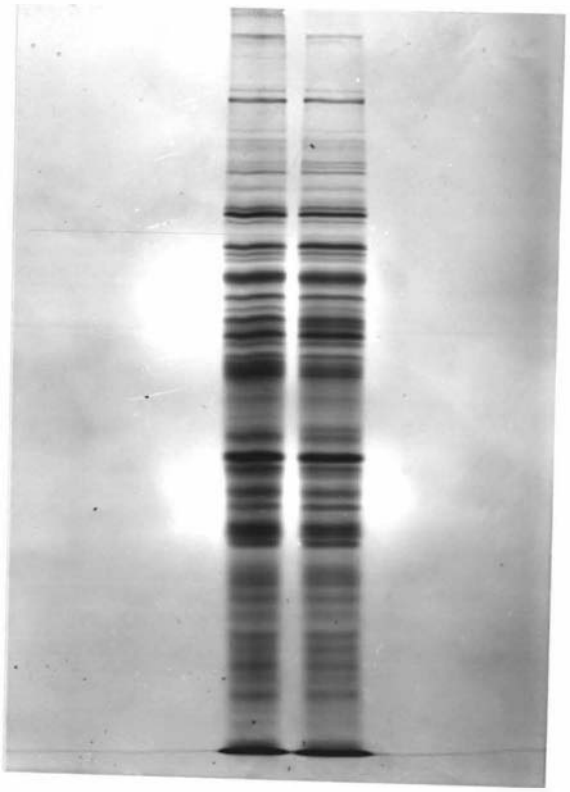


Figure 64

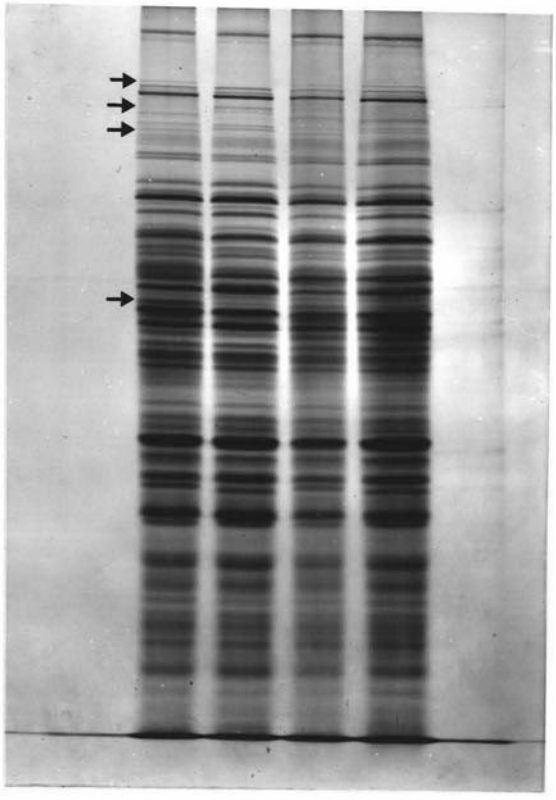
SDS-PAGE examination of four *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 3). The proteins were separated in a 10% acrylamide gel. Lane 1: L3T4; Lane 2: L3I6; Lane 3: L3I8; Lane 4: L3I9.

The first three isolates show slight differences in their protein banding pattern (see arrows) however, lane 4 is indistinguishable from lane 3.

Figure 65

SDS-PAGE examination of four *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 4). The proteins were separated in a 10% acrylamide gel. Lane 1: L4I8; Lane 2: L4A5; Lane 3: L4A8; Lane 4: L4T9.

Note the differences (see arrows) in the banding patterns in all four lanes. While all four lanes differ, lanes 1 and 3 and lanes 2 and 4 show close resemblance to each other than to the other two lanes.



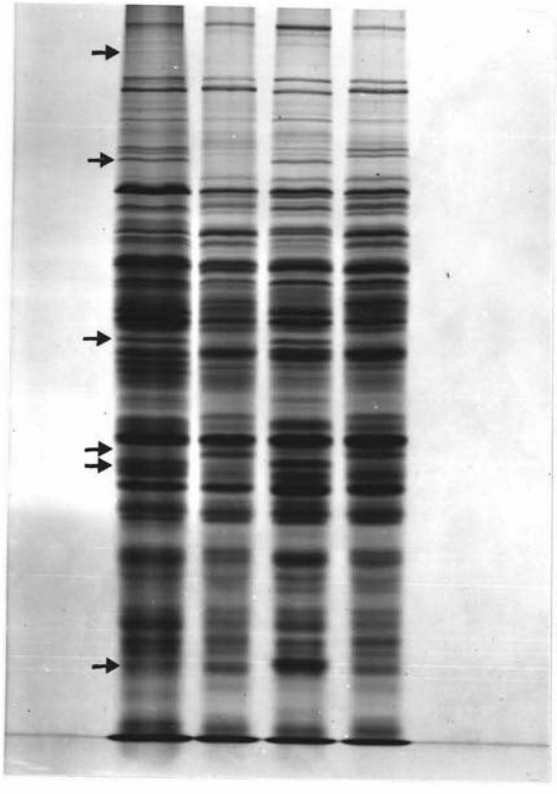


Figure 66

SDS-PAGE examination of three *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 5). The proteins were separated in a 10% acrylamide gel. Lane 1: L5T7; Lane 2: L5I8; Lane 3: L5T6.

Note that lane 3 differs from the other two lanes. Lanes 1 and 2 differ slightly in band density only (see arrows).

Figure 67

SDS-PAGE examination of four *M. ovipneumoniae* isolates (which differed in BRENDA patterns) obtained from a single pneumonic sheep lung (i.e. lung 6). The proteins were separated in a 10% acrylamide gel. Lane 1: L6I3; Lane 2: L6I8; Lane 3: L6A6; Lane 4: L6T3.

Note that lanes 3 and 4 differ from the other two lanes. However, lanes 1 and 2 appear identical.

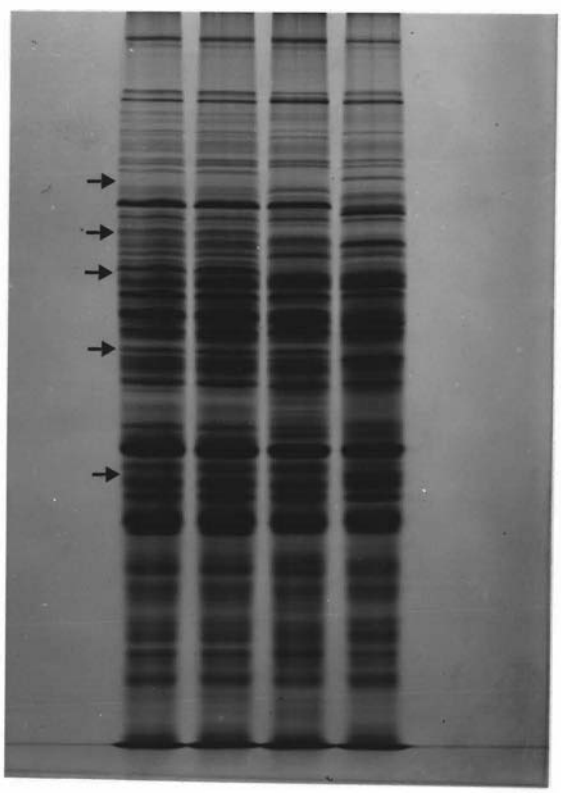


Figure 68

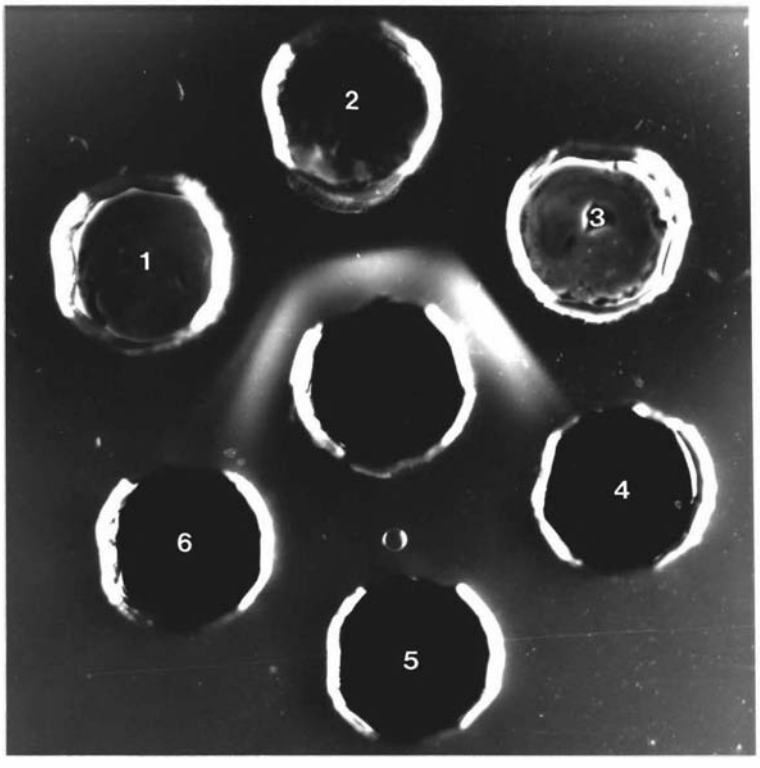
Gel precipitin test for the identification of a mycoplasma isolated from a pneumonic goat lung. The central well contains antiserum to *M. ovipneumoniae* strain 1. The peripheral wells contain *M. ovipneumoniae* strain 1 (well 1); goat isolate GT10 (well 2); *M. ovipneumoniae* strain 10 (well 3); wells 4, 5 and 6 contain FM4 Medium and were used as a control.

Note that the isolate shows a line of identity with the standard strains of *M. ovipneumoniae.*

Figure 69

A comparison of the total protein of an *M. ovipneumoniae* isolate from a pneumonic goat lung with three *M. ovipneumoniae* strains. Lane 1: strain 1; Lane 2: strain 5; Lane 3: strain 10; Lane 4: GT10 (goat isolate).

Note that the goat isolate has most of its protein bands in common with the other three *M. ovipneumoniae* strains.


2
1
3
4
6
5

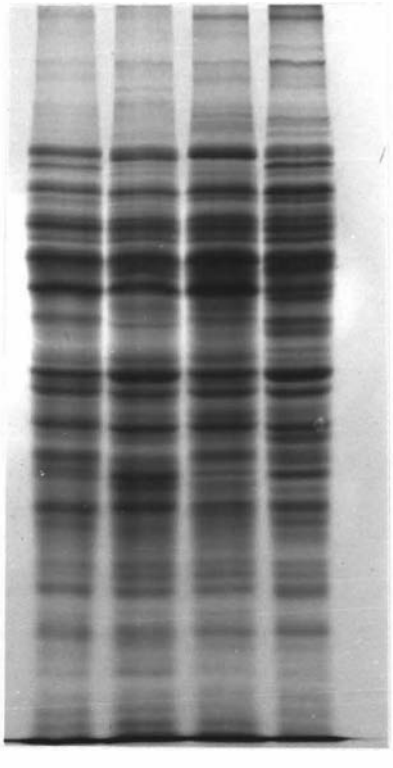


Figure 70a

Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates which were recovered simultaneously from a single pneumonic goat lung by the "normal" isolation method. The DNA was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Lane 1: GI1; Lane 2: GI2; Lane 3: GI3; Lane 4: GI4; Lane 5: GI5; Lane 6: GI6; Lane 7: GI7; Lane 8: GI8; Lane 9: GI9; Lane 10: GI10.

Note that two patterns are present. Lanes 1 through 6, 8 and 9 are identical or almost identical. Lanes 7 and 10 show a significantly different restriction pattern although many bands common to all 10 isolates are seen especially in the lower molecular weight range.

Figure 70b

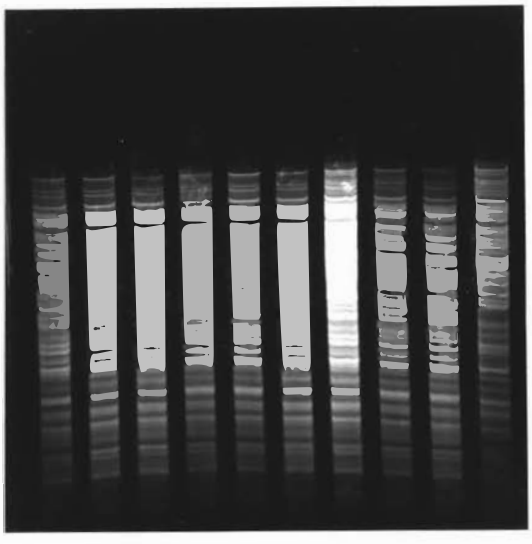
Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates recovered from a single pneumonic goat lung by selecting ten colonies derived from an inoculum of original lung material on FM4 agar. The DNA was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Lane 1: GA1; Lane 2: GA2; Lane 3: GA3; Lane 4: GA4; Lane 5: GA5; Lane 6: GA6; Lane 7: GA7; Lane 8: GA8; Lane 9: GA9; Lane 10: GA10.

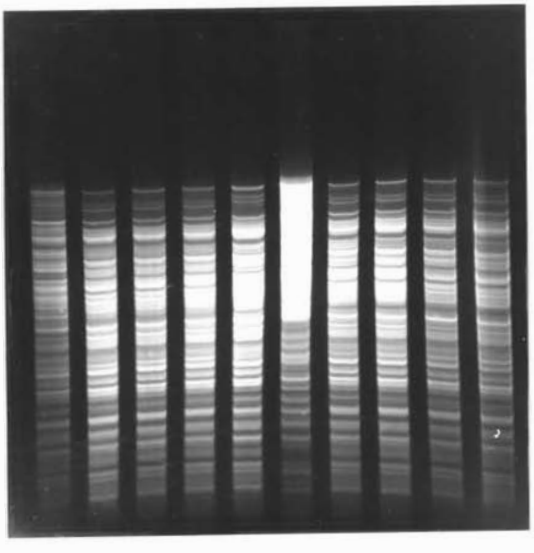
Note that nine lanes are similar to the DNA restriction pattern seen in Figure 70a, lanes 1 to 5, 7, 8, 9 and 10. Lane 6 of Figure 70b is similar to lanes 7 and 10 in Figure 70a.

Figure 70c

Bacterial restriction endonuclease DNA analysis of ten *M. ovipneumoniae* isolates recovered from a single pneumonic goat lung by limit dilution. The DNA was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Lane 1: GT1; Lane 2: GT2; Lane 3: GT3; Lane 4: GT4; Lane 5: GT5; Lane 6: GT6; Lane 7: GT7; Lane 8: GT8; Lane 9: GT9; Lane 10: GT10.

Note that two general patterns corresponding to lanes 1, 4, 7, 8 and 10 and lanes 2, 3, 5, 6 and 9 are seen. These two patterns are similar to the two patterns shown in Figures 70a and 70b.





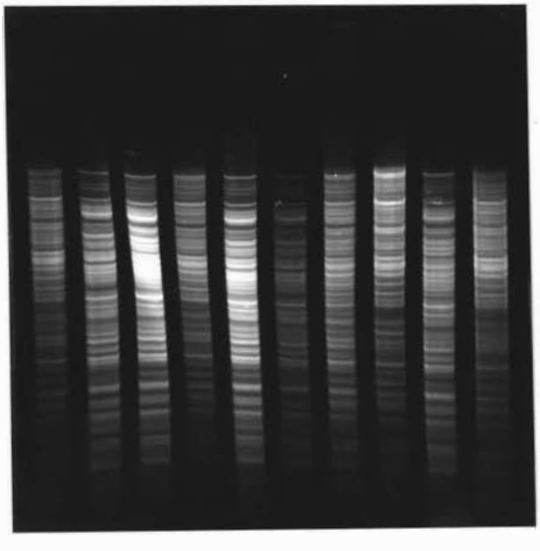


Figure 71a

SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered simultaneously from a single pneumonic goat lung by the "normal" isolation method. The proteins were separated in a 10% acrylamide gel. Lane 1: GI1; Lane 2: GI2; Lane 3: GI3; Lane 4: GI4; Lane 5: GI5; Lane 6: GI6; Lane 7: GI7; Lane 8: GI8; Lane 9: GI9; Lane 10: GI10.

Note that all ten isolates are similar.

Figure 71b

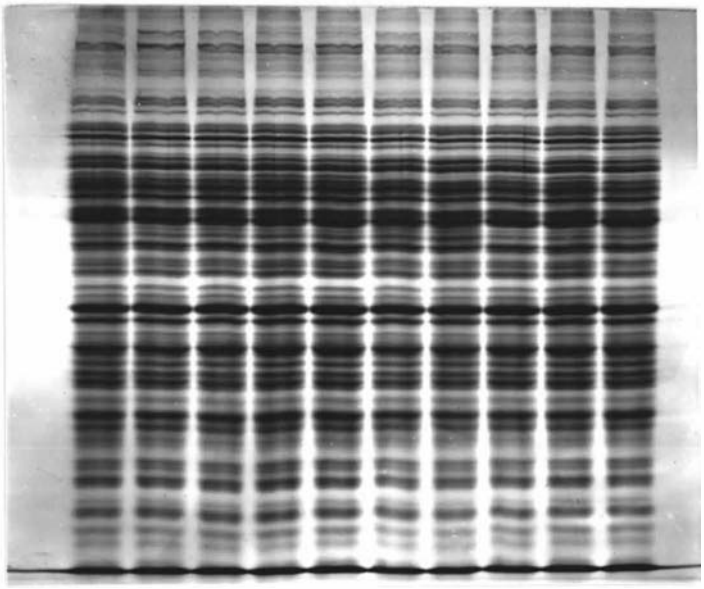
SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered from a single pneumonic goat lung by selecting ten colonies derived from an inoculum of original lung material on FM4 agar. The proteins were separated in a 10% acrylamide gel. Lane 1: GA1; Lane 2: GA2; Lane 3: GA3; Lane 4: GA4; Lane 5: GA5; Lane 6: GA6; Lane 7: GA7; Lane 8: GA8; Lane 9: GA9; Lane 10: GA10.

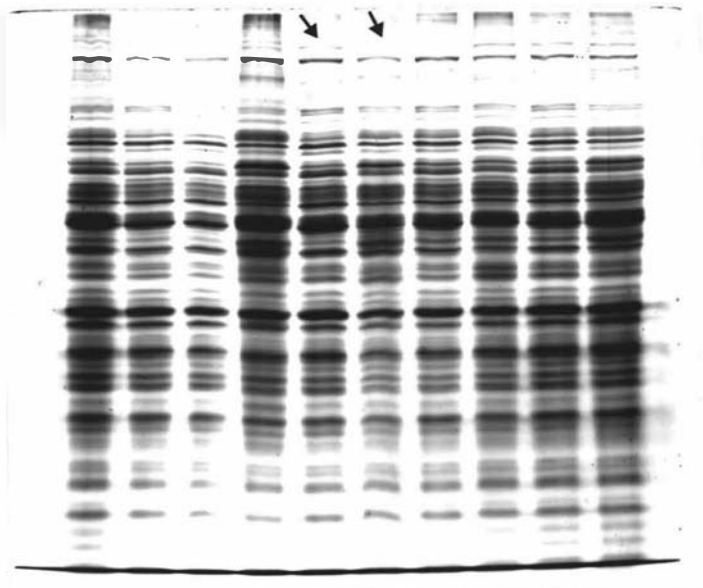
The protein banding patterns for all ten *M. ovipneumoniae* isolates are similar. Nevertheless, two patterns can be detected because of a variation (arrowed) in the high molecular weight range. These variations correlate with the BRENDA results e.g. compare lanes 5 and 6, Figure 71b with lanes 5 and 6 Figure 70b (these represent the same two isolates).

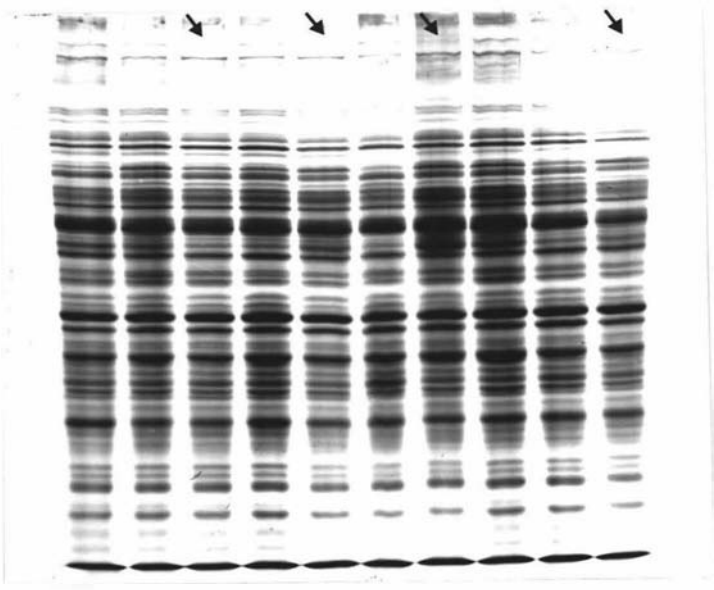
Figure 71c

SDS-PAGE examination of ten *M. ovipneumoniae* isolates recovered by ten parallel limit dilution titrations from a single pneumonic goat lung. The proteins were separated in a 10% acrylamide gel. Lane 1: GT1; Lane 2: GT2; Lane 3: GT3; Lane 4: GT4; Lane 5: GT5; Lane 6: GT6; Lane 7: GT7; Lane 8: GT8; Lane 9: GT9; Lane 10: GT10.

All protein banding patterns in each lane appear similar, except for the presence of a high molecular weight protein band (arrowed) in some lanes.







4.4 Discussion.

Mew *et al*, (1985) first demonstrated the heterogeneity of *M. ovipneumoniae* by examining *Eco*RI digests of the DNA. This study was limited to eight isolates and did not rule out the possibility that the heterogeneity observed is a function of the restriction enzyme used to observe the cleavage patterns. Such a possibility would imply that the base sequence cut by the restriction enzyme was subject to a much higher rate of mutation than other sites. To exclude this possibility, we re-examined the eight isolates of *M. ovipneumoniae* using *Eco*RI plus three other restriction enzymes (*viz*, *Dra*I; *Hpa*II; *Msp*I). The cleavage patterns observed were unique to each isolate and since the enzyme recognition sites bore no resemblance to that of *Eco*RI, we conclude that the original observation which the present author made in association with Mew (Mew *et al*, 1985), represents a general heterogeneity of the DNA sequence and is not merely a function of the enzyme originally used to demonstrate this phenomenon.

To further examine the extent of heterogeneity, the present author obtained three isolates of *M. ovipneumoniae* from each of twenty farms and examined them for the heterogeneity of protein and in association with a colleague (N. Norman), for the heterogeneity of DNA. The proteins were heterogeneous and Norman (1985) found that the DNA of fifty-eight of the sixty isolates gave markedly different patterns. Thus, each isolate, with three exceptions, was unique, even when isolates from sheep of the same flock were compared. This extreme heterogeneity led us to ask the question: "Is it possible that individual sheep, or more specifically, individual lung lesions can be colonized by more than one strain of *M. ovipneumoniae* simultaneously?"

If multiple strains colonize a lung, the method of isolation may favour the detection of one particular strain e.g the incubation time and hence the number of generations required to obtain a culture from which colonies of *M. ovipneumoniae* could be obtained may allow selection of the fastest growing strains. Consequently, in trying to find if multiple strains are present within a single pneumonic lung, we attempted to minimize this problem as follows: *M. ovipneumoniae* was isolated using three different methods *viz*, "normal" isolation procedure; direct plating of homogenized pneumonic lung tissue on agar and isolation from limit dilution titrations. Thirty isolates from each of six pneumonic sheep lungs were examined by BRENDA and in all cases major differences were seen between the isolates of any one lung i.e. lung 1, two patterns; lung 2, two patterns; lung 3, four patterns; lung 4, four patterns; lung 5, three patterns; lung 6, four patterns.

The lesions from lungs 1 and 2 were more limited in extent than the lesions seen in lungs 3 to 6 i.e. there may be a relationship between the number of strains colonizing the lung and the severity of the lesion produced. This point is returned to in the discussion at the end of this thesis.

While we have already demonstrated that the heterogeneity of *M. ovipneumoniae* isolates from sheep on different farms was not dependent on the restriction enzyme used, the unexpected finding that multiple, distinguishable isolates could be obtained from one lung lesion made it desirable to confirm that our earlier conclusions also applied to these isolates. Our results show that the heterogeneity demonstrated with *Eco*RI (see Figures 36 to 47) was also demonstrated when the following restriction enzymes were used: *Xba*I; *Bgl*II; *Hin*dIII; *Msp*I; *Dra*I; *Hpa*II; *Kpn*I; *Sal*I; *Bam*HI; *Sma*I; *Xho*I and *Hae*III. A new consideration, however, arose because the last six of these enzymes gave only partial digests (Figures 40 to 47). This observation is investigated in chapter five.

When two or more markedly different BRENDA patterns were detected with *Eco*RI, within any one of these patterns minor differences were seen when different isolates were examined (e.g. Figure 48, compare lanes 2 and 3). As noted above, the major differences remained when investigated by restriction enzymes other than *Eco*RI. However, minor differences were not detected by any other restriction enzyme.

The extreme variation in BRENDA patterns of the isolates was unexpected and seems to imply heterogeneity of coding capacity of different isolates. However, since the genetic code is redundant, it is possible, at least in theory, to change many bases in a DNA sequence, including the restriction sites, in such a way as to markedly alter the cleavage patterns while at the same time allowing the proteins to remain unaltered. This in turn would imply that isolates, especially those from one lung, could differ in their BRENDA patterns, while maintaining identical protein patterns as shown by SDS-PAGE. This was examined in the case of isolates for all six pneumonic and in general it was found that isolates with markedly different BRENDA patterns also differed in their SDS-PAGE protein patterns. This is illustrated in lung 6, thus taking the ten isolates shown in Figure 29b, two patterns are shown in the following order (from left to right) i.e. lanes 3, 5, 7, 8 and 9 (pattern Q) and lanes 1, 2, 4, 6 and 10 (pattern R). These same ten isolates were examined by SDS-PAGE

and two patterns are seen in Figure 61b (from left to right) i.e. lanes 3, 5, 7, 8 and 9 show one pattern and lanes 1, 2, 4, 6 and 10 showed the second pattern. This correlates with the BRENDA results. Therefore, the general conclusion is that different BRENDA patterns and different SDS-PAGE patterns are correlated. Nevertheless, the correlation is not perfect because in one case (lung 6) isolates with two different BRENDA patterns gave indistinguishable SDS-PAGE patterns [in Figure 29a, lane 7 (pattern P) differs from lane 8 (pattern Q), whereas these isolates gave identical protein patterns (see Figure 61a, lanes 7 and 8)].

The results obtained from multiple goat isolates from a single pneumonic lung gave similar results to that obtained from the pneumonic sheep lungs. Thus, one goat yeilded *M. ovipneumoniae* isolates which gave two different restriction patterns by BRENDA. These isolates gave very similar SDS-PAGE patterns although some minor variations did allow the patterns to be distinguished.

CHAPTER FIVE

The Detection of Methylated Bases in the DNA of Some Isolates of *M. ovipneumoniae*.

5.1 Introduction.

This general investigation was undertaken in the expectation that some heterogeneity could be detected in *M. ovipneumoniae* isolates with the consequent possibility that the heterogeneity would lend itself to the establishment of meaningful groups e.g. serovars or perhaps groupings based on a limited number of different BRENDA patterns or total protein patterns based on SDS-PAGE. However, the heterogeneity seen with both SDS-PAGE and restriction endonuclease digests, turned out to be much greater than we had anticipated. Differences in BRENDA patterns of some isolates, against a background of some common bands, would not have been unexpected, but the lack of common bands between different isolates, including those derived from sheep on one farm and even between some multiple isolates from one pneumonic lung was unexpected. This variation is not due to an exceptionally high mutation rate, since long term propagation of the organism *in vitro* left the BRENDA pattern unchanged or with an occasional single band difference (Norman, 1985).

Another observation, which also requires an explanation, is the partial digestion of DNA by some restriction endonucleases (Figures 41 through 47), particularly those which cleave a cytosine-rich sequence (see section 5.4). It therefore occurred to us, albeit late in the work, that the partial digests, if not due to some unidentified technical problem, could be due to methylation of some bases and, if so, this might invalidate our earlier conclusions *viz*, that *M. ovipneumoniae* isolates differ in base sequence. Thus, if there is variation in the methylation of DNA bases, it might be possible to explain the heterogeneity of the BRENDA patterns on the basis of differences in methylation of the bases, even against a background of identical base sequences.

Should methylation occur it would presumably be adenosine or cytosine methylation since, these are the only bases which are commonly modified. The adenine methylation is common in procaryotes, whereas cytosine methylation is found mainly in eucaryotes (Razin, 1985). If methylation of either adenosine or cytosine was the cause of the heterogeneity of the restriction patterns, it would follow that (on the assumption that only one of these bases

is methylated) the heterogeneity of the DNA digest would disappear if an endonuclease was used which cleaves at sequences which did not include the methylated bases e.g. an endonuclease which cleaves a sequence with only thymidine and adenosine (not affected by cytosine methylation) or one which cleaves sequences with only guanosine and cytosine (not affected by adenosine methylation).

This section therefore had several aims:

a) To ensure that partial digests were not due to technical problems.

b) To investigate the presence of 6-methyladenosine by comparing the digests produced by *Mbo*I and *Dpn*I. The former does not cleave DNA if the adenosine is methylated i.e. it cleaves the sequence 5'-^{v}GATC-3', whereas the latter will cleave only if the adenosine is methylated i.e. cleaves the sequence 5'-G^{m}A^{v}TC-3' (but not 5'-GA^{v}TC-3').

c) To investigate the presence of 5-methylcytosine by comparing the digests produced by two restriction endonucleases, *Hpa*II and *Msp*I. The former cleaves the sequence 5'-C^{v}CGG-3' but, does not cleave DNA if either of the two cytosines is methylated i.e. 5'-^{m}C^{v}CGG-3' and 5'-C^{vm}CGG-3'are not cleaved, whereas *Msp*I will cleave the sequence whether or not the internal cytosine is methylated will cleave the sequence 5'-C^{vm}CGG-3' and 5'-C^{v}CGG-3'.

d) To examine the DNA of *M. ovipneumoniae* for the presence of methylated bases using high-performance liquid chromatography (HPLC) of nucleosides derived from *M. ovipneumoniae* DNA.

5.2 Materials and Methods.

5.2.1 Materials

A) For Bacterial Restriction Endonuclease DNA Analysis to Detect Base Sequence Methylations.

1)**Restriction Endonucleases:**

*Xho*I (10units/ul) Amersham International, (Buckinghamshire, UK)
*Dpn*I (10units/ul) Bethesda Research Laboratories, (Maryland USA)
*Mbo*I (10units/ul) Bethesda Research Laboratories, (Maryland USA)
*Sau*3a1 (6units/ul) New England Biolabs™, (Massachusetts, USA)
*Msp*I (12units/ul) Amersham International, (Buckinghamshire, UK)
*Hpa*II (12units/ul) Amersham International, (Buckinghamshire, UK)

All digests were conducted using the manufacturers buffers and conditions of digestion.

B) For Using High-Performance Liquid Chromatography to Examine *M. ovipneumoniae* DNA for the Presence of 5-Methylcytosine.

i. For the Preparation of Nucleoside Standards.

1)**0.1% Phosphoric Acid**

Phosphoric Acid (1.685 sp.g)	4.0ml
Distilled Water to	4000.0ml

This solution was filtered through a 0.2u Millipore filter before use.

2)**Nucleoside Standards**

a. **2'-Deoxycytidine** (United States Biochemical Corp)
b. **5-Methyl-2'-Deoxycytidine** (Sigma)
c. **2'-Deoxyadenosine** (United States Biochemical Corp)
d. **2'-Deoxyguanosine** (United States Biochemical Corp)
e. **Thymidine-2-Deoxyriboside** (United States Biochemical Corp)
f. **6-Methylaminopurine-9-Ribofuranoside** (Sigma)

ii. For the Enzymatic Digestion of *M. ovipneumoniae* DNA.

1)**Tris/$MgCl_2$ Buffer** (pH 7.9)

Trizma Base (Sigma)	2.42g
$MgCl_2$ (anhydrous)	0.10g
HCl to pH 7.9	
Distilled Water to	1000.0ml

2)**DNase Solution** (10mg/ml)

Deoxyribonuclease I (Sigma)	0.01g
Distilled Water to	1.0ml

Stored at -20^{o}C.

3)**Phosphodiesterase** (10mg/ml)

Crotalus Atrox (Snake Venom) (Sigma)	0.01g
Distilled Water to	1.0ml

Stored at -20^{o}C.

4)**Alkaline Phosphatase** (10mg/ml)

Alkaline Phosphatase (from calf intestine) (Sigma)	0.01g
Distilled Water to	1.0ml.

Stored at -20^{o}C.

5.2.2 Methods

A) Bacterial Restriction Endonuclease DNA Analysis to Detect Base Sequence Methylations.

M. ovipneumoniae strain L6T3 DNA was isolated, purified, digested with restriction endonucleases and electrophoresed in an agarose gel as described in chapter four.

The concentration of agarose was varied depending on the restriction endonuclease used to cleave the DNA i.e. 0.7% agarose gel concentration was used for DNA digested with *Xho*I (recognizes six-base sequences) and 1.6% agarose gel concentrations were used to digest DNA digested with *Dpn*I; *Mbo*I; *Sau*3aI; *Msp*I; *Hpa*II (all recognise four-base sequences and give rise to smaller fragments than do six-base "cutters").

B) The Use of High-Performance Liquid Chromatography to Examine *M. ovipneumoniae* DNA for the Presence of 5-Methylcytosine.

5.2.2B.i Preparation of Nucleoside Standards.

Each nucleoside standard was dissolved in 0.1% phosphoric acid to give a final concentration of 1%. The solutions were each placed into an Eppendorf microfuge tube and centrifuged for five minutes at 15 500 g to ensure that no particulate matter was present in the sample.

5.2.2B.ii Enzymatic Digestion of *M. ovipneumoniae* DNA.

One strain of *M. ovipneumoniae* (L6T3) was propagated in 2000ml of FM4 Medium. The cells were collected, DNA extracted and purified by ultracentrifugation as described in sections 4.2.2A.ii and 7.2.2A.iii respectively. The DNA was then dialysed against four 2000ml volumes of Tris/$MgCl_2$ Buffer for two days at $4^{o}C$ and assayed for purity and concentration as described in section 4.2.2A.iii.

The method for enzymatic digestion of mycoplasma DNA to nucleosides was a modification of the method used by Ford *et al*, (1980) to digest eukaryotic DNA to nucleosides. Briefly, an aliquot of DNA which contained 250ug of CsCl purified DNA was placed in a 10ml

Nalgene^{TM} centrifuge tube. The volume was adjusted to 1ml with Tris/$MgCl_2$ Buffer. The DNA was initially digested with 10ul of DNase for 60mins at 37^{o}C. It was then reduced to nucleotides by adding 10ul of Phosphodiesterase (snake venom) and incubated for 2hrs at 37^{o}C. 40ul of Alkaline Phosphatase was then added to the solution which was incubated for a further 60mins at 37^{o}C. This removed phosphates from the nucleotides to produce nucleosides. The nucleosides were heated to 65^{o}C for 10mins to inactivate the enzymes. 2.5ml of cold absolute ethanol was added and the tubes were left overnight at -20^{o}C. The precipitated nucleosides were collected by centrifugation at 16 500g for 30mins at 4^{o}C. The supernatant was discarded and the surface or the pellet was washed with 1ml of cold absolute ethanol. The pellet was dried under vacuum for 20mins and resuspended in 250 ul of 0.1% phosphoric acid to give a final nucleoside concentration of 1ug/ul. The nucleosides were placed in a 1.5ml Eppendorf microfuge tube, centrifuged at 15 500g for 3mins and separated on a high performance liquid chromatography column.

5.2.2B.iii High-Performance Liquid Chromatography (HPLC).

All enzymatically produced nucleosides and nucleoside standards were examined using a Shimadzu (LC-4A) high performance liquid chromatograph. A reverse phase 8MBC18u (0.8cmx10cm) radial pak liquid chromatography cartridge (Waters Associates Inc) was compressed in a Radial Compression Module-100 (Waters Associates Inc) and the column was used to separate the nucleosides under isocratic (non-gradient) conditions.

The nucleosides were diluted in 0.1% phosphoric acid to a concentration of 10ng/ul and 25ul samples were injected into the 0.1% phosphoric acid-equilibrated column using a Shimadzu (SIL-2AS) auto injector. The samples were eluted with 0.1% phosphoric acid with a flow rate of 1ml/min at ambient temperature. The ultra-violet spectra of each nucleoside was measured using a Shimadzu (SPD-2AS) spectrophotometric detector set at 273nm and the results were recorded on a Shimadzu Chromatopac (C-R3A) integrator.

5.3 Results.

To determine if technical problems were responsible for causing incomplete digests, or in some cases non-digestion, of *M. ovipneumoniae* DNA (see chapter four), DNA from *M. ovipneumoniae* strain L6T3, which earlier experiments showed was not digested with *Xho*I (Figure 47, lane 5) was mixed with Bacteriophage Lambda DNA and exposed to *Xho*I. The results from this digest are illustrated in Figure 72. Lane 1: Lambda DNA digested with

*Xho*I which produced two DNA fragments (33 498bp and 15 004bp), indicating that the restriction endonuclease and the reaction conditions were satisfactory. Lane 3: strain L6T3 DNA exposed to *Xho*I. No cleavage occurred. Lane 2: Lambda DNA mixed with DNA from L6T3 and digested with *Xho*I. Only the Lambda DNA was cleaved (the higher molecular weight fragment of the cleaved Lambda DNA is obscured by the undigested mycoplasma DNA). These results confirm that *Xho*I fails to cleave *M. ovipneumoniae* strain L6T3, but that this failure is not due to technical problems. It could however, be explained by methylation of bases.

The presence of methylated adenine was investigated using *Mbo*I and *Dpn*I and the results are shown in Figure 73. Lane 1: 1kb ladder (i.e. molecular weight marker); Lane 2: *M. ovipneumoniae* strain L6T3 DNA digested with *Dpn*I (this restriction endonuclease will only cleave DNA if the internal adenine of the recognition sequence in methylated i.e. it cleaves 5'-G^{m}A^{v}TC-3'). Note that no cleavage of the DNA has occurred indicating the absence of methylated adenine at least from the recognition sequence. Lane 3: *M. ovipneumoniae* strain L6T3 DNA digested with *Mbo*I (this restriction endonuclease will only cleave DNA in the absence of methylated adenine in its recognition sequence i.e. it cleaves 5'-^{v}GATC-3'). The DNA has been digested into small fragments indicating that the adenine of the recognition sequence is not methylated. Lane 4: *M. ovipneumoniae* strain L6T3 DNA digested with *Sau*3a1 (this restriction endonuclease digests DNA whether or not the recognition sequence is methylated i.e. it cleaves 5'-^{v}GATC-3'or 5'-^{v}G^{m}ATC-3'). Note that the DNA cleavage pattern in lane 4 is the same as the cleavage pattern in lane 3. This implies the absence of 6-methyladenine.

The methylation of cytosine was investigated using *Msp*I and *Hpa*II. The results are shown in Figure 74. Lane 1: 1kb ladder (i.e.molecular weight marker); Lane 2: *M. ovipneumoniae* strain L6T3 DNA digested with *Msp*I (cleaves the sequences 5'-C^{v}CGG-3' and 5'-C^{vm}CGG-3' irrespective of whether the internal cytosine is methylated or not). Lane 3: *M. ovipneumoniae* strain L6T3 DNA digested with *Hpa*II (this cleaves the sequence 5'-C^{v}CGG-3' only in the absence of cytosine methylation). Note that the pattern resembles that of lane 2 but, the DNA is incompletely digested i.e. a high molecular weight fragment remains. *Msp*I (lane 2) cleaved the DNA into similar fragments but, without the uncleaved upper band, suggesting that (if there is no technical reason for the result) 5-methylcytosine is present in some (probably only a small minority) of the recognition sequence in the DNA.

To confirm that the partial digestion of the DNA digested with *Hpa*II was not due to a technical problem e.g. low restriction endonuclease activity. DNA (strain L6T3) was digested with a fresh batch of the same restriction endonucleases for a longer period. The results are shown in Figure 75. Lane 1: 1kb ladder (i.e. molecular weight marker); Lane 2: DNA from *M. ovipneumoniae* strain L6T3 digested completely with *Msp*I; Lane 3: strain L6T3 DNA digested with *Hpa*II for 60mins; Lane 4: strain L6T3 DNA digested with *Hpa*II, using twice the manufacturer's recommended concentration and twice the reaction time; Lane 5: DNA from strain L6T3 digested with both *Hpa*II and *Msp*I; Lane 6: undigested DNA from strain L6T3; Lane 7: undigested Lambda DNA used as a molecular weight marker. Note that increasing the reaction time and enzyme concentration did not change the result i.e. the uncleaved band in lanes 3 and 4 is unlikely to be due to technical reasons.

M. ovipneumoniae strain L6T3 DNA, which was totally uncleaved by *Xho*I (see above), was degraded to nucleotides by a combination of two enzymes i.e. DNase and phosphodiesterase (snake venom). The nucleotides were further degraded to nucleosides using alkaline phosphatase. The nucleosides were then examined by HPLC. The results are shown in Figure 76. Figure 76b is a tracing which represents a mixture of standards (2'deoxycytidine, 5-methyl-2'-deoxycytidine, 2'-deoxyadenosine, 2'-deoxyguanosine, thymidine-2-desoxyriboside and 6-methylaminopurine-9-ribofuranoside) which were identified in experiments (data not shown) which established their time of elution in the conditions used.

The tracing shown in Figure 76c represents a control with no DNA. The sensitivity of this method, combined with the contamination of the biological reagents used (i.e. the alkaline phosphatase and snake venom) produced peaks which do not correspond to nucleosides. These peaks contaminate the tracing in Figure 76a and should be disregarded.

Figure 76a represents those nucleosides of *M. ovipneumoniae* strain L6T3 (plus tracing contaminants from Figure 76c, which are disregarded). Note that a peak at 10.3mins (corresponding within experimental error to 10.7mins of the standard) is visible. This peak represents 5-methylcytosine.

Figure 72

Demonstration of the activity of *Xho*I and its inability to cleave L6T3 DNA. Lane 1: Lambda DNA cleaved with *Xho*I; Lane 2: L6T3 DNA mixed with Lambda DNA and exposed to *Xho*I. Lane 3: L6T3 DNA exposed to *Xho*I, but not cleaved.

Note that *Xho*I is active in lanes 1 and 2 although the upper Lambda fragment in lane 2 is obscured.

*Xho*I failed to cleave L6T3 DNA (lane 3). This is due to the presence of methylated bases.

Technical Note:

The digested DNA was separated on a 1.6% agarose gel.

Figure 73

BRENDA of *M. ovipneumoniae* strain L6T3 DNA digested with enzymes which discriminate between methylated and non-methylated adenine.

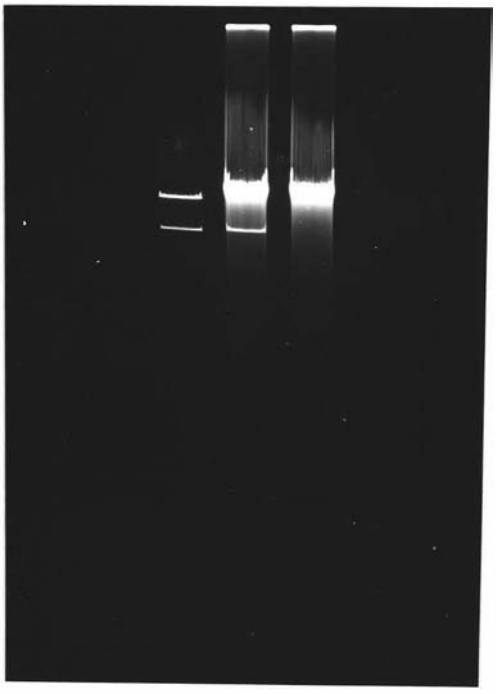
Lane 1: 1kb ladder (i.e. molecular weight marker); Lane 2: DNA exposed to *Dpn*I (cleaves 5'-G^{m}A^{v}TC-3' only); Lane 3: DNA cleaved with *Mbo*I (cleaves 5'-^{v}GATC-3' only); Lane 4: DNA cleaved with *Sau*3aI (cleaves 5'-^{v}GATC-3' and 5'-^{v}G^{m}ATC-3').

Note that *Dpn*I (Lane 2) did not cleave the DNA, hence methylated adenine is not present in the DNA.

Lanes 3 and 4 show identical digests which also indicates the absence of methylated adenine.

Technical Note:

The digested DNA was seperated on a 1.6% agarose gel.



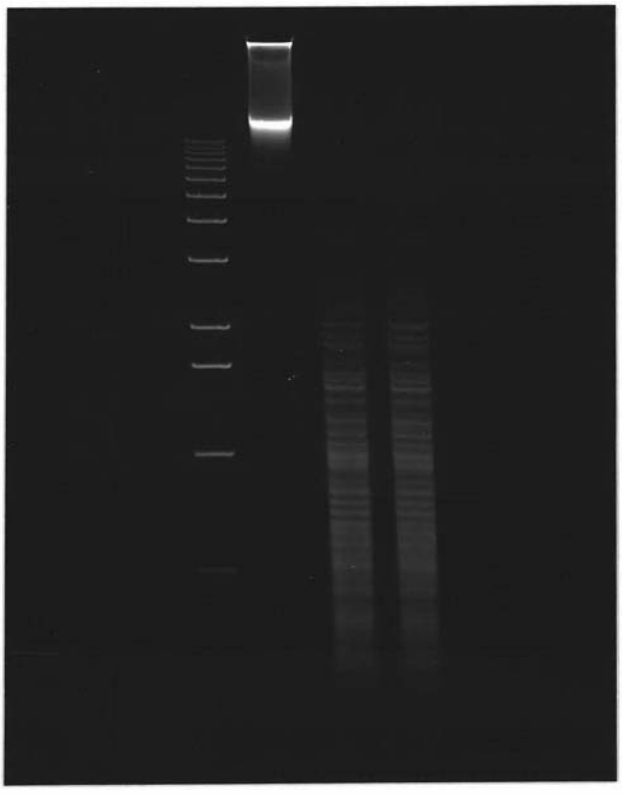


Figure 74

BRENDA of *M. ovipneumoniae* strain L6T3 DNA digested with enzymes which discriminate between methylated and non-methylated cytosine.

Lane 1: 1kb ladder (i.e. molecular weight marker); Lane 2: DNA totally cleaved with *Msp*I (cleaves 5'-C^{vm}CGG-3' and 5'-C^{v}CGG-3'); Lane 3: DNA cleaved with *Hpa*II (cleaves 5'-C^{v}CGG-3' only).

Note: The digests are identical apart from an uncleaved band in lane 3. This band represents DNA which contains 5-methylcytosine.

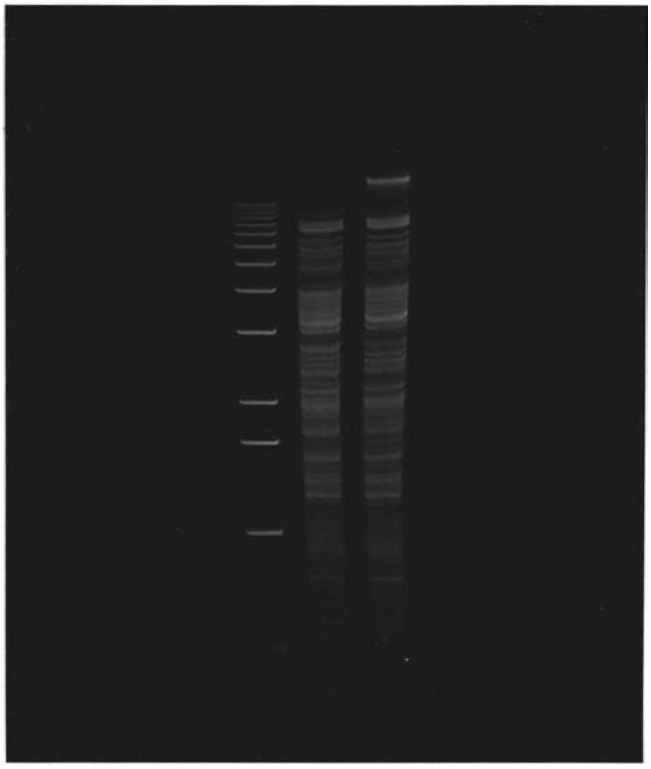
Technical Note:

The digested DNA was seperated on a 1.6% agarose gel.

Figure 75

Further investigation (see Figure 74) of *M. ovipneumoniae* strain L6T3 DNA cleaved by *Msp*I and *Hpa*II for longer periods and/or increased concentrations. Lane 1: 1kb ladder (i.e. molecular weight marker); Lane 2: L6T3 DNA cleaved with *Msp*I (cleaves 5'-C^{vm}CGG-3' and 5'-C^{v}CGG-3'); Lane 3: L6T3 DNA cleaved with *Hpa*II (cleaves 5'-C^{v}CGG-3' only); Lane 4: L6T3 DNA cleaved with double the concentration of *Hpa*II for twice the digestion period; Lane 5: L6T3 DNA cleaved with both *Hpa*II and *Msp*I; Lane 6: L6T3 DNA undigested; Lane 7: Lambda DNA undigested (molecular weight marker).

Note that the high molecular weight uncleaved band in lane 3 remains in lane 4. This confirms the results shown in Figure 74 and further shows that the high molecular weight band is not due to partial digestion of DNA.



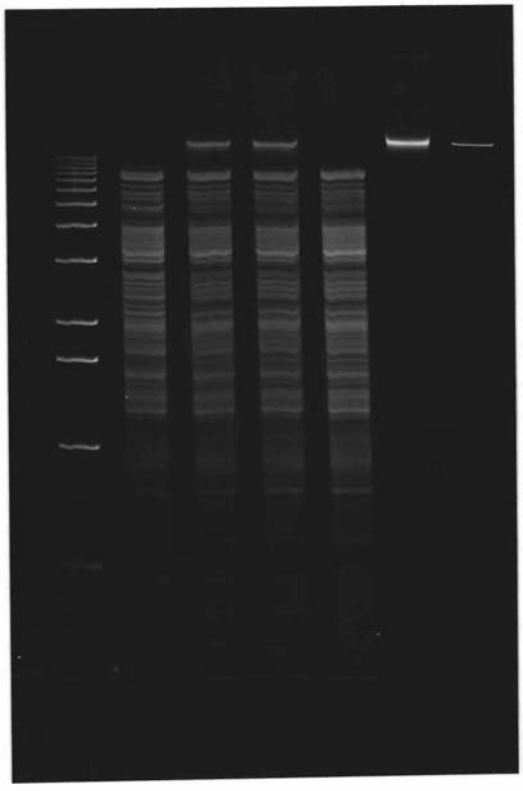


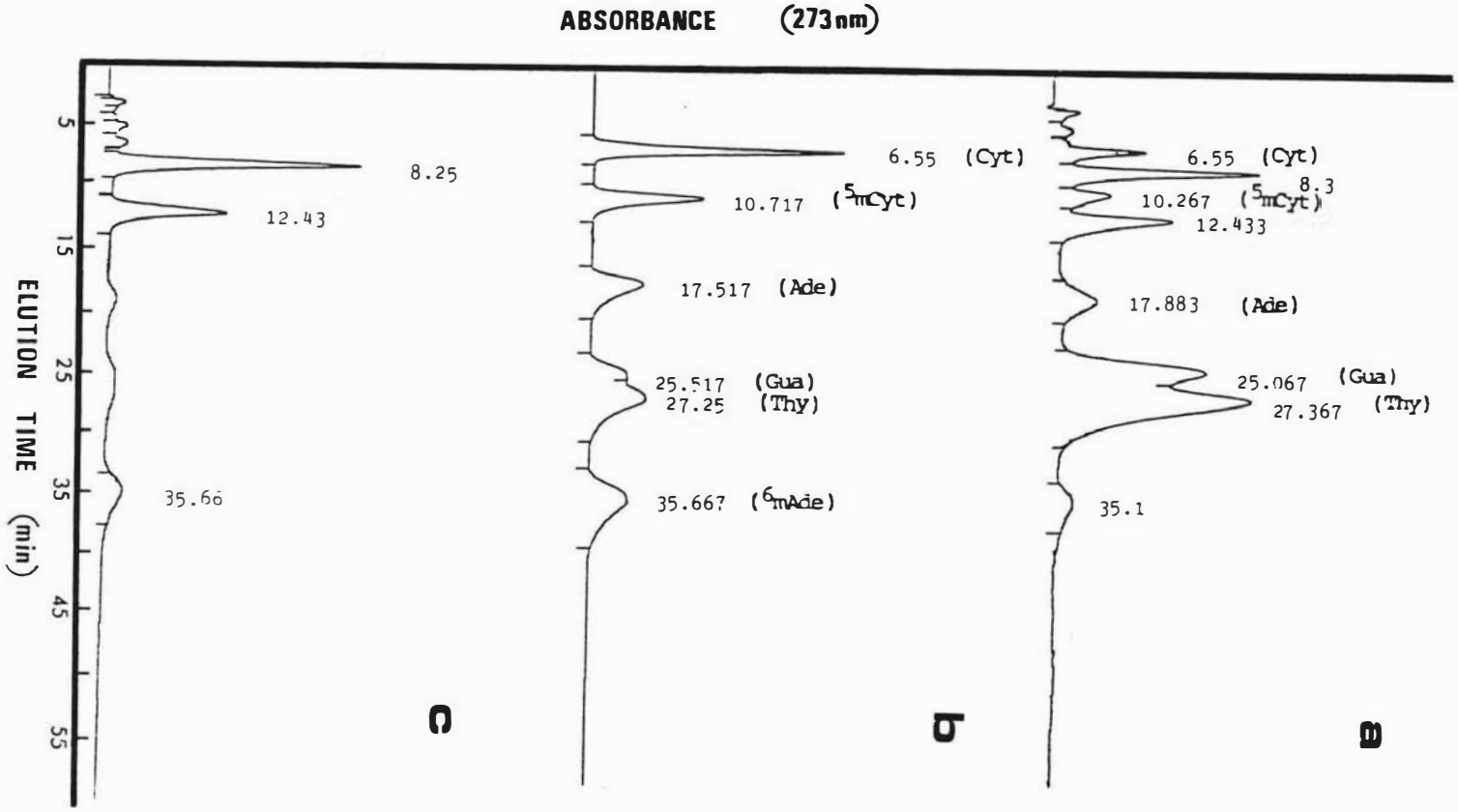
Figure 76 (a, b and c)

a) *M. ovipneumoniae* strain L6T3 DNA degraded to nucleosides and examined by HPLC. Note the elution of eight major peaks. Five of these peaks were identified as nucleosides which includes 5-methylcytosine.

b) Standard mixtures of six nucleosides [i.e. 2'-deoxycytidine (Cyt), 5-methyl-2'-deoxycytidine (^{5}mCyt), 2'-deoxyadenosine (Ade), 2'-deoxyguanosine (Gua), thymidine-2-desoxyriboside (Thy) and 6-methylaminopurine-9-ribofuranoside (^{6}mAde)] previously identified in experiments (data not shown) which established their time of elution in the conditions used.

c) This tracing represents a control with no DNA. Note that three peaks are detected which do not represent nucleosides but are contaminants of biological reagents used to degrade DNA to nucleosides. These contaminants are also seen in Figure 76a but not in the nucleoside standards (Figure 76b).

In particular note the 5-methylcytosine peak in the mycoplasma DNA (tracing a).


ABSORBANCE (273nm)
ELUTION TIME (min)
5
15
25
35
45
55
8.25
12.43
35.66
c
6.55 (Cyt)
10.717 (5mCyt)
17.517 (Ade)
25.517 (Gua)
27.25 (Thy)
35.667 (6mAde)
b
6.55 (Cyt)
8.3
10.267 (5mCyt)
12.433
17.883 (Ade)
25.067 (Gua)
27.367 (Thy)
35.1
a

5.4 Discussion.

Before proceeding with this discussion it might be useful to explain the recording of an endonuclease recognition system. The recognition is always double-stranded DNA. The sequence however is recorded as one strand only with the 5' end at the left of the recognition sequence e.g. *Hpa*II is recorded as: 5'-CCGG-3' which means:

5'-CCGG-3'
3'-GGCC-5'

Bases which are not at either end of this sequence are referred to as the internal bases.

Note that the recognition sequence for *Hae*III is: 5'-GGCC-3' which written in full is:

5'-GGCC-3'
3'-CCGG-5'

This represents a totally different sequence from 5'-CCGG-3'.

In original studies of *M. ovipneumoniae* DNA using restriction endoneclease (Ionas, 1985), *Eco*RI only was used to compare isolates. All isolates apparently gave complete digestion and the question of the methylation of bases was therefore not considered. The sequence involved (5'-GAATTCC-3') contains (but is not rich in) cytosine and *Eco*RI will not cut if the cytosine, or for that matter the adenosine, is methylated. Clearly not all cytosine or adenosine bases are methylated.

Results obtained with *Xba*I (5'-T^{V}CTAGA-3'), *Bgl*II (5'-A^{V}GATCT-3') and *Hind*III (5'-A^{V}AGCTT-3'), indicates complete "cutting" of DNA. All of these enzymes have recognition sites containing cytosine. Apparently complete "cutting" also occurred with *Msp*I (5'-C^{V}CGG-3') which cleaves a cytosine-rich sequence (see below).

Extension of this study by the use of other enzymes gave what at first sight were confusing results i.e. complete digests of some isolates (see Tables VI and VII) and partial digests of others. We originally attributed these partial digests to technical problems but, following extensive investigations (see chapter four), we were ultimately forced to consider other possibilities.

The recognition sequence of enzymes which gave partial digests were 5'-CC^{V}GG-3'; 5'-GGTAC^{V}C-3'; 5'-G^{V}TCGAC-3'; 5'-G^{V}GATCC-3'; 5'-CCC^{V}GGG-3'; 5'-C^{V}TCGAC-3'and 5'-GG^{V}CC-3'. In contrast to this, the enzymes which in our hands always gave apparently complete digestion had a recognition sequence which contained one cytosine only i.e. 5'-G^{V}AATTC-3'; 5'-T^{V}CTAGA-3'; 5'-A^{V}GATCT-3'and 5'-A^{V}AGCTT-3'. This suggests that cytosine methylation of some sequences in the DNA might occur, because (at least for the enzymes listed above) this would inhibit DNA cleavage and account for partial digestion of the DNA.

The apparent exception to the above statement that enzymes which had a cytosine-rich recognition sequence gave partial digests is *Msp*I which recognizes 5'-CC^{V}GG-3' and gave complete digestions. *Msp*I however, digests not only unmethylated 5'-C^{V}CGG-3' but also cleaves the sequence 5'-C^{vm}CGG-3' i.e. it is indifferent to the methylation of the internal cytosine. The fact that this enzyme gave complete digestion of DNA in contrast to enzymes which cleave a cytosine-rich sequence but are inhibited by cytosine methylation gives strong support to the idea that cytosine methylation occurs in some isolates.

*Msp*I and *Hpa*II both recognize the sequence 5'-C^{V}CGG-3', but *Hpa*II, unlike *Msp*I is inhibited by methylation. Figure 74 shows that *M. ovipneumoniae* isolate L6T3, while giving similar digests with *Hpa*II and *Msp*I, nevertheless shows that *Hpa*II gives only a partial digest. This result is unchanged when higher concentrations of *Hpa*II were used for longer periods (>60mins) and in concentrations where *Hpa*II was shown to be not inhibited (i.e. digesting bacteriophage lambda DNA). We conclude that technical reasons do not account for this partial digest and confirms the conclusion that cytosine methylation occurs in some *M. ovipneumoniae* strains especially L6T3.

The conclusion that cytosine methylations occur in *M. ovipneumoniae* does not imply that all strains have cytosine methylations or that strains which have cytosine methylation have it to an equal extent. Thus, when four *M. ovipneumoniae* isolates were examined by *Hpa*II, two showed complete and two showed partial digestion (Figure 41 and Table VI).

Only one *M. ovipneumoniae* isolate (L6T3) showed no cleavage by restriction enzymes e.g. by *Xho*I and *Hae*III, so the DNA of this isolate probably represents the maximum methylated cytosine detected in this study. It was used to examine *M. ovipneumoniae* DNA by HPLC and thus to demonstrate the presence of 5-methylcytosine.

We now consider evidence that adenine methylation does not occur at least in the strains tested and then point out evidence based on restriction endonuclease cleavage that the heterogeneity of DNA restriction patterns does not depend upon methylation but must represent base sequence differences.

*Dpn*I cleaves 5'-G^{m}A^{v}TC-3' but only if the adenine is methylated, *Mbo*I cleaves 5'-^{v}GATC-3' provided the adenine is not methylated and *Sau*3aI cleaves 5'-^{v}GATC-3' whether or not the adenine is methylated. The strain of *M. ovipneumoniae* (L6T3) which failed to cleave with *Xho*I and *Hae*III was examined by the above three enzymes. The results (Figure 73) showed that since *Dpn*I failed to cleave the DNA, adenine methylation is absent. The other two enzymes (*Mbo*I and *Sau*3aI) gave identical patterns. This could only occur if no adenine methylation was present in any 5'-GATC-3' sequence i.e. this confirms the absence of adenine methylation.

Much of the work of this thesis is based on the concept that different restriction digests reflect differences in base sequence. However, if a variable proportion of the cytosine is methylated and the cytosine of different sequences is methylated in different isolates, it follows that different restriction endonuclease digests could represent variation in methylation patterns, rather than different base sequences. We have several pieces of evidence to the contrary (see later) but one of these is considered now.

As noted above, adenine methylation was not detected and this was confirmed by HPLC (see later). If methylated adenosine is absent, differences in restriction endonuclease digests must reflect differences in base sequences. This was tested using *Dra*I which cleaves 5'-TTT^{v}AAA-3' and it was found that the isolates were (as with other enzymes) totally different (Figure 40). We conclude that different restriction endonuclease patterns do indeed represent different DNA sequences and not a function of variations in the methylation of the DNA of different isolates. This point is returned to in the final discussion.

DNA from *M. ovipneumoniae* isolate L6T3 showed the maximum methylation as assessed by restriction endonucleases analysis. This isolate was therefore chosen for assaying for the presence of methylated bases using HPLC analysis. The DNA was degraded to nucleotides using DNase and phosphodiesterase and then to nucleosides using alkaline phosphatase. This procedure introduced three contaminant peaks into the DNA (Figure 76c). These peaks

should be ignored in Figure 76a. Figure 76b represents several standards including 5-methylcytosine and 6-methyladenine. Figure 76a represents *M. ovipneumoniae* DNA. Note that it contains a peak corresponding closely to 5-methylcytosine and furthermore no 6-methyladenine was detected i.e. the peak at 35.1mins in Figure 76a is less than the contaminant peak Figure in 76c.

The proportion of cytosines which are methylated can be estimated from the peak height. This suggests that 25 to 30% of the cytosines of this strain are methylated. This is an approximate figure and is to some extent affected by different absorbances of U.V. light by different nucleosides.

CHAPTER SIX

Examination of *M. ovipneumoniae* Strains for the Presence of Plasmids.

6.1 Introduction.

Heterogeneity of DNA cleavage patterns of *M. ovipneumoniae* could be due to differences in base sequence or (at least in part) to the presence of different plasmids. This section examines eight *M. ovipneumoniae* isolates, which differ totally in their cleavage patterns, for the presence of extrachromosomal DNA.

6.2 Materials and Methods.

6.2.1 Materials

A) For the Detection of Small Plasmids.

All materials required for the extraction of DNA from *M. ovipneumoniae* strains are described in chapter four, with the exception of Ribonuclease-A:

1)**Ribonuclease-A** (RNase)

Ribonuclease (Type 1-A) (from bovine pancrease)	2.0mg
Distilled Water to	10.0ml.

This stock solution was incubated at 90^{o}C for 10mins to destroy deoxyribonucleases and was stored at -20^{o}C.

2)**Bromophenol Blue Tracking Dye**: see appendix

B) For the Detection of Large Plasmids.

1)**FM4 Medium**: see appendix

2)**Phosphate Buffered Saline**: see appendix

3)**Brain-Heart Infusion Broth** (BHI Broth)

Brain-Heart Infusion (Difco)	3.7g
Distilled Water to	100.0ml.

The broth was autoclaved at 121^{o}C for 15mins.

4)**BHI Agar**

Agar (Davis)	0.8g
BHI Broth (prepared fresh) to	100.0ml

This solution was autoclaved at 121^{o}C for 15mins and 20ml aliquots were dispensed into standard size sterile petri dishes.

5)**Tris Borate Buffer** (10xTBB) pH 8.2

Trizma Base (Sigma)	108.0g
EDTA (sodium salt)	9.3g
Boric Acid	55.0g
Adjust to pH 8.2	
Distilled Water to	1000.0ml

Stored at room temperature and diluted 10 fold for use (1xTBB).

6)**Ribonuclease-A** (RNase) 10mg/ml: see above

7)**Solution #1**

Ficoll	1.0g
Bromophenol Blue	5.0mg
RNase Solution (10mg/ml)	0.01ml
Lysozyme (Egg White-Sigma)	2.0mg
1xTBB to	10.0ml

Stored at 4oC and warmed to room temperature before use.

8)**Solution #2**

Sodium Dodecyl Sulphate	20.0mg
Ficoll	1.0g
1xTBB to	10.0ml

Stored at room temperature.

9)**Solution #3**

Sodium Dodecyl Sulphate	20.0mg
Ficoll	0.5g
1xTBB to	10.0ml

Stored at room temperature.

10)**Tris-EDTA Buffer** (TEB) pH 8.0

Trizma Base (Sigma)	1.21g
EDTA (sodium salt)	0.34g
Adjust to pH 8.0	
Distilled Water to	1000.0ml

11)**Ethidium Bromide** (10mg/ml)

Ethidium Bromide (Bio-Rad)	100.0mg
Distilled Water to	10.0ml

This solution was stored in a dark bottle at room temperature. 25ul of this solution was diluted to 500ml with distilled water to give a final concentration of 0.5ug/ml for staining DNA in agarose gels.

6.2.2 Methods

A) The Detection of Small Plasmids.

DNA from eight *M. ovipneumoniae* strains and one *E. coli* (V517) strain (contains eight plasmids) was extracted, assayed for concentration and purity (260nm:280nm ratio) as described in chapter four.

An aliquot of DNA containing 15ug from each of the eight *M. ovipneumoniae* strains and from the *E. coli* V517 strain was incubated at 37^{o}C for 30mins with 2ul of RNase (10mg/ml). 10ul of Bromophenol Blue Tracking Dye (containing 10% glycerol) was then added to the DNA solution which was placed in the wells of a 0.8% agarose gel (see chapter four-gel electrophoresis).

The samples were electrophoresed at 80 volts until the tracking dye moved a distance of 160cm. The results were photographed as described in chapter four.

B) The Detection of Large Plasmids.

General:

The method used was basically that of Eckhardt (1978). However, since a number of technical modifications were made and a succession of minor difficulties were encountered and overcome, the technique used here is recorded in detail.

6.2.2B.i The Gel System.

A horizontal slab-gel was used

The agarose gel slab was poured between two perspex spacers set inside the gel apparatus which kept the agarose solution in place until it set. Wells in the gel solution were made by inserting a "comb" at the cathode end of the gel. After the agarose had set, the spacers and "comb" were removed. Buffer was then poured into the reservoir tanks at both ends, up to the level of the gel.

6.2.2B.ii Preparation of a 0.6% agarose gel.

0.6g of agarose (Ultrapure grade, Bio-Rad) was added to 100ml of TBB and boiled for 5mins in a 250ml round-bottomed flask fitted with a 40cm reflux column. The agarose was left to cool to approximately 50^{o}C before being poured into an electrophoresis tank (see Figure 77). A "comb" was inserted in the agarose solution prior to gelation, forming the wells for sample application. The cast gel was approximately 12.0cm x 10.0cm x 0.8cm in dimension.

Figure 77

Preparation of Eckhardt gels for the detection of large plasmids in *M. ovipneumoniae* lysates.

a) The electrophoresis apparatus.

b) Two lenghts of perspex were positioned at opposite ends of the raised portion of the electrophoresis tank. These strips were tight fitting.

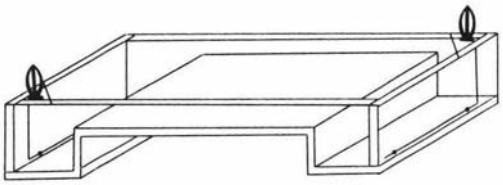
c) Molten agarose was poured between the two perspex strips and a "comb" to cast sample application wells was inserted into the molten agarose at the cathode end of the electrophoresis tank. Notches in the perspex held the "comb" in position.

d) Once the agarose had solidified the "comb" and the two perspex strips were removed leaving an agarose gel approximately 12cm x 10cm x 0.8cm in dimension.

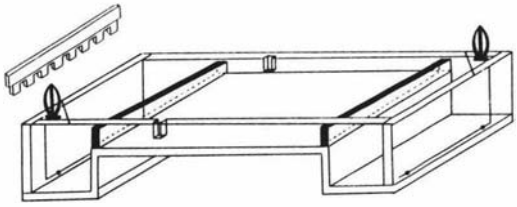
e) Washed mycoplasma cells were then loaded into each well and lysed (see text for procedure).

f) The gel was then transferred to a cold-room ($4^{o}C$) and 1xTBB was carefully added to the electrophoresis tank until the gel was submerged to a depth of 1-2mm. The samples were electrophoresed initially at 20 volts for 60mins and then at 100 volts until the dye marker present in the lysate reached the anode end of the gel.

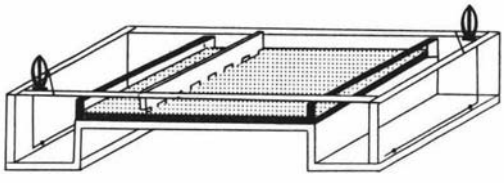
a



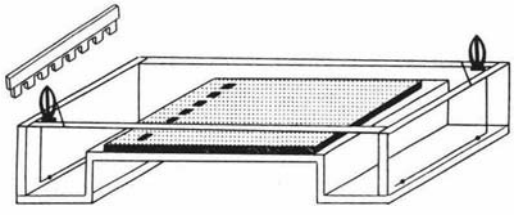
b



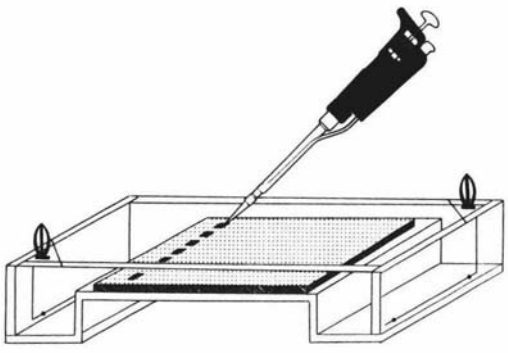
c



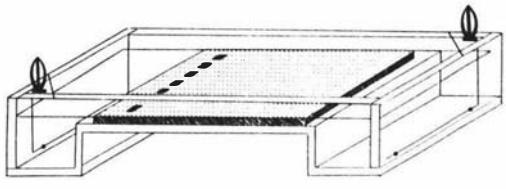
d



e



f



6.2.2B.iii Propagation of Isolates.

A 1ml aliquot (stored at -80^{o}C) from each of eight *M. ovipneumoniae* strains derived from sheep on different farms was inoculated into 10ml of FM4 Medium and incubated overnight at 37^{o}C. An *E. coli* strain V517 (containing eight plasmids) was used as a control. An isolated colony from a BHI plate was inoculated into 3ml of BHI broth and incubated for 2hrs at 37^{o}C.

6.2.2B.Iv Preparation of Samples.

1ml from each of the *M. ovipneumoniae* cultures and 0.2ml from the *E. coli* culture were placed in a 1.5ml Eppendorf microfuge tube and centrifuged at 14 500g for 60sec. The supernatants were discarded and the pellets resuspended in 1ml PBS and re-centrifuged. This washing procedure was repeated once more. The cells were then resuspended in 20ul of Solution #1 and the pellet was carefully suspended using a glass rod. The cell suspension was added to the wells in the 0.6% agarose and left to stand for 10mins. 20ul of Solution #2 was added to each of the wells and carefully mixed with a rounded pasteur pipette tip, using only a single back-and-forth motion. An aliquot of Solution #3 was carefully layered over the top of the wells, so that the contents of each well was level with the top of the gel.

The apparatus was transferred to a cold-room (4^{o}C) and 1xTBB buffer was carefully added to submerge the gel to a depth of 1-2mm without disturbing the contents of the wells.

6.2.2B.v Electrophoresis Conditions.

The 0.6% agarose gel was electrophoresed at 20 volts (samples at the cathode end) for one hour, the voltage was increased to 100 volts and run until the bromophenol blue dye (present in Solution #1) reached the anode end of the gel.

6.2.2B.vi Staining the Gel.

After electrophoresis of the sample, the gel was removed from the apparatus and stained by immersing in a Ethidium Bromide for 30mins. The gel was then placed in 500ml of distilled water to remove excess ethidium bromide which had not entered into the gel. The gel was placed on a UV transilluminator and the DNA bands were observed and photographed.

6.2.2B.vii Gel Photography.

The results were recorded on 12.5cm x 10.0cm Tri-X film and developed as described in section 4.2.2A.vi.

6.3 Results.

The examination of eight *M. ovipneumoniae* strains for the presence of small plasmids is shown in Figure 78. Lane 1: 1kb ladder (i.e. a molecular weight marker); Lanes 2 to 9: *M. ovipneumoniae* strains (1, 2, 4, 5, 10, 37E, MPP74 and L3/C3 respectively). All strains were obtained from sheep on different farms in New Zealand, except for strain 10 (lane 6) which is an Australian strain. No plasmids were detected in any of these *M. ovipneumoniae* strains; Lane 10: *E. coli* strain V517 (contains eight plasmids) was used as a control. Note that more than eight plasmid bands are shown in the *E. coli* lane, this was caused by the nicking of one of the plasmid DNA strands in the processing of the DNA. This causes a proportion of the plasmids to change their configuration from covalently closed circular (CCC) to open circular (OC), which changes the migration rate. Note that two faint bands adjacent to the wells are present in Lanes 2 and 10. These are not plasmids but are artefacts which commonly occur.

The examination for the presence of large plasmids, using the Eckhardt method is shown in Figure 79. Lanes 1 to 8: *M. ovipneumoniae* strains (1, 2, 4, 5, 10, 37E, MPP74 and L3/C3), no large plasmids were detected; Lane 9: *E. coli* strain V517 used as a control which shows all eight plasmids.

Figure 78

Examination of eight *M. ovipneumoniae* strains for the presence of small plasmids. Lane 1: 1kb molecular weight ladder; Lane 2: strain 1; Lane 3: strain 2; Lane 4: strain 4; Lane 5: strain 5; Lane 6: strain 10; Lane 7: strain 37E; Lane 8: strain MPP74, Lane 9: strain L3/C3; Lane 10: *E. coli* strain V517.

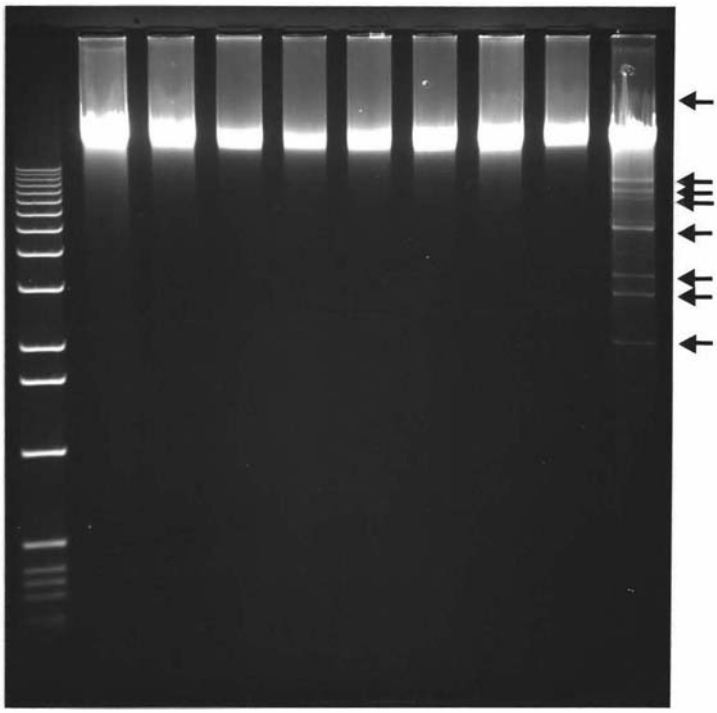
Note that no small plasmids were observed in any of the eight *M. ovipneumoniae* strains examined. The *E. coli* strain, used as a control, shows plasmids (see arrows), some fainter bands seen in this lane represent plasmids that have changed their configuration from a covalently closed supercoiled circle to an open non-supercoiled circle. This changes the migration rate.

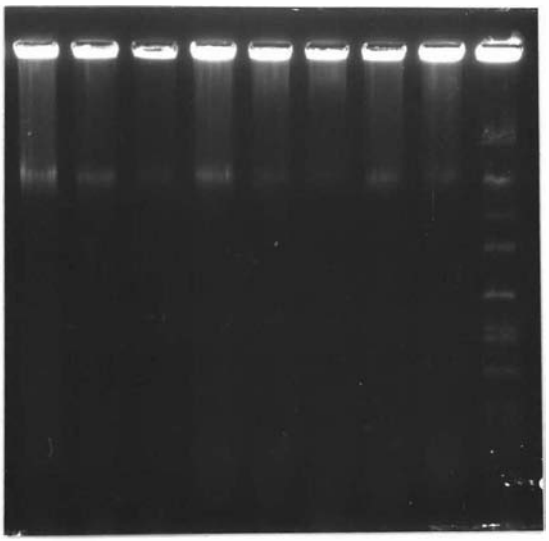
A band present at the top of lanes 2 and 10 are not plasmids but are common artefacts.

Figure 79

Examination of eight *M. ovipneumoniae* strains for the presence of large plasmids using the Eckhardt method. Lane 1: strain 1; Lane 2: strain 2; Lane 3: strain 4; Lane 4: strain 5; Lane 5: strain 10; Lane 6: strain 37E; Lane 7: strain MPP74, Lane 8: strain L3/C3; Lane 9: *E. coli* strain V517.

The single band seen (lanes 1 to 9) represents chromosomal DNA, thus no plasmids were detected in any of the eight *M. ovipneumoniae* strains examined. The *E. coli* strain, used as a control, shows all eight of its plasmids.





6.4 Discussion.

No small or large plasmids were detected in any of the *M. ovipneumoniae* strains. This implies that the heterogeneity seen in the BRENDA cleavage patterns is not due to the presence of extrachromosomal elements.

CHAPTER SEVEN

Investigation of the Relatedness of *M. ovipneumoniae* using DNA-DNA Hybridization.

7.1 Introduction.

This thesis is concerned largely with the heterogeneity of *M. ovipneumoniae* isolates and much of the work examines the heterogeneity of DNA using restriction endonucleases. Unfortunately, the heterogeneity appears to be so extreme that the relatedness of the DNA of many isolates might appear to be zero in the sense that no common bands were seen. However, this is not plausible because, by definition, much DNA relatedness must occur within a species. The standard way to measure the relatedness of DNA both between related species or within a species, is by DNA homology which can be estimated by DNA-DNA hybridization. In this section we investigate DNA-DNA homology between *M. ovipneumoniae* isolates and between *M. ovipneumoniae* and some other micro-organisms i.e. *M. pneumoniae*, *M. arginini*, *A. laidlawii* and *E. coli*.

7.2 Materials and Methods.

7.2.1 Materials

A) For DNA-DNA Hybridization of *M. ovipneumoniae* Isolates and Other Members of the Class *Mollicutes*.

i. For the Propagation of *M. ovipneumoniae*, *M. arginini*, *M. pneumoniae* and *A. laidlawii* Strains.

1)**FM4 Medium**: see appendix

2)**FM4A Medium**: see appendix

3)**Modified SP-4 Medium**

Brain-Heart Infusion	3.5g
Bacto-Peptone	5.3g
Bacto-Tryptone	10.0g
Glucose	5.0g
RPMI 1640 Tissue Culture Supplement	1x sachet
Glutamine	0.3g
Yeast Autolysate	10.0g
Bovine Serum	170.0ml
Benzyl Penicillin	10^6 units
NAD	0.1g
Phenol Red (0.1% aqueous)	20.0ml
Distilled Water to	1000.0ml

The pH of the medium was adjusted to pH 7.5 and filter sterilized through a sterile 0.2um pore size filter and stored at 4^{o}C.

4)**Brain-Heart Infusion Broth** (Difco)

BHI	37.0g
Distilled Water to	1000.0ml

Autoclaved at 121^{o}C for 15mins and stored at 4^{o}C.

5)**Phosphate Buffered Saline** (PBS): see appendix

6)**Tris-EDTA Solution**: see appendix

ii. For DNA Extraction.

1)**Sodium Dodecyl Sulphate** (25%)

SDS	25.0g
Distilled Water to	100.0ml

Stored at room temperature.

2)**Lysozyme** (10mg/ml)

Lysozyme (from egg white)	0.1g
Distilled Water to	10.0ml

Stored at -20^{o}C. Lysozyme was used only in the lysis of *E. coli* strain V517 which was used as a negative control.

3)**Protease** (10mg/ml)

Protease (Type XIV)	0.1g
Distilled Water to	10.0ml

This stock solution was incubated at 37^{o}C for 2hrs to self-digest impurities (e.g. DNase) and was stored at -20^{o}C.

4)**Ribonuclease A** (2mg/ml)

Ribonuclease (Type 1-A) (from bovine pancrease)	2.0mg
Distilled Water to	10.0ml

This stock solution was incubated at 90^{o}C for 10mins to destroy deoxyribonucleases and was stored at -20^{o}C.

5)**5M Sodium Perchlorate**

Sodium Perchlorate	70.23g
Distilled Water to	100.0ml

This solution was stored at room temperature.

6)**Saline Tris-EDTA Buffer (STE)** : see appendix

7)**Tris-EDTA Solution**: see appendix

8)**TE Buffer**: see appendix

9)**Extraction Mixture**

Phenol/Chloroform/Isoamyl Alcohol were mixed in a ratio of 25:24:1 respectively. This solution was saturated by adding 1/10 its total volume of (1x) Saline Tris-EDTA Buffer. It was freshly prepared before use.

III. For Purification of DNA by CsCl Density Gradient Ultracentrifugation.

1)**1.4M Phosphate Buffer**: see appendix

2)**0.2M Phosphate Buffer**

1.4M Phosphate Buffer	100.0ml
Distilled Water to	500.0ml

3)**TE Buffer**: see appendix

4)**Ethidium Bromide Solution** (10mg/ml)

Ethidium Bromide (Boehringer Mannheim)	0.01g
Distilled Water to	10.0ml

Stored in a dark bottle at room temperature.

5)**1M Tris Buffer**: see appendix

6)**Isopropanol Solution** (for extracting ethidium bromide)

A saturated solution of CsCl in 1M Tris Buffer pH 7.5 was prepared. Isopropanol was added and mixed vigorously. Three layers separated: CsCl (bottom), tris buffer (middle) and isopropanol on top. The isopropanol layer was removed and used to extract ethidium bromide from the centrifuged DNA preparation.

Iv. For the Determination of:

a. **The Tm of *M. ovipneumoniae* DNA and Other Micro-organisms.**
b. **The Purity of the DNA by Measuring its Hyperchromatic Shift.**

1)**20xSSC Buffer**

Sodium Chloride (Analytical Grade)	8.77g
Sodium Citrate (Analytical Grade)	0.44g
Adjust to pH 7.0	
Distilled Water to	1000.0ml

Dilute 20-fold before use.

v. For the Preparation of Radioactive ("Labelled") DNA Using the Nick-Translation Method.

1)**Stock Solutions**

a. **0.2M EDTA:** see appendix

b. **1M Trls Buffer:** see appendix

c. **1M $MgCl_2$**

$MgCl_2$ (anhydrous)	9.52g
Distilled Water to	100.0ml

d. **1M NaCl**

NaCl	5.84g
Distilled Water to	100.0ml

2)**10x Reaction Buffer** (for *Hae*III digestion)

1M Tris	1.2ml
1M $MgCl_2$	2.0ml
β-Mercaptoethanol	0.14ml
Distilled Water to	20.0ml

3)**Deoxynucleotide Triphosphates***

dATP 50ug/ml

dGTP 50ug/ml

dTTP 50ug/ml

*In 50mM Tris-HCl (pH 7.5) and stored at -20°C.

4)**DNase I Solution**

Deoxyribonuclease I	0.05mg
0.1M $MgCl_2$ to	10.0ml

Stored at -20°C.

5)**DNase Dilution Buffer**

Trisma Base (Sigma)	0.16g
Nuclease-free Bovine Serum Albumin	0.10g
Adjust to pH 7.5	
Distilled Water to	100.0ml

Stored at 4°C.

6)**10mCi/ml dCTP [alpha-^{32}P]** (New England Nuclear).

Stored at -20°C.

7)**DNA Polymerase I.**

3 units/ul in 0.1M Sodium Phosphate Buffer (pH 7.2), 50% Glycerol (v/v), 1.0M Dithiothreotol (Boeringer Mannheim).

Stored at -20°C.

8)**0.2M EDTA**: see appendix

vi. For the Removal of Unincorporated dCTP [alpha-^{32}P] Following "Nick Translation" Using a "Minispin Column".

1)**Column Buffer**

1M Tris-HCl (pH 8.0)	1.0ml
0.2M EDTA	0.05ml
β-mercaptoethanol	0.03ml
Distilled Water to	100.0ml

2)**Pre-Swollen Sephadex G40-50**

Sephadex G40-50	1.0g
Column Buffer	20.0ml

Sephadex G40-50 was suspended in Column Buffer and left overnight at 4^{o}C to swell. If longer storage was required, 0.02g sodium azide was added. If so, sodium azide was removed before use by flushing the column with excess Column Buffer. It was stored at 4^{o}C.

3)**Acid-Washed Glass Wool**

Glass wool was soaked in 30% nitric acid and thoroughly rinsed in distilled water, followed by one rinse in Column Buffer, excess fluid was drained and the glass wool was stored in a screw capped bottle.

vii. For the Determination of the Specific Activity of Labelled DNA.

1)**Polyethyleneimine (PEI) Cellulose Ion Exchange Resin** (obtained from Schleicher and Schull).

2)**2M HCl**

viii. For the Preparation of Unlabelled DNA for Hybridization.

See Methods (Section 7.22, Subsection A; part viii.).

Ix. For DNA Reassociation.

1)**0.28M Phosphate Buffer**: see appendix

x. For the Calculation of the Concentration of Labelled and Unlabelled DNA to be Used In Hybridization Experiments.

See Methods (Section 7.22, Subsection A; part x.).

xi. For the Separation of Single and Double Stranded DNA on Hydroxylapatite.

1)**1.4M Phosphate Buffer** (PB): see appendix

2)**25% Sodium Dodecyl Sulphate** (25% SDS)

Sodium Dodecyl Sulphate (Sigma)	25.0g
Distilled Water to	100.0ml

3)**Low Molarity Phosphate Buffer**

To make a total volume of 500ml:

a. 0.08M PB + 0.4% SDS = [28.57ml (1.4M PB) + 8ml (25% SDS)]
b. 0.09M PB + 0.4% SDS = [32.14ml (1.4M PB) + 8ml (25% SDS)]
c. 0.10M PB + 0.4% SDS = [35.71ml (1.4M PB) + 8ml (25% SDS)]
d. 0.11M PB + 0.4% SDS = [39.29ml (1.4M PB) + 8ml (25% SDS)]
e. 0.12M PB + 0.4% SDS = [42.86ml (1.4M PB) + 8ml (25% SDS)]
f. 0.13M PB + 0.4% SDS = [46.43ml (1.4M PB) + 8ml (25% SDS)]
g. 0.14M PB + 0.4% SDS = [50.00ml (1.4M PB) + 8ml (25% SDS)]
h. 0.15M PB + 0.4% SDS = [53.57ml (1.4M PB) + 8ml (25% SDS)]
i. 0.16M PB + 0.4% SDS = [57.14ml (1.4M PB) + 8ml (25% SDS)]
j. 0.20M PB + 0.4% SDS = [71.42ml (1.4M PB) + 8ml (25% SDS)]
k. 0.21M PB + 0.4% SDS = [75.00ml (1.4M PB) + 8ml (25% SDS)]
l. 0.22M PB + 0.4% SDS = [78.58ml (1.4M PB) + 8ml (25% SDS)]

4)**High Molarity Phosphate Buffer** (made from 1.4M Phosphate Buffer)

To make a total volume of 500ml:

0.4M PB = 142.86ml (1.4M PB).

B) For the Estimation of DNA Relatedness.

See Methods (Section 7.22, Subsection B).

7.2.2 Methods

A) DNA-DNA Hybridization of *M. ovipneumoniae* Isolates and Other Members of the Class *Mollicutes*.

7.2.2A.i Propagation of *M. ovipneumoniae*, *M. arginini*, *M. pneumoniae* and *A. laidlawii* strains.

Seven *M. ovipneumoniae* isolates and one *A. laidlawii* strain were propagated in FM4 Medium. One *M. arginini* strain was propagated in FM4A Medium which is supplimented with arginine and adjusted to pH 7.0 and one *M. pneumoniae* strain was propagated in Modified SP-4 Medium. All cultures were inoculated into 3000ml of medium and incubated on a rotating platform at 37^{o}C until the medium reached approximately pH 6.8, except for *M. arginini* which was incubated until the medium reached approximately pH 8.3. The cells were collected by centrifugation at 15 500g for 15mins at 4^{o}C, washed once in PBS and resuspended in the minimum volume of Tris-EDTA Solution to give a thick suspension of approximately 20ml.

7.2.2A.ii DNA Extraction.

The procedures for the extraction and purification of DNA were modified slightly from those used in the restriction endonuclease analysis of *M. ovipneumoniae* isolates.

The mycoplasma cells suspended in approximately 20ml of Tris-EDTA Solution were lysed by the addition of 25% Sodium Dodecyl Sulphate to give a final concentration of 1%.

Protease Type XIV was also added to give a final concentration of 0.5mg/ml and for the *E. coli* V517 lysozyme was added to give a final concentration of 0.5mg/ml. The suspension was incubated overnight at 50^{o}C to ensure total cell lysis. Ribonuclease (Type 1-A) was added to a final concentration of 50ug/ml. The lysate was mixed by gently inverting the centrifuge tube a number of times to ensure that the RNase was evenly mixed (at this stage the lysate is extremely viscous). It was then incubated at 50^{o}C for 60mins and a quarter volume of 5M Sodium Perchlorate was added to the lysate which was incubated for a further hour at 50^{o}C. This disassociates proteins from the DNA.

An equal volume of extraction mixture (Phenol/Chloroform/Iso-amyl alcohol saturated with one-tenth its volume with STE Buffer) was added to the lysate and mixed by inverting the centrifuge tubes several times. The extraction mixture and the aqueous phase were separated by centrifugation for 10mins at 5000g at room temperature. The upper aqueous phase, containing the DNA, was carefully removed by using a 5ml serological pipette which had its tip cut off so as to ensure that the DNA was not sheared. The DNA was placed in another centrifuge tube and the purification procedure was repeated several times of until no lipopolysaccharides and/or proteins were visible at the interphase.

The DNA was dialysed four times against 8 litres of TE Buffer for 48hrs, removed from the dialysis bag and stored in sterile bottles at 4^{o}C. Experiments indicated (data not shown) that further purification of the DNA was required to achieve an adequate level of incorporation of radioactive nucleotides.

7.2.2A.iii Purification of DNA by CsCl Density Gradient Ultracentrifugation.

29ml of DNA in TE Buffer was placed in a 50ml screw capped SS34 Sorval centrifuge tube and 29g of CsCl (Var Lac Oid Chem Co^{TM}) was added. The CsCl was dissolved and the tube was transferred to a photographic darkroom where 2ml of Ethidium Bromide Solution was added. Normal laboratory lighting includes some short wavelength UV light which, in the presence of ethidium bromide, "nicks" the DNA, so while ethidium bromide was present all manipulations were conducted in a photographic darkroom under red safety lights.

The refractive index of the CsCl was measured using a refractometer and adjusted to 1.382 by the addition of either TE Buffer or CsCl crystals for *M. ovipneumoniae* and *M. arginini* strains. The refractive index was adjusted to 1.388 for *M. pneumoniae, A. laidlawii* and

E. coli V517 strains. 45ml of this solution (DNA/CsCl/ethidium bromide) was added to a TV 850 (Sorval^{TM}) vertical ultracentrifuge rotor and centrifuged at 315 000g for 48hrs at 15^{o}C using a Beckman^{TM} L5-75 Ultracentrifuge. The DNA, viewed by long-wave UV light, was removed from the ultracentrifuge tubes by puncturing the side of the centrifuge tube with a 20 gauge needle with syringe just below the DNA band. The DNA was drawn off slowly to avoid convection currents and to avoid dislodging any contaminating lipopolysaccharide which adhered to the wall of the tube.

Approximately 5ml of DNA/CsCl was removed and placed in a 10ml Nalgene^{TM} centrifuge tube. To remove the ethidium bromide from the DNA, an equal volume of the Isopropanol Solution (described above) was added. The solutions were mixed by inverting the tubes and left for 5mins for an interface to appear. The upper phase, containing the isopropanol and ethidium bromide, was removed and discarded. This extraction process was repeated until no more ethidium bromide (as detected by long-wave UV) was extracted from the DNA. At this stage the DNA could be exposed to normal laboratory lighting.

The DNA was dialysed using 3x1000ml volumes of 0.28M Phosphate Buffer at room temperature with two-hourly changes to remove residual isopropanol. The DNA was removed from the dialysis tube and placed in a bijou bottle. A 100ul aliquot of DNA was spectrophotometrically assayed for purity and concentration (see chapter four). The remaining DNA was stored at 4^{o}C.

7.2.2A.iv The Determination of:

a. **The Tm of *M. ovipneumoniae* DNA and Other Micro-organisms.**
b. **The Purity of the DNA by Measuring its Hyperchromatic Shift.**

A 2ml aliquot of DNA purified by CsCl gradient ultracentrifugation was dialysed against 800ml of 1xSSC buffer. The DNA concentration was determined as described in chapter four, except that the spectrophotometer was zeroed against 1xSSC Buffer. The Tm and the purity of the DNA to be used in hybridization experiments was determined as follows: A 3ml aliquot of *M. ovipneumoniae* DNA (50ug/ml) dialysed in 1xSSC buffer was placed in a quartz cuvette with a 1cm light-path and fitted with a Teflon^{TM} stopper. The DNA sample was placed in a cuvette holder which can be heated *via* a Cecil^{TM} Linear Temperature Program Controller. Two quartz cuvettes containing 1xSSC buffer were used as zero controls. The temperature was initially 25^{o}C and the optical density of the DNA at 260nm

was recorded. The temperature of the cuvettes was then raised 1^{o}C/min to 50^{o}C while recording the optical density of the DNA at 5^{o}C intervals. The rate of heating was then reduced to 0.5^{o}C/min and the optical density was recorded at 0.5^{o}C intervals until the solution reached approximately 105^{o}C (this is just below the boiling point of the solution). The tight-fitting Teflon^{TM} stopper avoided significant water loss due to evaporation. The optical density recorded at each temperature was corrected for thermal expansion of the solution using the table shown by Mandel and Marmur (1968). The corrected optical density at 25^{o}C and the ratio (i.e. the relative absorbance) was plotted against the temperature of the DNA solution. The temperature corresponding to half the maximum increase in the relative absorbance is designated as the Tm.

The Tm values obtained were used (a) to determine the hybridization temperature at which *M. ovipneumoniae* DNA would be allowed to reassociate. This temperature was 25^{o}C below Tm for low stringency and 10^{o}C below Tm for high stringency hybridizations and (b) to determine and compare the GC ratio between *M. ovipneumoniae* and other micro-organisms within the same genus.

The hyperchromatic shift could also be used to determine if the purity of the DNA was adequate for the purpose required. Thus, a hyperchromatic shift of >40% is universally deemed to be pure enough for DNA-DNA hybridization experiments (Mandel and Marmur, 1968).

7.2.2A.v Preparation of Radioactive ("Labelled") DNA Using the "Nick-Translation" Method.

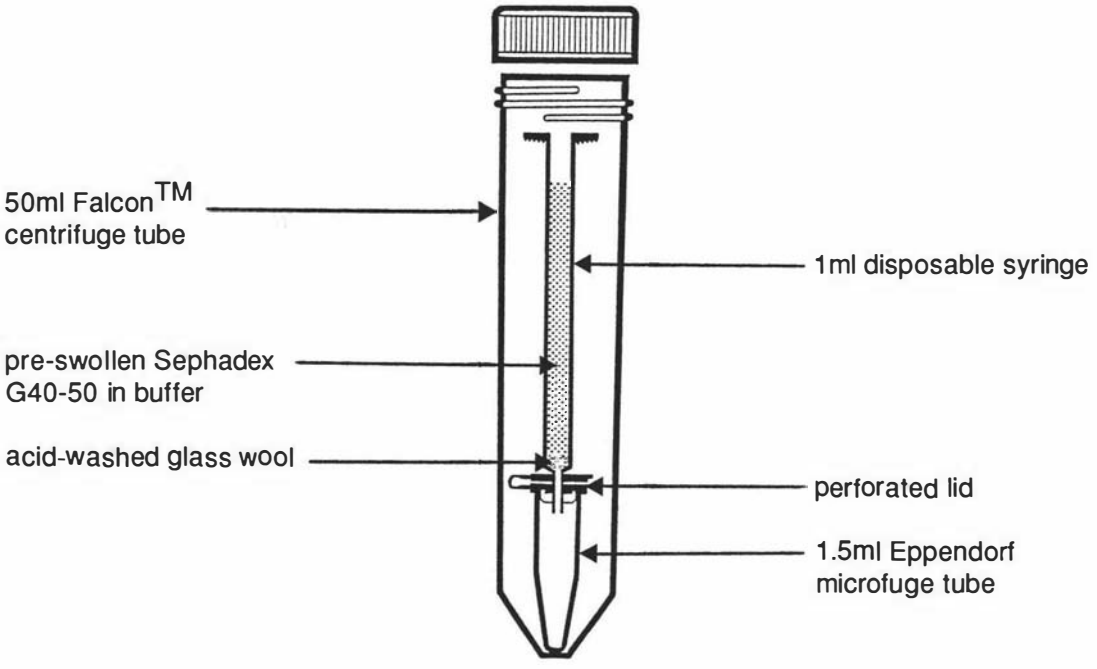
This procedure was based on the method of Rigby *et al*, (1977) but with some modifications. Briefly, a 3ul aliquot containing 1ug of DNA was pipetted into a 1.5ml Eppendorf microfuge tube, placed on ice and the following reagents were added; 2.5ul of 10x Reaction Buffer; 2.5ul NaCl (1M); 2.0ul *Hae*III restriction enzyme (Bethesda Research Laboratories) containing 20 units plus distilled water to make a final volume of 25ul. This mixture was used for labelling up to 1ug of DNA. If more DNA was labelled it was increased proportionally. Note that the labelling of 1ug of DNA is adequate for only one hybridization experiment with excess cold DNA. The reaction mixture was incubated in a waterbath at 37^{o}C for 60mins to allow the *Hae*III restriction enzyme to digest the DNA. This reaction was terminated by incubating the reaction mixture at 65^{o}C for 10mins. The following solutions

were then added; 2.5ul distilled water; 2.5ul (10x) Reaction Buffer; 3ul of each of the following deoxynucleotide triphosphates: dTTP, dATP, dGTP; 3ul dCTP [alpha-^{32}P]; 4ul DNA polymerase I (12 units) and finally 4ul of DNase Solution (diluted 1/2000 in DNase Dilution Buffer immediately prior to use, giving a final concentration of 0.25ng/ul). The reaction mixture was incubated at 14^{o}C for 2hrs and the reaction terminated by the addition of 5ul of 0.2M EDTA. After terminating the reaction, the enzymes (i.e. DNA polymerase and DNase) were inactivated by incubation at 65^{o}C for 10mins. The unincorporated dCTP [alpha-^{32}P] was separated from the double stranded DNA by Sephadex G40-50 fractionation using a "mini-spin" column.

7.2.2A.vi The Removal of Unincorporated dCTP [alpha-^{32}P] Following "Nick-Translation" Using a "Minispin Column".

A 1ml disposable syringe was plugged with acid-washed glass wool (to approximately the 0.05ml mark) and filled with pre-swollen Sephadex G40-50. The nozzle of the syringe was pressed through a 1.5ml Eppendorf microfuge tube lid (making a hole in the process). The syringe, now attached to the microfuge tube, was placed into another centrifuge tube (50ml Falcon^{TM}) and centrifuged at 1500rpm for 3mins to compact the Sephadex G40-50, this process was repeated with extra Sephadex until 0.9-1.0ml of the syringe was filled with Sephadex.

The 1.5ml Eppendorf microfuge tube was replaced with a fresh one following Sephadex compaction and the nick-translated reaction mixture was added to the top of the syringe. A 0.2ml aliquot of Column Buffer was added after the reaction mixture had entered the Sephadex and the column was centrifuged for 5mins at 3 000g. Following centrifugation the "nick-translated" DNA had collected at the base of the 1.5ml Eppendorf microfuge tube. This was detected using a hand-held Gieger counter. The unincorporated dCTP[alpha-^{32}P] remained in the column.



7.2.2A.vii The Determination of the Specific Activity of Labelled DNA.

Following the separation of unincorporated dCTP[alpha-^{32}P] from nick-translated DNA, 2ul of probe was spotted on to a PEI cellulose strip (1.0cm wide x 5.0cm high) approximately 1.0cm from the base. The probe was allowed to dry and then stood in 2M HCl, ensuring that the probe was not in contact with the acid. The 2M HCl was allowed to ascend to the top of the PEI cellulose strip which was cut into two equal lengths. The upper and lower portions were placed into scintillation vials containing 10ml of distilled water and assayed for radioactivity by Crenkov counting (Clausen, 1968) using a LS-7000 Beckman™ scintillation counter.

The specific activity of the labelled DNA was calculated as described by Brenner *et al*, (1982).

$$\frac{\text{counts/minute/ml of DNA}}{\text{DNA concentration (ug/ml)}} = \text{counts/minute/ug of DNA} \quad [1]$$

In our hands, a typical specific activity for labelled mycoplasma DNA was 0.5 to 1.0 x 10^6 cpm/ug of DNA.

The presence of unincorporated dCTP[alpha-^{32}P] in the labelled DNA following the "minispin column" was also determined.

$$\frac{\text{counts/minute/ul in top portion of the PEI cellulose}}{\text{counts/minute/ul spotted on to the PEI cellulose}} \times \frac{100}{1} = \%\text{ of unincorporated dCTP[alpha-}^{32}\text{P]} \quad \textbf{[2]}$$

If the unincorporated dCTP[alpha-^{32}P] was greater than 5% of the total counts, the labelled DNA was passed through a second "minispin column" and the percentage of unincorporated DNA was recalculated.

7.2.2A.viii The Preparation of Unlabelled DNA for Hybridization.

A 5ml aliquot of unlabelled DNA (200ug/ml) was sonicated at 20k/c (Soniprep 150 sonicator) in an ice-bath using a 0.9cm probe for various time intervals. 37.5ul aliquots containing 7.5ug DNA were removed after each ten-second interval and placed on a 1.2% agarose gel, where the fragmented DNA was electrophoresed and photographed (see Figure 83, insert). The sonicated DNA produced streaks of various lengths and their positions were compared with molecular weight markers. From this comparison a sonication time was chosen which gave DNA fragments 400 to 500bp long.

7.2.2A.ix DNA Reassociation.

A volume containing 1ug of the labelled DNA from a reference strain was added to unlabelled, sonically sheared DNA (150ug/ml) of a strain of mycoplasma. This was repeated in parallel (using the same labelled DNA) for each of the strains to be tested. A "label only" control was also used to determine if a detectable level of self-hybridization had occurred. The labelled and unlabelled DNA was mixed in 1x10cm screw-capped glass Kimax tubes and the volume adjusted to 1ml with 0.28M phosphate buffer. The DNA was denatured by heating to 100^{o}C for 10mins and then immediately plunged into an ice-bath. This ensured that both the labelled and unlabelled DNA were single-stranded. The above procedures

were done in duplicate. Then one of the DNA aliquots was incubated at 56^{o}C and the other at 70^{o}C (these temperatures were 25^{o}C and 11^{o}C below Tm) for 16hrs.

The above-mentioned reassociation conditions (see above) allowed the reaction for unlabelled DNA to reach 100 Cots (Cot is defined as the product of DNA concentration and the time of incubation in reassociation reactions), this is sufficient for almost complete reassociation of the single stranded DNA.

Estimation of 100 Cot depends on the phosphate buffer concentration. In these standard reassociation conditions (0.28M PB), the rate of reassociation occurs 3.5 times faster than in 0.12M PB (Britten *et al*, 1974).

7.2.2A.x Calculation of the Concentration of Labelled and Unlabelled DNA to be Used in Hybridization Experiments.

When labelled DNA is mixed with homologous unlabelled DNA and hybridized, it is necessary to choose conditions such that at the end of the process, substantially all of the labelled DNA has hybridized i.e. becomes double-stranded. This in principle requires that a high concentration of total DNA must be used, most of which will be unlabelled.

However, where the labelled DNA is mixed with unlabelled heterologous DNA (e.g. from a species with no homology with the labelled DNA) it is necessary that the conditions used above are such that self-hybridization (i.e. hybridization of labelled DNA to labelled DNA) is negligible. In all such experiments however a concentration of labelled DNA must be used such that an adequate number of counts are obtained to allow analysis of the collected data. With regard to the degree of hybridization required (approximately 100%) it is generally accepted that this is achieved when the Cot value is not less than approximately 100. Conversely, if the Cot value is less than about 0.1 then the degree of hybridization is negligible. It follows from this that, when labelled DNA is mixed with unlabelled homologous DNA, the conditions (namely DNA concentration and time) should allow a Cot value of 100. Whereas the Cot value for self-hybridization (labell only) should be less than 0.1.

In the experiments described above, the unlabelled DNA was used at a concentration of 150ug/ml and the labelled DNA at 0.1ug/ml.

Calculation:

$$\text{Cot} = \frac{\left(\begin{array}{c}\text{DNA concentration} \\ \text{ug per ml}\end{array}\right)\left(\begin{array}{c}\text{absorbance of sheared} \\ \text{DNA per ug at 260nm}\end{array}\right)\left(\begin{array}{c}\text{incubation} \\ \text{times in hrs}\end{array}\right)}{2} \quad [3]$$

The Cot for the unlabelled DNA was calculated as follows:

$$\text{Cot (0.12M PB)} = \frac{(150\text{ug/ml})\,(0.024)\,(16)}{2} = 28.8$$

This Cot refers to 0.12M PB. When 0.28M PB was used, the Cot must be amended by a factor of 3.5 (Britten *et al*, 1974). i.e.:

$$\text{Cot (0.28M PB)} = \text{Cot (0.12M PB)} \times 3.5 = 100.8$$

A Cot of 100 is sufficient for almost complete reassociation of the labelled DNA with the unlabelled DNA.

The Cot for the labelled DNA was calculated as follows:

$$\text{Cot (0.12M PB)} = \frac{(0.1\text{ug/ml})\,(0.024)\,(16)}{2} = 0.019$$

However, used 0.28M PB.

$$\text{Cot (0.28M PB)} = \text{Cot (0.12M PB)} \times 3.5 = 0.067$$

A Cot of 0.067 for the labelled DNA was small enough to preclude significant self-reassociation.

7.2.2A.xi The Separation of Single and Double Stranded DNA on Hydroxylapatite.

Following incubation of the labelled and unlabelled DNA, done in duplicate i.e. at 56^{o} and 70^{o}C, the hybridization reaction was quenched by adding an equal volume of distilled water and placing the reassociated DNA on ice.

The batch separation method, as described by Brenner *et al*, (1969) was used to separate single and double-stranded DNA. Briefly, 0.7g of DNA-grade hydroxylapatite (BioRad^{TM}) was placed in 1.3cm x 12.5cm thick-walled test tubes and 8ml of Low Molarity* Phosphate Buffer + 0.4% SDS was added and mixed using a variable speed overhead stirrer. The hydroxylapatite was raised to the hybridization temperature in a circulating waterbath and immediately placed in a Sorval^{TM} bench centrifuge (fitted with a type A rotor) held at the hybridization temperature. The hydroxylapatite was centrifuged at 1000g for 60secs and the rotor stopped quickly by hand. The supernatant was discarded and the hydroxylapatite was re-washed in the appropriate Low Molarity Phosphate Buffer. 0.5ml of reassociated labelled DNA was then added to the hydroxylapatite and 8ml of Low Molarity Phosphate Buffer was added, the reassociated DNA was mixed thoroughly into the hydroxylapatite and the above washing procedure (which includes heating to the hybridization temperature) was repeated. The supernatant was collected in scintillation vials and the hydroxylapatite was washed three more times (with heating to the hybridization temperature) in Low Molarity Phosphate Buffer + 0.4% SDS. This ensured that substantially all of the single-stranded (i.e. unhybridized) DNA was eluted, therefore leaving only the double-stranded, reassociated DNA bound to the hydroxylapatite. The reassociated DNA was removed by washing the hydroxylapatite with a high molarity phosphate buffer (0.4M without SDS and without heating). It was left for 5mins at room temperature before centrifugation and collection of the supernatant into scintillation vial. This procedure was repeated thrice to ensure that all of the reassociated DNA was removed from the hydroxylapatite. All eluants were assayed by Cerenkov counting (Clausen, 1968) using a Beckman^{TM} LS 7000 liquid scintillation counter. The proportion of reassociated labelled DNA, bound to the hydroxylapatite, was expressed as a percentage of the total label.

*Note: A range of different low-molarity phosphate buffers were used with each new batch of hydroxylapatite to determine the optimal conditions for eluting unhybridized single-stranded DNA from the hydroxylapatite. The optimal phosphate buffer molarity was determined experimentally and is recorded in the result section (see Figure 85).

B) Estimation of DNA Relatedness.

Hybridizations were carried out with a standard amount of labelled DNA from a strain of *M. ovipneumoniae* which was added to an excess amount of unlabelled DNA from the same and different isolates of *M. ovipneumoniae* and other organisms including *M. arginini, A. laidlawii* and *E. coli* V517. The apparent hybridization obtained in the label-only DNA was regarded as background and was subtracted from all results. If the apparent hybridization exceeded 5% the experimental results were disregarded.

The relatedness of reassociated DNA was expressed as the Relative Homology (%). This was calculated as follows:

$$\% \text{ of ds-DNA} = \frac{\text{cpm of DNA removed with 0.4M PB}}{\text{total number of cpm removed}} \qquad [4]$$

$$\% \text{ homology} = (\% \text{ ds-DNA}) - (\% \text{ self-hybridized DNA}) \qquad [5]$$

Where:

$$\frac{\% \text{ homology of homologous DNA}}{\% \text{ homology of heterologous DNA}} = \frac{100}{\text{Relative Homology}}$$

Or:

$$\text{Relative Homology (\%)} = \frac{\% \text{ homology heterologous DNA}}{\% \text{ homology homologous DNA}} \times \frac{100}{1} \qquad [6]$$

7.3 Results.

In DNA reassociation experiments, typically a high concentration of unlabelled DNA is incubated under a set of conditions such that single-stranded DNA fragments may collide and anneal with (low concentration) labelled DNA fragments in free solution to form double-stranded DNA. The extent and specificity of the reassociation of the labelled DNA with the unlabelled DNA is affected by a number of conditions. These conditions must be experimentally determined for each system before undertaking a study of DNA-DNA hybridization.

For optimal reassociation, the first condition or requirement is that only pure nucleic acid should be used. Purity was determined by measuring the 260:280 absorption ratio and the hyperchromatic shift i.e. the increase in the optical density of the DNA at 260nm following heat denaturating of the DNA. Spectrophotometric analysis of both CsCl purified and "standard" DNA gave similar 260:280 ratios (data not shown) indicating that the DNA was not contaminated by protein. However, other impurities (e.g. polysaccharide) can affect the result, so "standard" DNA and DNA further purified by ultracentrifugation in a CsCl gradient were compared by thermal denaturation. The result is shown in Figure 80. The purified DNA showed a hyperchromatic shift of about 50% (1.015 to 1.525 OD units), while the "standard" DNA showed a lesser shift i.e. about 20%. A hyperchromatic shift of >40% is generally deemed to be satisfactory for DNA reassociation experiments. Thus, the CsCl DNA but not the original DNA preparation met the standard for hybridization experiments.

The second condition required is a knowledge of the midpoint of thermal denaturation (Tm) of the DNA i.e. the temperature at which 50% of the DNA duplex is single stranded. The Tm for *M. ovipneumoniae* was determined in standard conditions i.e. 1xSSC buffer and is compared with *M. arginini* and *A. laidlawii* in Figure 81. The Tm for *M. ovipneumoniae* was 81.2^{o}C compared with 83.0^{o}C and 84.1^{o}C for *M. arginini* and *A. laidlawii* respectively. These Tm values allow us to define the optimum temperature for the reassociation of *M. ovipneumoniae* DNA hybridization. This is approximately 25^{o}C below Tm i.e. 56^{o}C for low stringency and approximately 10^{o}C below Tm for high stringency i.e. 70^{o}C. Note that the results from the Tm can be used to calculate the GC ratio of an organism (De Lay, 1970)

i.e. %GC = 2.44 (Tm - 69.4)

Thus, the GC ratio for *M. ovipneumoniae* strain L6I1 is 28.8%, *M. arginini* strain M30/C4 is 33.2% and *A. laidlawii* (ATCC 23206) is 35.9%.

The Tm was determined in standard conditions i.e. 1xSSC buffer. However, the hybridization experiments are carried out in 0.28M phosphate buffer (PB). The Tm determination was repeated using phosphate buffer and the results (Figure 82) show that in this buffer the Tm of *M. ovipneumoniae* is 82.2^{o}C i.e. a 1.0^{o}C increase compared with 1xSSC Buffer. This small difference is not significant.

The third condition is to obtain an optimal fragment size of the DNA to be hybridized. Fragment sizes commonly employed are approximately 300 to 400 nucleotides of single

stranded DNA. DNA from *M. ovipneumoniae* strain L6I1 was sonicated for various times and from each, an aliquot was electrophoresed in a 1.2% agarose gel. The results are shown in Figure 83 (insert). The "average" value (half way between top and bottom) from each sonication period was measured from the gel and plotted as shown in Figure 83. A sonication period of 50secs was chosen. This gave fragment sizes of approximately 600bp to 800bp (i.e. 300 to 400 nucleosides/single strand).

The fourth and final condition, the incubation time allowed for the strands to reassociate, is governed by the DNA concentration and is calculated as described in the materials and methods section of this chapter [equation 3].

After all experimental parameters were determined, labelled DNA from *M. ovipneumoniae* strain L6I1 was reassociated with a measured excess of unlabelled DNA, of the same strain, at five different temperatures. This was done to confirm the optimum temperature for hybridization. These results are shown in Figure 84. The maximum percent homology, as was expected, occurred at 56^{o}C i.e. 25^{o}C below Tm. Note the decrease in percent homology with the increase in hybridization temperature. A decrease in apparent homology also occurred at temperatures below 56^{o}C. This is attributed not only to an incomplete reaction, because of a slower reaction rate at lower temperatures in the time allowed, but also to the poor binding capacity of the hydroxylapatite at lower temperature. A control i.e. label only, gave a constant "background" of 2-3% homology between 45^{o}C and 70^{o}C and represents the labelled DNA annealing to itself. This has to be allowed for in calculating the apparent percent reassociation.

Each batch of hydroxylapatite used was tested with various concentrations of phosphate buffer to determine which concentration would best allow single stranded DNA to be eluted from the hydroxylapatite. Twelve aliquots of annealed DNA from one reassociation experiment were added to the hydroxylapatite and the labelled single-stranded DNA was eluted by the batch process (see materials and methods) using a range of different phosphate buffer concentrations. The results from this are shown in Figure 85. Note that the maximum percent annealing (79%), as measured by the method used, was attained when 0.1M phosphate buffer was used to elute the single stranded DNA. At higher phosphate concentrations, lower values were recorded, suggesting that some double-stranded DNA was also eluted from the hydroxylapatite. The control sample i.e. label only, showed only 2-3% self-annealing.

The homology of *M. ovipneumoniae* DNA with DNA from the same and other species at stringencies giving the maximum hybridization, i.e. Tm minus 25^{o}C or 56^{o}C and at higher stringencies i.e. Tm minus 10^{o}C or 70^{o}C, is shown in Table IX. Note that the homology with *E. coli*, *A. laidlawii* and *M. arginini* is low.

M. ovipneumoniae homology with test strains is normalized to 100% and the extent (normalized value) of homology with other *M. ovipneumoniae* strains varied from 100% to 79.6% (low stringency) and from 90.1% to 68.1% (high stringency). Isolates from one lung which were identical by restriction endonuclease cleavage patterns, gave the same degree of hybridization i.e. 98.7% as the homologous DNA (Table IX).

If two organisms are identical then reciprocal results, e.g. labelling "X" and probing with excess "Y" or alternatively labelling "Y" and probing with excess "X", should give results which agree within experimental error. Note that this is true with strains 1 and 10 (at high stringency), but other reciprocal results are less symmetrical (see Table IX). The greatest discrepancy is between strain L6I1 and strain 1 (Table IX). L6I1 labelled probe gave (at high stringency) a homology of 71.7% whereas the reciprocal figure was 89.3%. The figures quoted in the table are the mean of four separate reciprocal experiments and gave (within the experimental limits of 5%) consistant results. Such non-reciprocal results are common with micro-organisms and are normally due to a segment of DNA which is absent from one (say "X") but present in "Y". This would allow a probe of "X" DNA tested with excess "Y" to have a higher homology than is found in the reverse ("Y" with "X"). Sometimes this indicates the presence of a plasmid in one strain only. However, we have failed to detect plasmids in *M. ovipneumoniae* (see chapter six).

Some of the low stringency results of Table IX are presented in graphical form (Figure 86). Note that the four closely related (if not identical) isolates from one lung form a group whereas other *M. ovipneumoniae* isolates are more heterogeneous.

Figure 80

The increase in hyperchromatic shift as a measure of DNA purity. DNA from *M. ovipneumoniae* strain L6I1 purified by the "standard" method (○———○) and further purified by CsCl-gradient ultracentrifugation (●———●) were denatured by heating in 1xSSC buffer and their optical densities were recorded at various temperatures. The CsCl-purified DNA shows a hyperchromatic shift of about 50% (i.e. 1.015 OD units to 1.525 OD units) whereas the standard DNA increased by about 20% (i.e. 1.065 OD units to 1.285 OD units). Note that while this gives an indication of DNA purity (important in hybridization experiments), the midpoint of thermal denaturation (Tm) is almost the same for CsCl-purified DNA (81.2^{o}C) and "standard" DNA (81.3^{o}C).

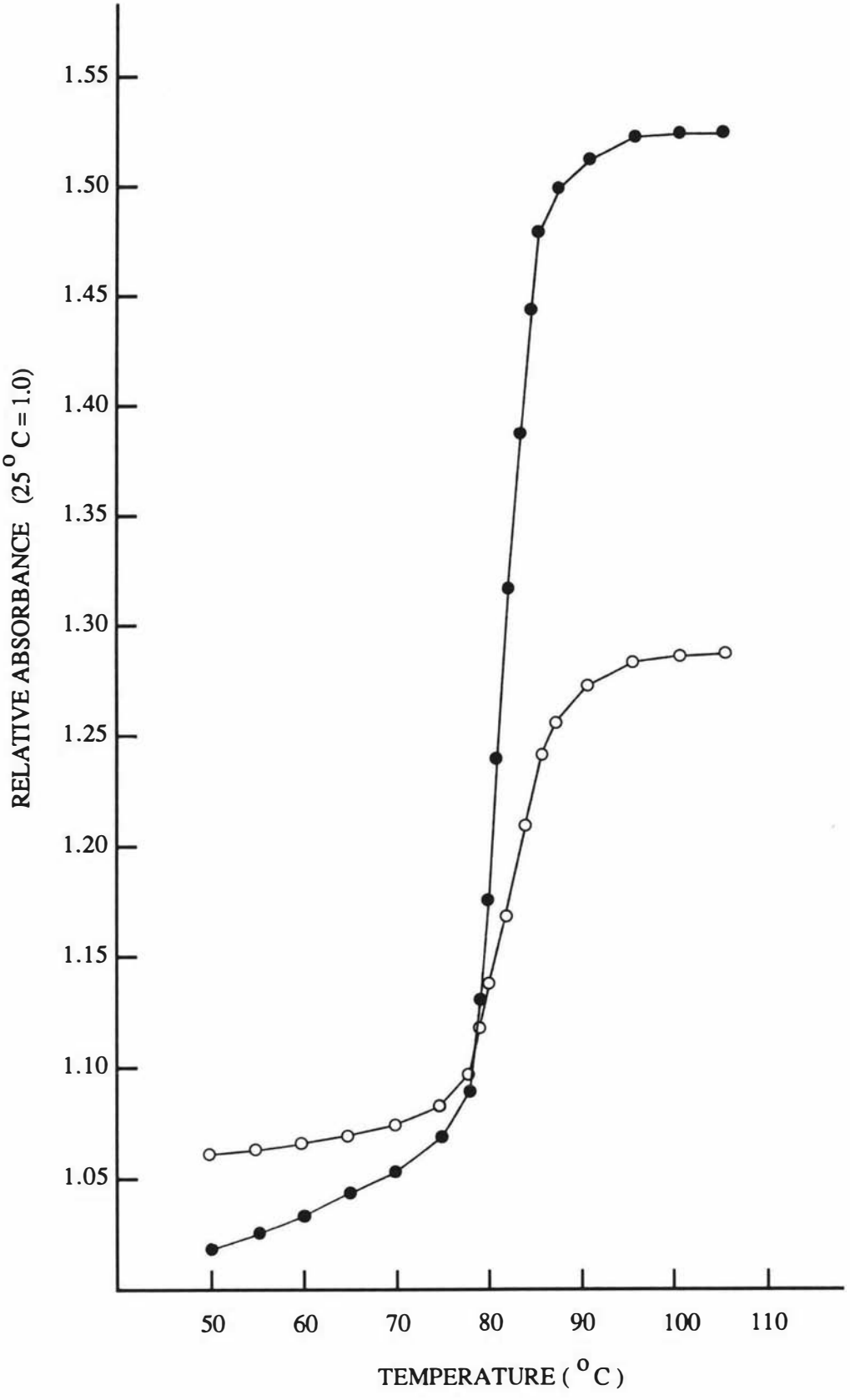

1.55
1.50
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
RELATIVE ABSORBANCE (25 °C = 1.0)
50
60
70
80
90
100
110
TEMPERATURE (°C)

Figure 81

Determination of the midpoint (Tm) of thermal denaturation of the DNA from three mycoplasma species. *M. ovipneumoniae* strain L6I1 (●———●), 81.2^{o}C; *M. arginini* strain M30/C4 (■———■), 83.0^{o}C and *A. laidlawii* strain PG8 (▲———▲), 84.1^{o}C.

These determinations were made to calculate the appropriate hybridization temperatures, which are Tm - 25^{o}C.

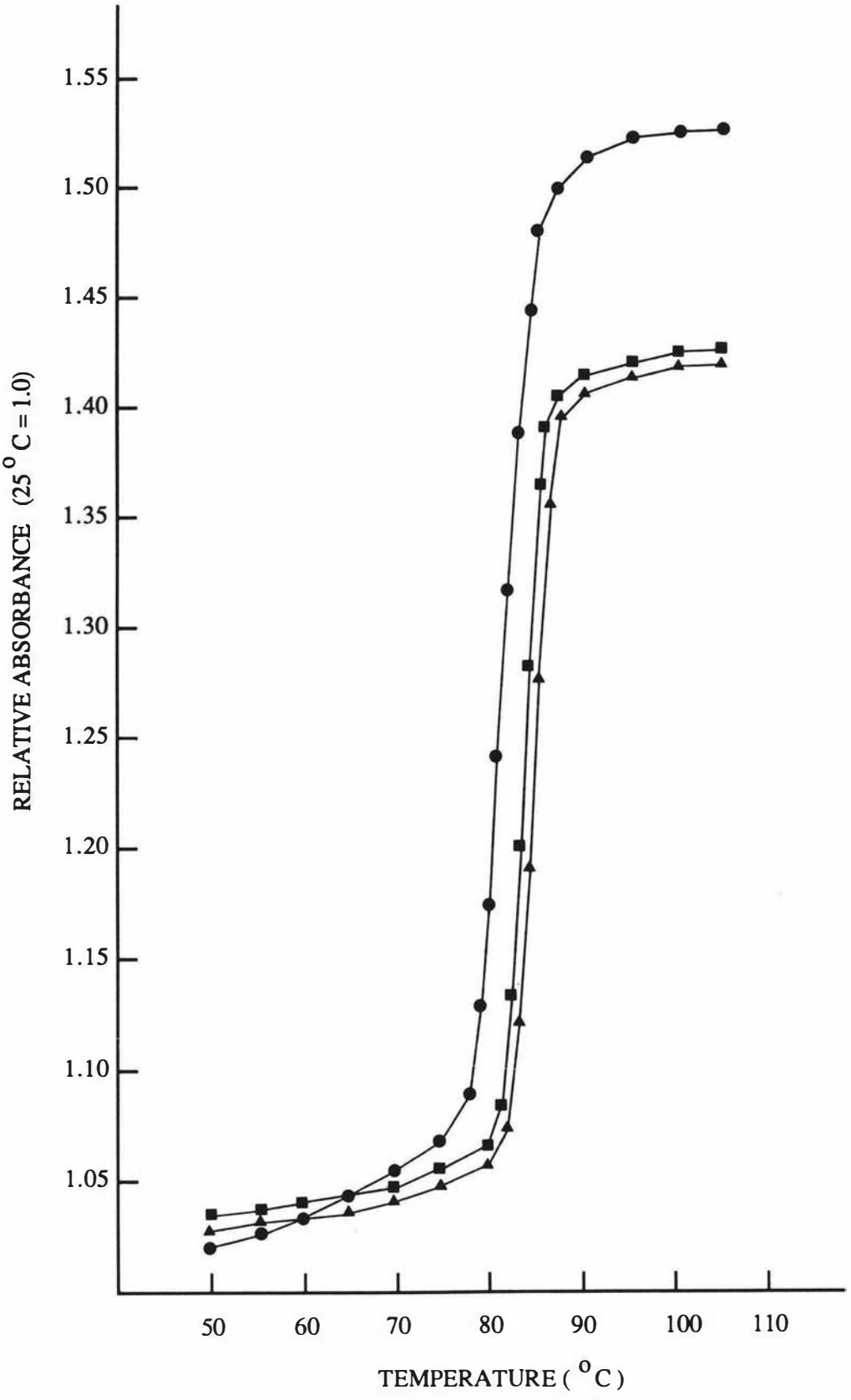

1.55
1.50
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
RELATIVE ABSORBANCE (25 °C = 1.0)
50
60
70
80
90
100
110
TEMPERATURE (°C)

Figure 82

A comparison of the midpoint of thermal denaturation (Tm) of DNA from *M. ovipneumoniae* strain L6I1 in two different buffers i.e. 1xSSC buffer (●——●) and 0.28M phosphate buffer (○——○). The Tm for DNA in 1xSSC buffer is 81.2^{o}C whereas in 0.28M phosphate buffer it is 82.2^{o}C.

1xSSC is the standard buffer for Tm determination, but hybridizations are carried out in 0.28M phosphate buffer at a temperature of **Tm** $_{\text{(in phosphate buffer)}}$ **- 25^{o}C**.

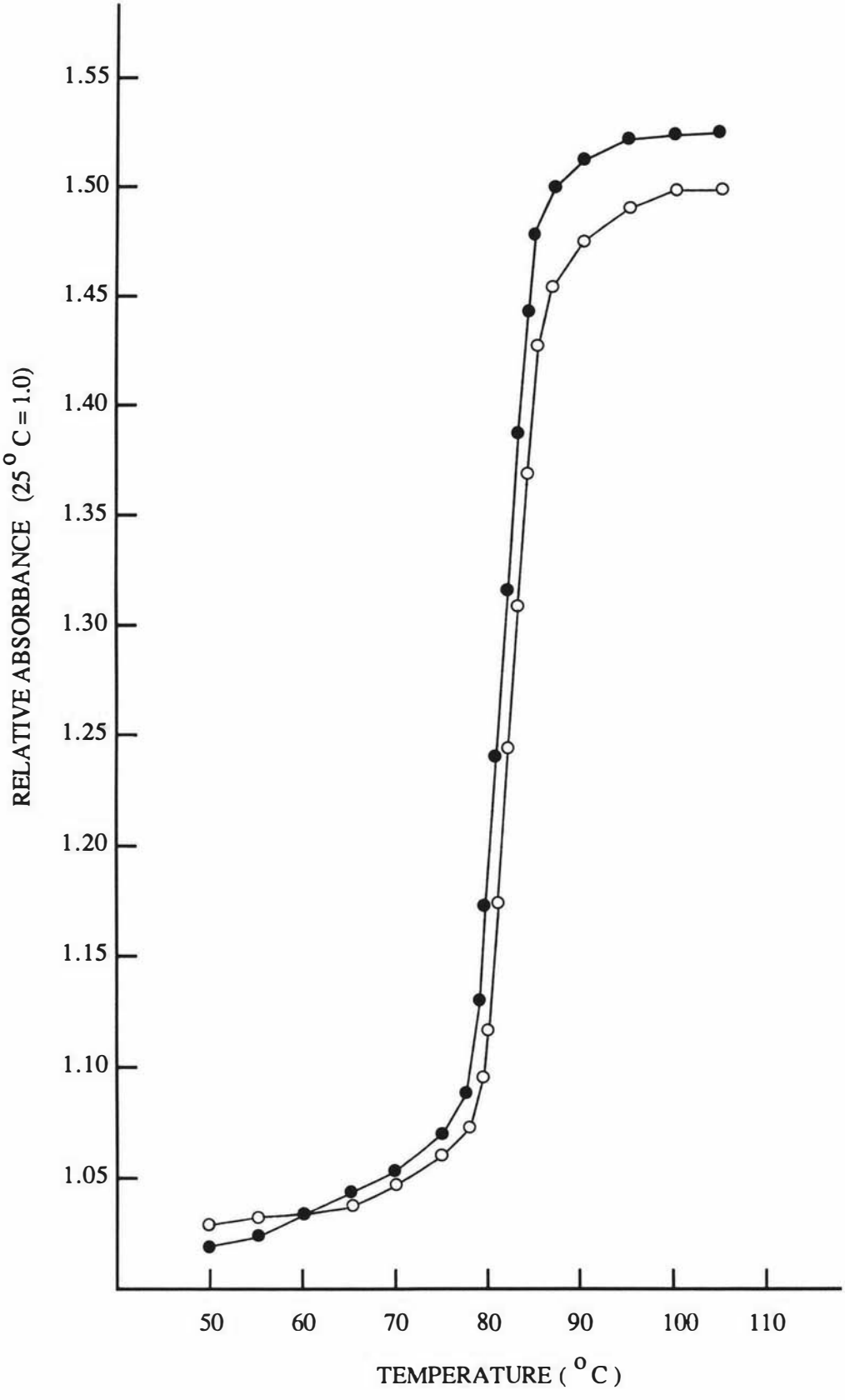

1.55
1.50
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
RELATIVE ABSORBANCE (25 °C = 1.0)
50
60
70
80
90
100
110
TEMPERATURE (°C)

Figure 83

Determination of the sonication period required to fragment DNA to approximately 700bp for use in DNA-DNA hybridizations. DNA from *M. ovipneumoniae* strain L6I1 was sonicated for varying periods and electrophoresed in a 1.2% agarose gel (see insert). The "average" value (half-way between the top and the bottom) from each DNA track was estimated using the 1kb molecular weight "ladder" marker (lane 1) and the average fragment sizes were plotted against the sonication time. Fragment sizes of approximately 700bp (i.e. about 350 nucleosides/single strand of DNA) correspond to a sonication time of about 50secs (see arrow).

Insert:

Lane 1: 1kb ladder (DNA molecular weight marker); Lane 2: DNA not sonicated; Lanes 3 to 10: DNA sonicated for 10, 20, 30, 40, 50, 60, 70 and 80 seconds respectively. Note the DNA used was not purified by CsCl gradient-ultracentrifugation (RNA can be detected at the base of the lanes). Subsequent experiments (not shown) using purified DNA gave similar results.

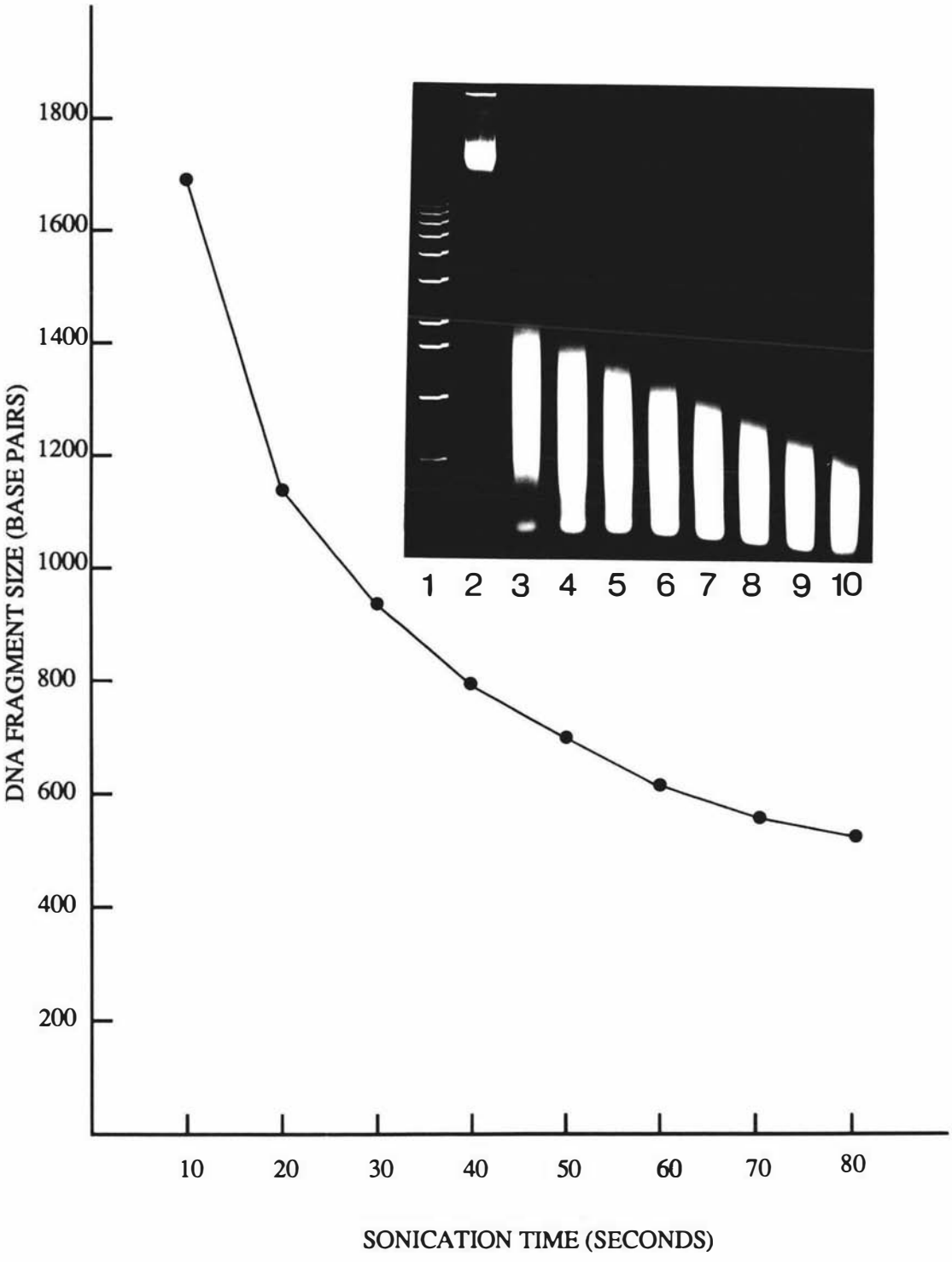

DNA FRAGMENT SIZE (BASE PAIRS)
1800
1600
1400
1200
1000
800
600
400
200
1 2 3 4 5 6 7 8 9 10
10
20
30
40
50
60
70
80
SONICATION TIME (SECONDS)

Figure 84

The effect of temperature on the hybridization of ^{32}P-DNA from *M. ovipneumoniae* strain L6I1 to an excess of unlabelled DNA from the same strain (●———●). A maximum apparent homology of 79% occurred at 56^{o}C (i.e. 25^{o}C below Tm). A decrease in homology occurred at higher temperatures because of the higher stringency requirement. At lower temperatures, the apparent decrease in homology is due in part to an incomplete reaction, in the time allowed but in part is also due to the diminished binding capacity of hydroxylapatite at temperatures of around 45^{o}C and lower.

Hybridization controls (○———○) containing only labelled DNA gave an apparent self-reassociation of 2-3% between 45^{o}C and 70^{o}C.

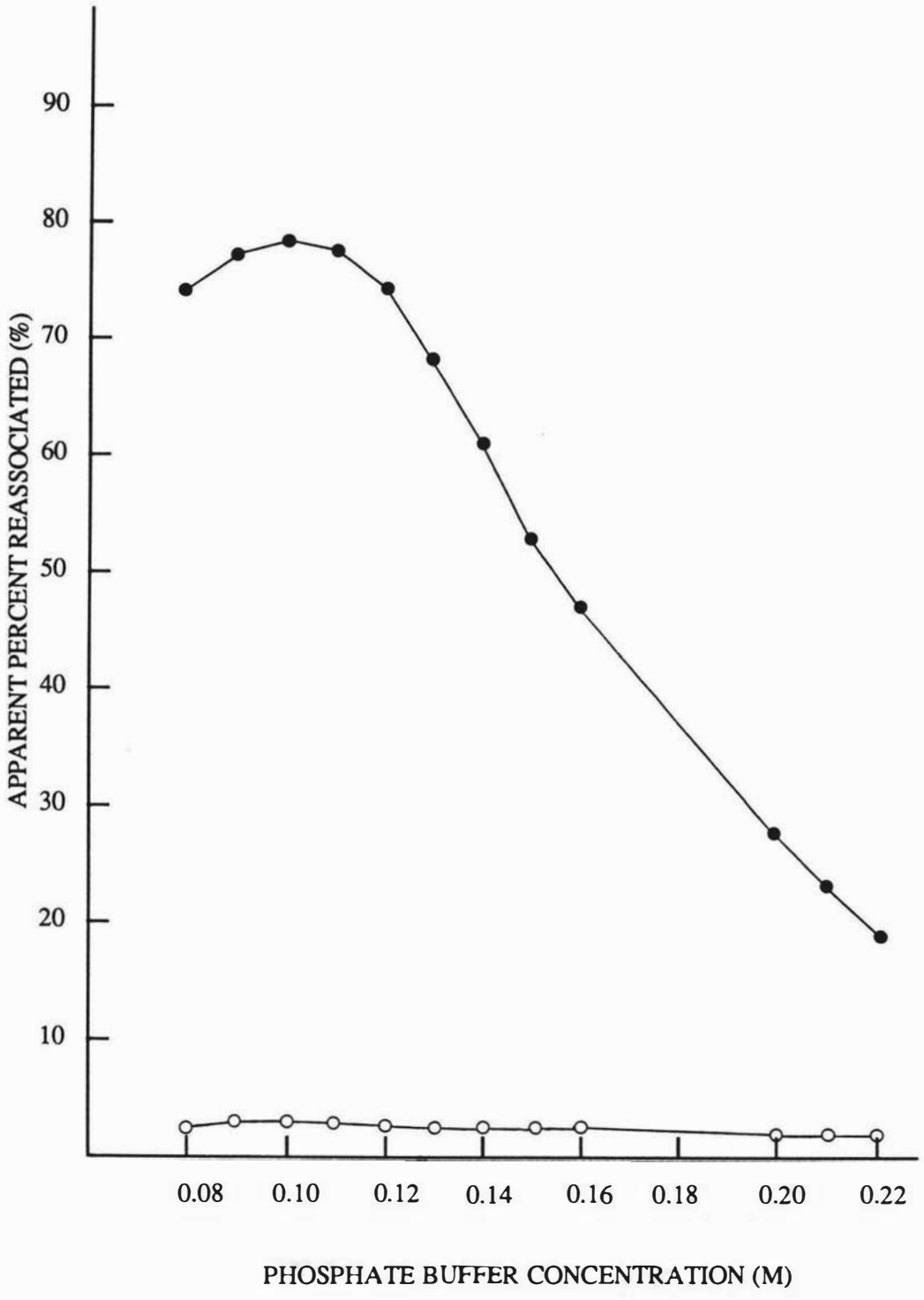

APPARENT PERCENT REASSOCIATED (%)
90
80
70
60
50
40
30
20
10
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0.22
PHOSPHATE BUFFER CONCENTRATION (M)

Figure 85

Investigation of the optimum buffer concentration for separating single and double stranded DNA using hydroxylapatite.

Labelled DNA was hybridized with a large excess of unlabelled homologous DNA in conditions calculated to give (almost) total hybridization of the probe which has thus become double stranded DNA.

This hybridized DNA was added to hydroxylapatite in various molarities of phosphate buffer. At an appropriate molarity of buffer (which differs for each batch of hydroxylapatite) the double stranded DNA adsorbs while any remaining single stranded DNA does not absorb. The unabsorbed DNA was removed by three washings with the same initial molarity of phosphate buffer. This was followed by removal of all DNA using 0.48M phosphate buffer.

Then:

$$\text{Apparent \% reassociation} = \frac{\text{final eluate}}{\text{initial + final eluate}} \times \frac{100}{1}$$

When this figure is maximum it represents the optimum condition (i.e. optimum initial phosphate buffer concentration) to seperate single stranded DNA from double stranded DNA.

The optimum phosphate buffer molarity to maximize separation was 0.10M (see graph, ●——●).

The original concentration of labelled DNA should be low enough to minimize self-hybridization in the absence of added unlabelled DNA. This was investigated in parallel (○——○). Note that some self annealing does apparently occur. This has to be allowed for in later calculations.

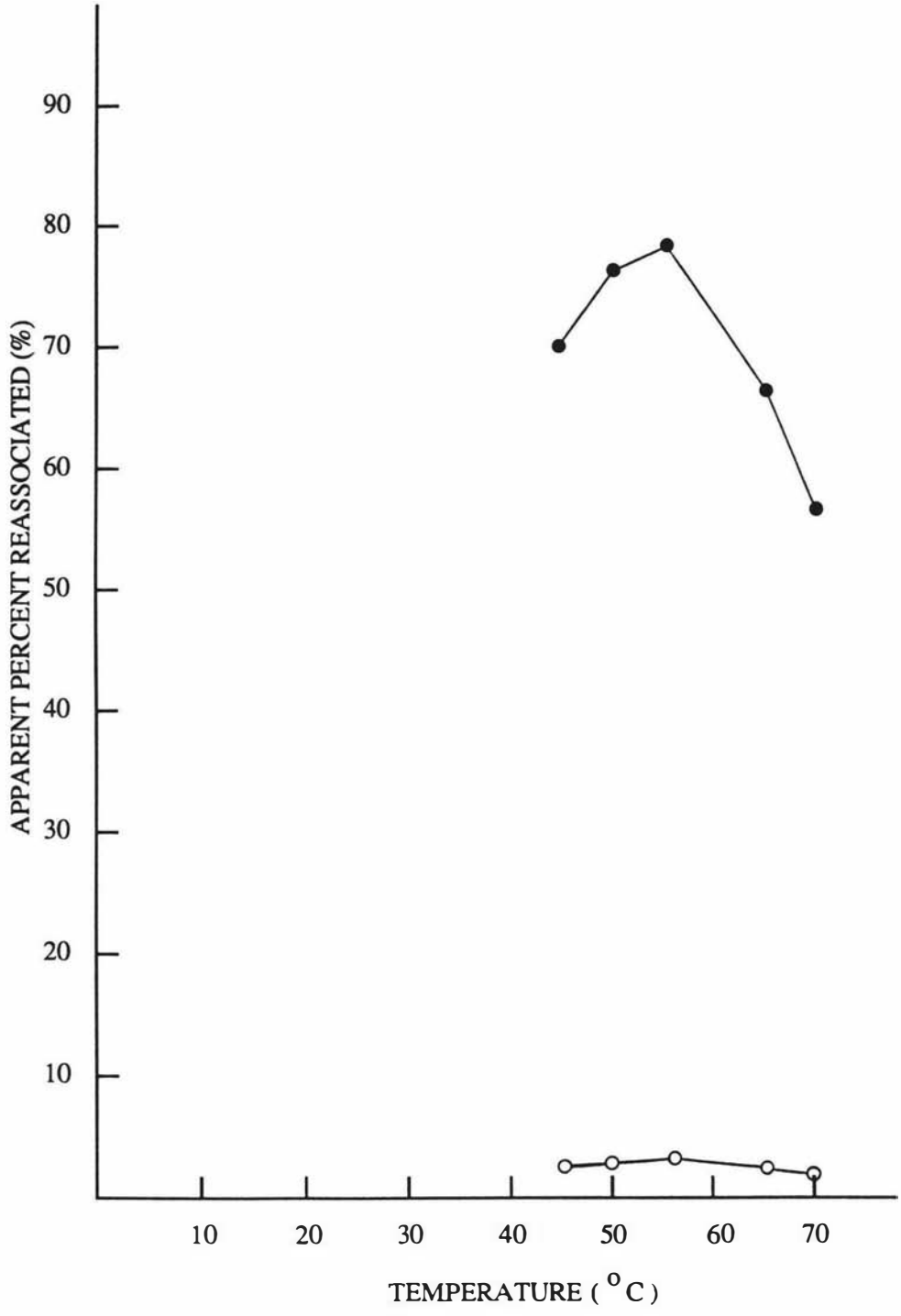

APPARENT PERCENT REASSOCIATED (%)
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
TEMPERATURE (°C)

Figure 86

Investigation of the DNA homology of *M. ovipneumoniae* with different isolates of the same species and with other species of mycoplasma.

Two labelled reference strains of *M. ovipneumoniae* (strain 1 and L6I1) were hybridized with the organisms listed. For each organism, the % of hybridization with each reference DNA is plotted.

Note:

a) *M. ovipneumoniae* strains L6I1, L6I2, L6I4 and L6I6 (derived from one lung and having closely similar but not identical BRENDA patterns) all form a tight cluster with essentially identical homologies.

b) All the *M. ovipneumoniae* strains tested fall within one group, with homologies of at least 80%. However, they are quite distinct from *M. pneumoniae* (45% homology), *M. arginini* strain M30/C4 (about 20% homology) and *A. laidlawii* strain ATCC 23206 (about 10-15% homology) with *M. ovipneumoniae*.

c) *E. coli* strain V517 was included as a control and showed a 4% homology (i.e. essentially zero) with the *M. ovipneumoniae* strains.

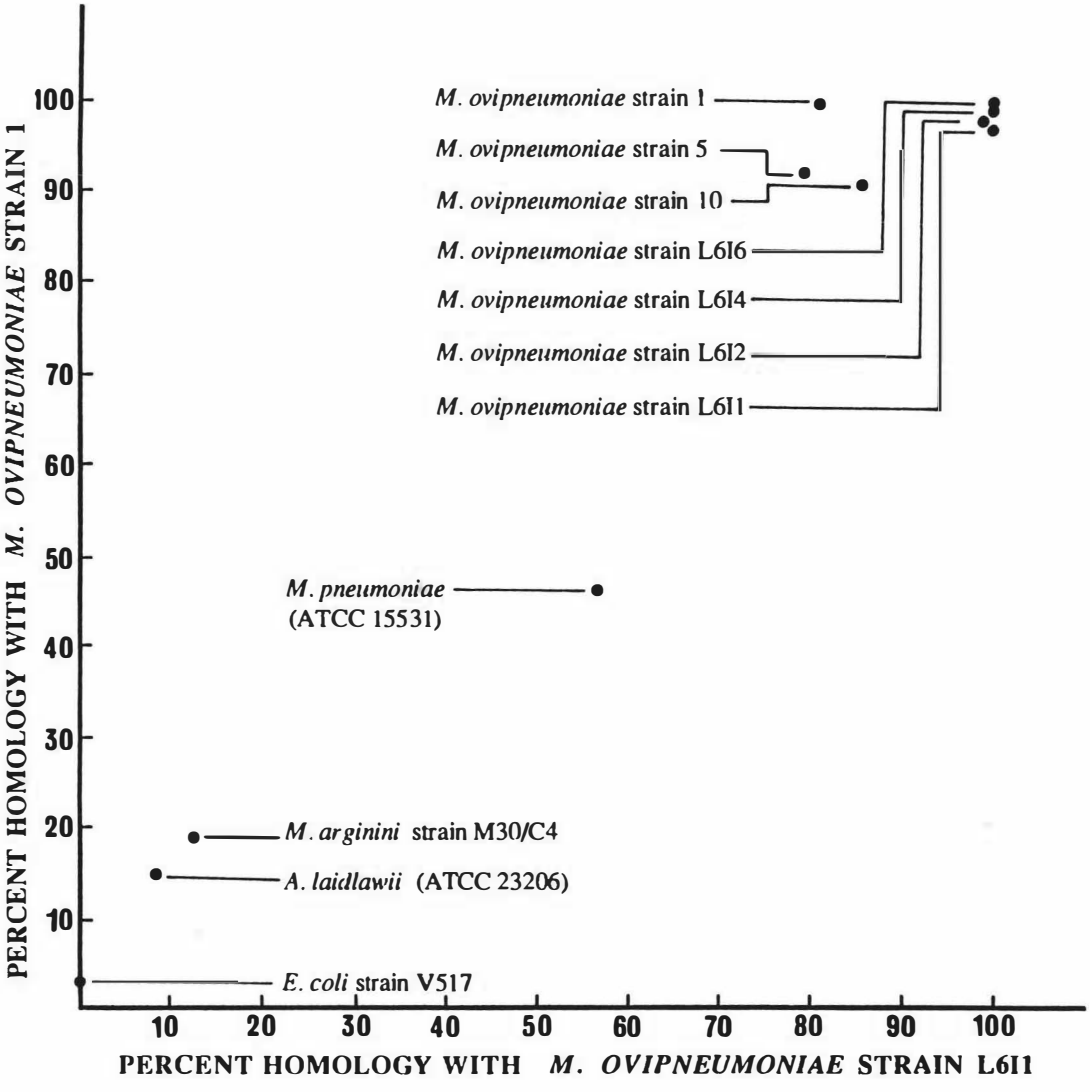

M. ovipneumoniae strain 1
M. ovipneumoniae strain 5
M. ovipneumoniae strain 10
M. ovipneumoniae strain L6I6
M. ovipneumoniae strain L6I4
M. ovipneumoniae strain L6I2
M. ovipneumoniae strain L6I1
M. pneumoniae (ATCC 15531)
M. arginini strain M30/C4
A. laidlawii (ATCC 23206)
E. coli strain V517
PERCENT HOMOLOGY WITH M. OVIPNEUMONIAE STRAIN 1
PERCENT HOMOLOGY WITH M. OVIPNEUMONIAE STRAIN L6I1
10
20
30
40
50
60
70
80
90
100

TABLE IX: **THE DEGREE of HYBRIDIZATION using ^{32}P-DNA PROBES for Four M. ovipneumoniae STRAINS with DNA FROM M. ovipneumoniae, M. pneumoniae, M. arginini and A. laidlawii STRAINS**

DNA hybridized at 56°C

% hybridization with excess unlabelled DNA[1]

Strain	1[2]	5[2]	10[3]	L6I1[4]	L6I2[4]	L6I4[4]	L6I6[4]	M. pneumoniae[5]	M. arginini[6]	A. laidlawii[7]	E. coli[8]
^{32}P 1	100[9](66.0)[10]	92.6 (61.1)	91.0 (60.1)	97.3 (64.2)	98.0 (64.7)	99.0 (65.3)	100 (66.0)	46.6 (30.8)	19.4 (12.8)	14.9 (9.8)	3.7 (2.4)
^{32}P 5	92.7 (66.5)	100 (71.7)	93.8 (67.3)	93.6 (67.1)	96.5 (69.2)	100 (71.7)	99.2 (71.1)	NT[11]	NT	NT	4.0 (2.9)
^{32}P 10	86.3 (59.6)	87.7 (60.6)	100 (69.1)	84.5 (58.4)	90.2 (62.3)	94.1 (65.0)	98.7 (68.2)	NT	NT	NT	2.7 (1.9)
^{32}P L6I1	81.0 (58.3)	79.6 (57.3)	85.9 (61.8)	100 (72.0)	98.7 (71.1)	100 (72.0)	100 (72.0)	56.7 (40.8)	12.5 (9.0)	8.5 (6.1)	0 (0)

DNA hybridized at 70°C

% hybridization with excess unlabelled DNA

Strain	1	5	10	L6I1	L6I2	L6I4	L6I6	M. pneumoniae	M. arginini	A. laidlawii	E. coli
^{32}P 1	100 (65.3)	74.0 (48.3)	72.9 (47.6)	89.3 (58.3)	84.0 (54.9)	89.3 (58.3)	90.1 (58.8)	NT	10.3 (6.7)	4.2 (4.0)	1.3 (0.85)
^{32}P 5	80.8 (53.7)	100 (66.5)	78.2 (52.0)	77.7 (51.7)	85.0 (56.6)	90.0 (59.9)	85.2 (56.7)	NT	NT	NT	4.1 (2.7)
^{32}P 10	72.5 (44.4)	69.4 (42.5)	100 (61.2)	77.7 (47.6)	82.4 (50.4)	88.6 (54.2)	88.4 (54.1)	NT	NT	NT	6.9 (4.2)
^{32}P L6I1	71.7 (47.9)	69.0 (46.1)	68.1 (45.5)	100 (66.8)	100 (66.8)	100 (66.8)	100 (66.8)	NT	15.4(10.3)	8.7 (5.8)	0 (0)

1 Each value represents the average from four separate experiments. Values from each experiment did not differ by more than 4 percent.
2 M. ovipneumoniae strains obtained from sheep on different farms in New Zealand.
3 M. ovipneumoniae strain 10 - Australian source.
4 M. ovipneumoniae strains recovered from a single pneumonic lung and having a closely similar but not an identical BRENDA pattern.
5 M. pneumoniae (ATCC 15531).
6 M. arginini strain M30/C4.
7 A. laidlawii (ATCC 23206).
8 E. coli strain V517.
9 % relative homology (i.e. represents the normalized values).
10 % hybridization with the ^{32}P-DNA probe.
11 NT - not tested.

7.4 Discussion.

Having found that the heterogeneity of DNA cleavage patterns might be explained by postulating varying degrees of methylation of the bases (note this work was undertaken before all the restriction results described earlier were to hand), it became desirable to use another technique, i.e. DNA-DNA hybridization, which is unafffected by base methylation, to investigate the heterogeneity of the DNA.

With this in mind we attempted to answer two questions:

a) Is *M. ovipneumoniae* DNA truly heterogeneous as indicated by DNA-DNA hybridizations and if so:

b) Could *M. ovipneumoniae* be subdivided into two or more species or subspecies?

DNA-DNA hybridizations conducted at low stringencies (i.e. Tm-25^{o}C) demonstrated that the *M. ovipneumoniae* isolates are heterogeneous (i.e. homology values range from 79.6% to 100%), as seen in Table IX. At higher stringencies (i.e. Tm-10^{o}C) the heterogeneity of *M. ovipneumoniae* strains becomes even more evident i.e. homology values range from 68.1% to 100%. This indicates that there are major differences in DNA base sequences, in different *M. ovipneumoniae* isolates, so we conclude that the DNA of *M. ovipneumoniae* is indeed heterogeneous. This confirms earlier conclusions (results with *Dra*I) that the restriction endonuclease cleavage pattern differences are not merely a function of differences in methylation of the bases.

We now consider if the heterogeneity observed indicates that *M. ovipneumoniae* should be divided into two or more species or subspecies. Obviously, when the DNA of two related organisms differ beyond a certain point, they will be assigned to different species. The critical question is how much they have to differ before they should be officially recognized as different species or subspecies.

Brenner and Falkow (1971) have discussed this at length, but without setting precise figures. However, the most authoritative guidelines are to be found in Bergey's Manual of Systematic Bacteriology (see Johnson, 1984). In brief, isolates which differ to the extent that their homology is between 70% and 100% can be accomodated within one species. This

might be extended to homologies as low as 60% provided the homologies are scattered between 60% and 100%. If however two groups of isolates show a "clustering" of homologies, then two subspecies might be defined.

Our results with *M. ovipneumoniae* indicate that it is not related to *E. coli* (*sic*), is little related to *A. laidlawii* (about 15%) or *M. arginini* (about 10%) but is related (about 50% homology) to *M. pneumoniae*. The latter figure falls outside the maximum scale of 60 to 100% relatedness which may define a species, so clearly, *M. ovipneumoniae* and *M. pneumoniae* do not belong to the same species. These conclusions merely confirm what is generally believed and accepted.

Returning to the question of whether or not *M. ovipneumoniae* represents one or more species or subspecies. Clearly, a minimum figure (low stringency) of 79.2% for the degree of hybridization of DNA of various isolates falls well within the 70% limit defining a species (Johnson, 1984). Furthermore, the spread of homologies of the (admittedly few) isolates tested do not cluster at any figure significantly different from 100%, so there is no evidence for two groupings of *M. ovipneumoniae* isolates. We conclude that *M. ovipneumoniae* represents a single species.

CHAPTER EIGHT

General Discussion.

The major object of this work was to investigate the extent of the heterogeneity of *M. ovipneumoniae* isolates.

Serological heterogeneity which can be linked to immunity to *M. ovipneumoniae* is ultimately the most critical area of heterogeneity, so our initial experiments used the metabolic inhibition test. This showed that *M. ovipneumoniae* is serologically heterogeneous, but did not allow us to distinguish groupings of isolates. This conclusion is similar to that of Jones *et al*, (1976) who could distinguish between some *M. ovipneumoniae* isolates but failed to recognize clear-cut groups. Jones *et al*, (1976) however, noted a "polarization" of isolates between "nasal" and "pneumonic" strains. We did not pursue this comparison because initial studies (Ionas *et al*, 1985) showed that nasal strains can colonize the lung and hence there is probably no meaningful distinction between them.

The inability of metabolic inhibition tests to distinguish well defined groupings of *M. ovipneumoniae* led us to explore several other approaches. The first of these approaches was to examine isolates by SDS-PAGE. The initial study with eight isolates (Figure 9) derived from sheep on different farms showed that all were different. These differences were meaningful because the patterns obtained were highly reproducible, even when fresh cultures were used (Figure 12). However, since all eight isolates differed, no recognizable groupings could be distinguished, so we extended the study and examined sixty isolates which we derived from the lungs of pneumonic sheep i.e. three isolates were examined from three pneumonic lungs from sheep from each of twenty farms. The results indicated, somewhat disconcertingly, that fifty-eight different patterns were seen (Figure 10a, b, c and d). Three identical patterns were found for three isolates from three sheep on one farm (Figure 10a, patterns 1a, 1b and 1c). This result clearly indicates that there is a large number of different strains of *M. ovipneumoniae* associated with sheep in New Zealand and confirms and extends the findings of Mew *et al*, (1985). But the heterogeneity seen in the SDS patterns of *M. oviponeumoniae* isolates contrasts with the results of other workers (Razin, 1968; Zola *et al*, 1970; Forshaw, 1972; Daniels and Meddins, 1973; Gois

et al, 1974, Jones *et al*, 1976; Asa *et al*, 1979 and Chandler *et al*, 1972) who have examined different species of mycoplasmas and have found that the SDS-PAGE patterns within one species were identical or closely similar.

To estimate the total number of *M. ovipneumoniae* strains would probably be a difficult task, since the number is clearly too large for *M. ovipneumoniae* isolates to be "typed" by exactly matching each SDS-PAGE pattern with a standard set. However, a modified approach to classifying *M. ovipneumoniae* isolates by their SDS-PAGE pattern could be to detect some prominent band or bands which were present in a proportion of isolates only. One such band was detectable in our original experiments (Figure 10a, b, c and d). To illustrate this, some isolates with and without this band were re-propagated and their proteins were extracted and re-examined on SDS-PAGE gels (Figure 11). Clearly, at least these eighteen isolates can be assigned to one or other group depending on the absence of this single band. However, the question arises: "how meaningful is this division?" If the band represents a surface protein then it would probably play a role in immunity. If not, its significance if any, would be less certain. We therefore concluded that examination of total proteins of SDS-PAGE to classify strains of *M. ovipneumoniae* is not, on its own, a useful or practical approach. But, if combined with another approach e.g. comparison of surface proteins only, it may have some potential. Anticipating some later results, we note here that our evidence suggests that the band in question is not a surface protein. Nevertheless, the above consideration led us to attempt to identify surface proteins. We attempted this initially by exposing intact cells to chemicals which were reported to be unable to cross membranes (Maddy, 1964 and Dockter, 1979). These experiments were unproductive and are not recorded here.

Another approach is to selectively remove surface proteins using proteolytic enzymatic enzymes. This requires that the enzymes act on surface proteins but, in the time and conditions used, do not lyse the cells. Comparison of trypsin, pronase and papain for various times and concentrations showed that high concentrations of pronase and papain rapidly lysed the cells (Figure 13b and d). Trypsin did not lyse the cells even at relatively high concentrations for a long period (30min). It did however selectively remove (or diminish in staining intensity) several proteins (Figure 13f) which we conclude are surface proteins. These proteins labelled A to F are best identified in Figure 14. Bands A, B, C and D were common to eight isolates. Band E, which shows diminished staining following trypsin treatment, was present on two isolates only and band F was unique to one isolate.

Surface proteins of micro-organisms are often glycoproteins and will therefore stain with silver following periodate treatment. To augment the results following proteolytic digestion, our original eight *M. ovipneumoniae* isolates were examined by this method. Of the four common surface proteins (Figure 14, bands A, B, C and D) three (Figure 15, bands B, C, and D) are glycoproteins. Band A is apparently not a glycoprotein.

Band E is also a glycoprotein (Figure 15) and was detected in only two of the eight isolates examined. None of the unique (surface) bands were stained using this method (compare Figure 14 with Figure 15) which suggests that although they are surface proteins, they are not glycoproteins.

The total number of bands which stained by periodiate-silver was high and the method is technically difficult. Consequently, it does not have much potential as a rapid means of classifying and distinguishing isolates of *M. ovipneumoniae.* A technique which examines a smaller number of bands, preferably surface proteins and also considers their serological relationship should be more valuable. We therefore turned to immuno-blotting using antisera prepared against five apparently single SDS-PAGE bands which were previously identified as surface proteins by proteolytic digestion. Of these five bands, two were common to all isolates tested (Figure 19, band C and Figure 20, band D) and are thus species specific (Figure 21). Band E, which was apparently shared (on SDS-PAGE, Figure 14) by only two of the eight isolates was found to represent at least two proteins which are common (in the serological sense) to all isolates but differ slightly in molecular weight. Band F was present in only one of the eight isolates as shown by SDS-PAGE (Figure 14) and antiserum to this band showed that it is unique serologically as well as in its molecular weight. More interestingly, antiserum to band B, which is apparently common to all eight isolates on SDS-PAGE gels (Figure 14), reacted with only five of the eight isolates (Figure 21). This means that antiserum to this surface protein has the potential to divide *M. ovipneumoniae* isolates into two groups, which may correlate to immunity. This line of investigation merits further work, but in our opinion the most appropriate way to proceed is to produce monoclonal antibody to individual proteins, especially to unique surface proteins and to surface proteins common to only a proportion of isolates. However, monoclonal antibody technology was not available to us when this investigation was in progress. We therefore turned out attention to DNA studies which have great potential to distinguish *M. ovipneumoniae* isolates.

Isolates which are of one species but which show diversity must differ to some extent in both their proteins and, even more fundamentally, in their DNA. Having shown that

M. ovipneumoniae isolates differ in a significant proportion of their proteins, but that this did not readily allow us to recognize groups of organisms within the species, we decided to concentrate on DNA studies. Initially we examined organisms by restriction endonuclease analysis, but in the light of these results, isolates were compared by DNA-DNA hybridizations.

Our initial experiments compared the DNA of eight isolates of *M. ovipneumoniae* using *Eco*RI digests. All gave totally different patterns (Figure 25).

Sixty isolates which we derived from sheep on twenty different farms were examined using bacterial restriction endonuclease DNA analysis by a colleague (Norman, 1985) who found all were different except for the three isolates from one farm which were identical when examined by SDS-PAGE (Figure 10a, b, c and d). This result further confirms the heterogeneity of *M. ovipneumoniae* strains and also indicates, as would be expected, that the heterogeneity of proteins reflects heterogeneity of the DNA. However, this is in marked contrast to the results obtained with other species of mycoplasma i.e. *M. gallisepticum* and *M. genitalium* (Razin *et al*, 1983c) and *M. pneumoniae* (Razin *et al*, 1983b) indicated a high degree of genetic homogeneity using bacterial restriction endonuclease DNA analysis.

It did however, occur to us that the apparent absence of common bands in the patterns might be a function of the endonucleases used and other restriction endonucleases might indicate a closer relationship between isolates. We therefore re-examined the eight isolates using *Msp*I (Figure 28), *Hpa*II (Figure 27) and later (for reasons described below) with *Dra*I (Figure 26). However, in all cased the eight isolates gave totally different DNA restriction cleavage patterns. Clearly, *M. ovipneumoniae* is a heterogeneous species! Razin *et al* (1983c) also used a range of restriction enzymes to examine *M. gallisepticum* and *M. genitalium*, they found that the DNA cleavage patterns indicates genetic homogeneity.

As outlined above, isolates from sheep on different farms differed, isolates from different sheep on one farm differed, so could it be that isolates from one pneumonic lung differ? We addressed this question by selecting six pneumonic lungs; four had severe i.e. large lesions, whereas two had small lesions. Thirty isolates were derived from each pneumonic lung, ten by each of three different methods ("normal" isolation; limit dilution and direct from colonies on agar). Three of the four lungs with large lesions contained four different *M. ovipneumoniae* strains (Table V) and the other one had three as indicated by restriction

endonucleases analysis of the DNA. The remaining two lungs with small lesions each had two different strains. Since we can not be certain that all strains present in the lungs were isolated, these represent minimum figures. The possible implication of this for disease is discussed later.

The heterogeneity of four strains from lung six, initially distinguished by *Eco*RI (Figure 25), was further examined using a range of enzymes and this gave unexpected results i.e. incomplete digestion in many cases. However, some restriction endonucleases used i.e. *Xba*I, *Bgl*I, *Hin*dIII, *Msp*I and *Dra*I (Figures 36 to and including 40) gave complete DNA digestion and all four isolates from the one lung were confirmed as being different. The DNA was also examined by *Hpa*II (Figure 41) which gave complete digestion of two of the four isolates but incomplete digestion of the other two isolates. *Kpn*I, *Sal*I, *Bam*HI and *Sma*I (Figures 42 to and including 45) all gave partial digests of the DNA. *Xho*I and *Hae*III (Figures 46 and 47) gave a complete digest, a partial digest or no digestion of DNA from the four isolates. However, in all cases (except where no digestion occurred) all four isolates gave different DNA cleavage patterns.

The recognition sequences of enzymes which gave only partial, or no digestion, were cytosine-rich (i.e. cytosine represented 50% or more of the bases) whereas those which gave complete digestion were not cytosine-rich (see Table VI). This observation suggested that cytosine-methylation, which usually prevents cleavage, may occur in *M. ovipneumoniae* strains, perhaps to differing extents and affecting different sequences in various isolates. This was a disconcerting concept because the different restriction endonuclease cleavage patterns on which we had placed much stress, might be explained on the relatively trivial basis that they represent different degrees and patterns of cytosine-methylation rather than different base sequences. This would cast doubt on some of our major conclusions. However, before this point is further considered, the evidence that cytosine-methylation does indeed occur, should be discussed. *Msp*I cleaves 5'-C^{V}CGG-3' independently of "internal" cytosine-methylation, whereas *Hpa*II cleaves only the unmethylated sequence. As indicated in Figure 74, *Hpa*II, in contrast to *Msp*I which gave complete digestion, failed to cleave a part of the DNA of the isolates tested indicating the presence of 5-methylcytosine. However, more definitive evidence was gained by HPLC examination of digested DNA (Figure 76a). The isolate which totally failed to be cleaved by *Xho*I and *Hae*III (i.e. the isolate which probably had the highest proportion of 5-methylcytosine) was estimated to have about 25 to 30% of its cytosines in the form of 5-methylcytosine.

While little has been published on the presence of modified nucleotides of mycoplasma DNA. Chan and Ross (1984) were able to use DNA methylation to distinguish betwee two mycoplasma species i.e. *M. hyopneumoniae* which contained 6-methyladenosine in its DNA, while *M. flocculare* contained no methylated bases.

Razin and Razin (1980) examined the hydrolysed DNA of five *Mycoplasma* and one *Acholeplasma* species by HPLC. They found that 6-methyladenosine was present in all isolates. This contrasts with our *M. ovipneumoniae* results where no 6-methyladenosine was detected. However, Razin and Razin (1980) found one species, *M. hyorhinis*, that contained both 6-methyladenosine and 5-methylcytosine.

Clearly since 5-methylcytosine does occur in *M. ovipneumoniae*, so two questions arise: To what degree, if any, does this invalidate the distinction made between strains of *M. ovipneumoniae* and does it invalidate our conclusion that the DNA of *M. ovipneumoniae* is heterogeneous?

It has been a consistent finding throughout this work that any isolate of *M. ovipneumoniae*, when repeatedly examined by any restriction endonuclease, gave the same banding pattern even when separate cultures were prepared and independently processed at different times. This constant banding pattern (which is almost always unique for any isolate) implies that a strain can be unequivocally identified. This conclusion is independent of the presence or absence of 5-methylcytosine. However, to return to the second question. The heterogeneity of DNA in terms of base sequence, may, because of variation in methylation between isolates, be less than is implied by the DNA restriction results. This possibility was investigated by DNA-DNA hybridization and we were able to show that *M. ovipneumoniae* DNA is heterogeneous. Thus, the extent of homology of isolates varied from 100% down to as low as 70% (Table IX).

We therefore conclude that DNA sequence differences account for much, (if not all) of the differences in restriction endonuclease cleavage patterns seen. Furthermore, if differences in cytosine methylation were the dominant factor in heterogeneous DNA digests, then digestion with an enzyme which recognizes a sequence not containing cytosine would show identity between isolates, because we have already shown that adenosine-methylation does not occur (Figure 73). The opposite, however, was found (Figure 26) and *Dra*I gave totally different patterns for the DNA of our original eight isolates.

We therefore conclude that although *M. ovipneumoniae* DNA can have 5'-methylcytosine, this does not interfere with the use of restriction endonuclease analysis to identify strains and it does not account for the heterogeneity of restriction endonuclease cleavage patterns seen.

While the DNA-DNA hybridization was undertaken, for the reason outlined above, our results justify some further comment. The heterogeneity of *M. ovipneumoniae* DNA i.e. 70 to 100% homology of various strains, though quite large, can be accommodated with one species. In view of our results with restriction endonuclease digests, it is important to state clearly that the heterogeneity observed in *M. ovipneumoniae* reflects differences within one species and our results do not suggest that *M. ovipneumoniae* should be divided into two or more species or subspecies.

The protein and DNA are legitimate foci for study but microbiologists interested in micro-organisms and disease might reasonably ask if it has any relevance in this area. Of course SDS-PAGE or REA patterns can be used for epidemiologically tracing of individual strains (Ionas *et al*, 1985) but, do our results have any relevance in the understanding of chronic non-progressive pneumonia?

Jones *et al* (1978) showed that CNP (which he called atypical pneumonia) could be transmitted by *M. ovipneumoniae* followed by *P. haemolytica*. Their experiments clearly demonstrate that infection with multiple strains of *M. ovipneumoniae* followed by *P. haemolytica* were more successful in transmitting severe lesions than was any one strain. This result has not been to our knowledge the subject of comment, but in our view it is a potentially important conclusion.

The classical concept of microbial pathogenesis is that one organism causes one disease. More recently it has become obvious that two different species can often be involved in one disease e.g. β-haemolytic streptococci in conjunction with staphylococci (impetigo) or *Bacteroides* in conjunction with *E. coli* (intra-abdominal abscesses), to give two of many possible examples.

Our results and those of Jones *et al* (1982a), go a stage further and suggest that multiple strains of one organism may be more pathogenic than any one strain on its own. If this

concept is not exclusive to CNP (an unlikely possibility), microbiologists interested in pathogens should now consider that several strains of one species may be involved in the maximum production of lesions of one disease. Thus, our demonstration of the heterogeneity of *M. ovipneumoniae* gave rise to a novel concept which should be investigated using other systems to confirm (or not as the case may be) its general validity.

APPENDIX I

Media and Reagents Commonly Used in this Thesis.

1) **Eagles Vitamin Solution** (x100)

Calcium Pantothenate	20.0mg
D-biotin	20.0mg
Calcium Chloride	20.0mg
Folic Acid	20.0mg
Riboflavin	2.0mg
Myo-inositol	40.0mg
Niacinamide (Nicotinamide)	20.0mg
Pyridoxine	20.0mg
Thiamine-HCl	20.0mg
Distilled Water to	200.0ml

This solution was stored in 25ml aliquots at -20^{o}C.

2) **0.2M EDTA**

Ethylaminediaminetetraacetic acid (EDTA)	7.44g
Distilled Water to	1000.0ml

3) **FM4 Agar**

BHI (Difco)	0.74g
Agar (Davis)	1.0g
Distilled Water to	20.0ml

This was autoclaved at 121^{o}C for 15mins, allowed to cool to approximately 50^{o}C and added to 80ml of FM4 Medium which had been preheated to 46^{o}C. This was then mixed thoroughly and 4ml aliquots were dispensed into Gelman^{TM} 50mm x 9mm petri dishes with tight fitting lids. The plates were left to solidify and stored at 4^{o}C.

4)**FM4 Medium** (Frey *et al*, 1968)

NaCl	5.0g
KCl	0.4g
$MgSO_4.7H_20$	0.2g
$Na_2HPO_4.12H_20$	4.03g
KH_2PO_4	0.1g
Glucose	10.0g
Peptone CS (ALBIMI)	10.0g
Yeast Autolysate (Pfizer)	5.0g
β-NAD	0.1g
L-Cysteine HCl	0.1g
Eagles Vitamin Solution (x100)	25.0ml
0.4% Phenol Red	2.5ml
Penicillin	10^6 Units
Thallium Acetate	0.5g
Distilled Water to	1000.0ml

Additional factors to take note of:

a. The Thallium Acetate was dissolved in 10ml of distilled water and added to the medium dropwise to prevent precipitation.

b. The basal medium was supplimented with 150ml of swine serum.

c. The pH of the medium was adjusted to 7.8 with 1.0M NaOH.

d. The complete medium was clarified by filtration through non-sterile 5.0um, 0.45um and 0.2um pore size membrane filters and sterilized by filtration through a sterile 0.2um Tuffryn^{TM} membrane filter.

5)**"Modifled" FM4 Medium**

Same as FM4 Medium, however, the swine serum was replaced with bovine serum; Albimi Peptone was replaced with Bacto Peptone and Yeast Autolysate (Albimi) was replaced with Pytone Peptone.

6)**1.4M Phosphate Buffer** (Stock solution)

Na_2HPO_4	198.8g
$NaH_2PO_4.2H_2O$	218.4g
Distilled Water to	2000.0ml

7)**Phosphate Buffered Saline (PBS)** pH 7.2

NaCl	8.5g
$Na_2HPO_4.12H_2O$	2.7g
$NaH_2HPO_4.2H_2O$	0.39g
Distilled Water to	1000.0ml

Autoclave for 15mins at 121^{o}C, store at room temperature.

8)**0.15M Saline**

NaCl	8.78g
Distilled Water to	1000.0ml

This solution was autoclaved at 121^{o}C for 15mins and stored at room temperature.

9)**Saline Tris-EDTA (STE) Buffer** (x10)

5.0M NaCl	20.0ml
1.0M Tris-HCl (pH 7.5)	50.0ml
0.2M EDTA (disodium salt) (pH 7.2)	5.0ml
Distilled Water to	100.0ml

Stored at room temperature and diluted 1/10 before use.

10)**1M Tris Buffer** (pH 7.5)

Trizma Base (Sigma)	12.11g
HCl to pH 7.5	
Distilled Water to	100.0ml

Stored at room temperature.

11)**TE Buffer** (Tris-EDTA)

1M Tris-HCl (pH 7.5)	40.0ml
0.2M disodium salt EDTA (pH 7.2)	20.0ml
Distilled Water to	4000.0ml

12)**Tris-EDTA Solution**

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

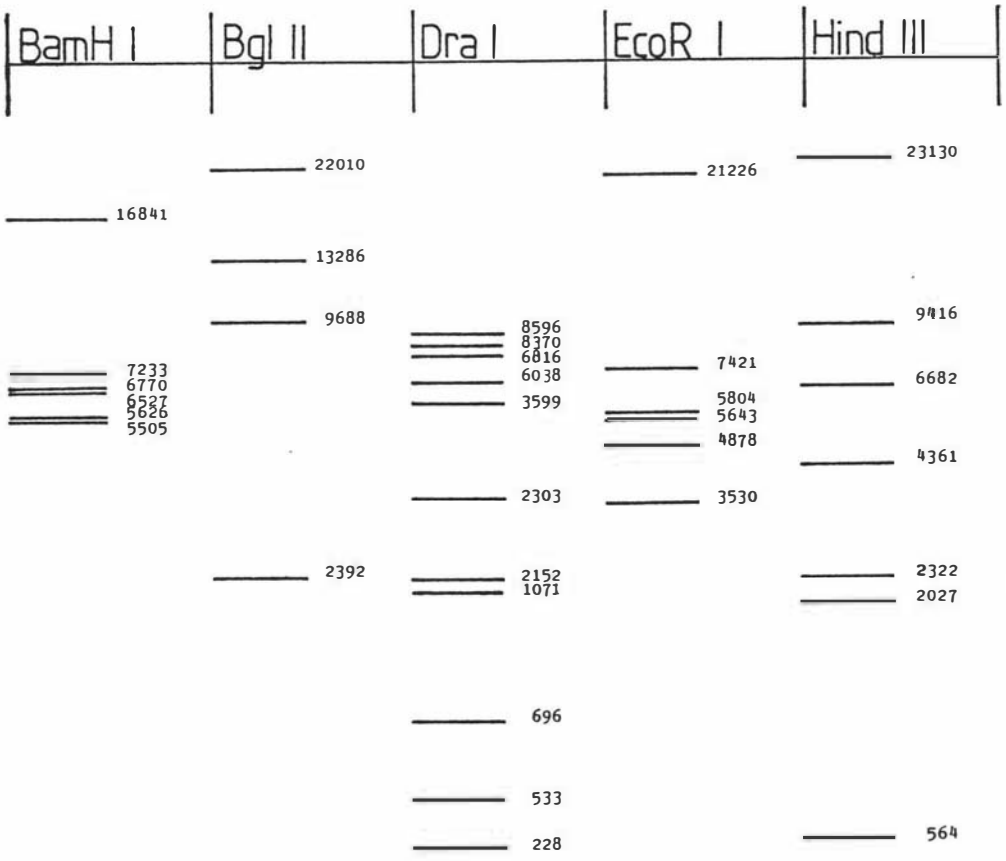
APPENDIX II

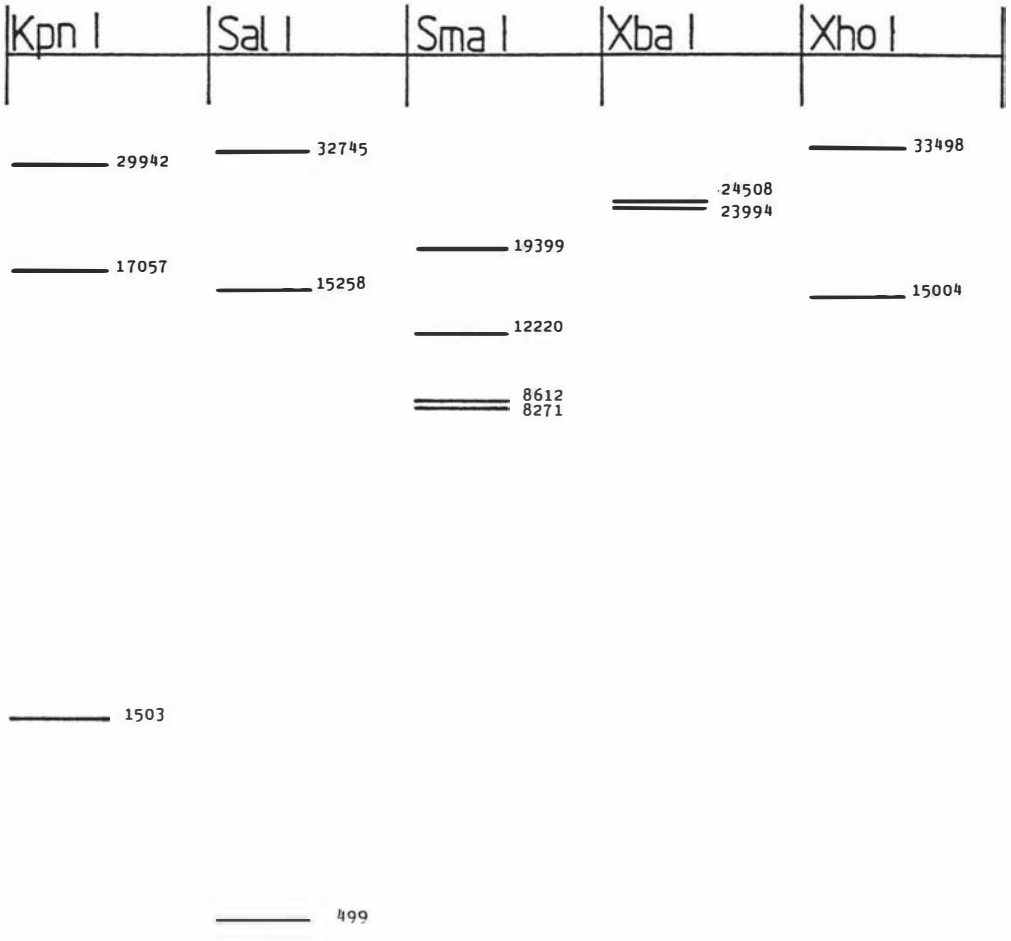
MOLECULAR WEIGHT MARKERS

Bacteriophage Lambda DNA was digested with restriction endonucleases to serve as both a control for Bacterial Restriction Endonuclease DNA Analysis and as a molecular weight marker. The molecular weights of the cleaved fragments were determined from a sequence analysis software package (Devereux *et al*, 1984).

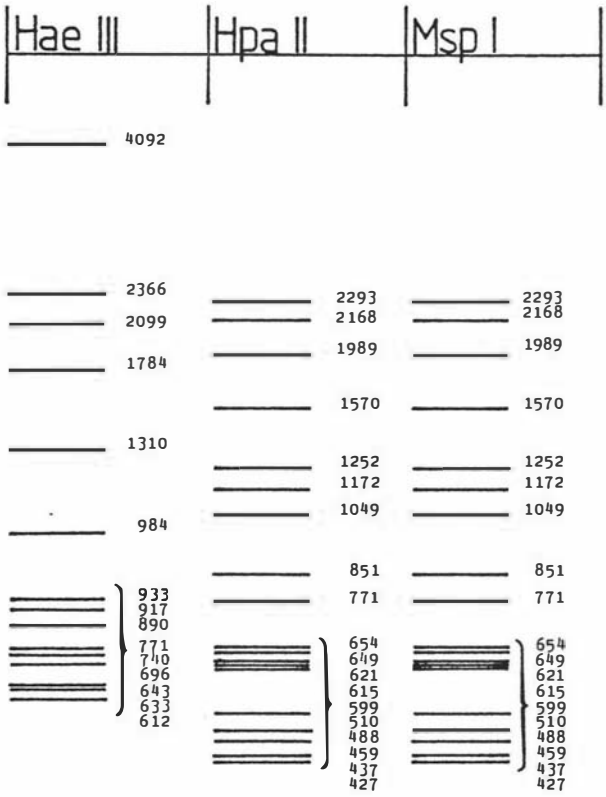
The molecular weights are adjacent to the cleavage fragment and are expressed in kilobases (kb). Molecular weight marker for proteins are expressed megadaltons.

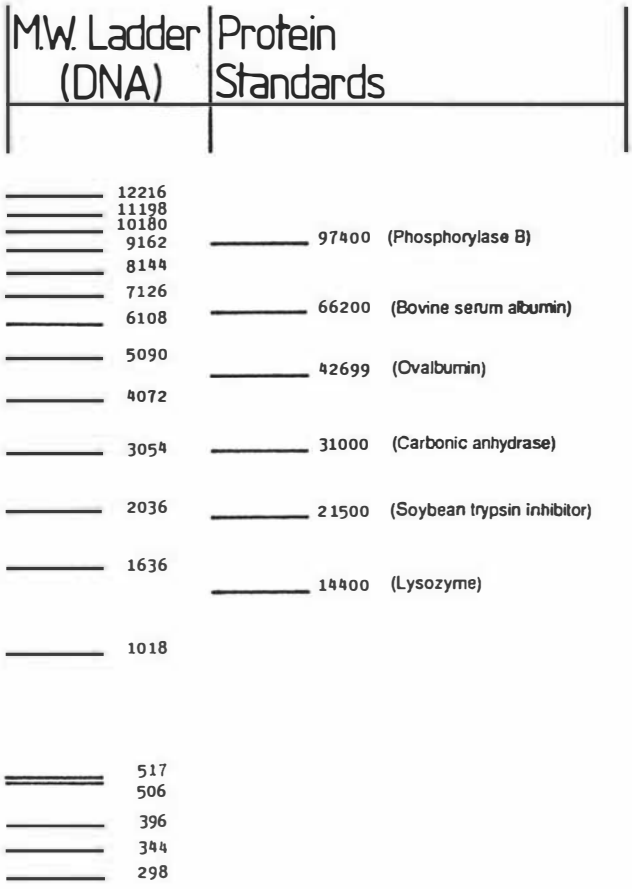
1. Restriction Endonucleases which recognize six base sequences.





2. Restriction endonucleases which recognize four base sequences.




M.W. Ladder (DNA)
Protein Standards
12216
11198
10180
9162
8144
7126
6108
5090
4072
3054
2036
1636
1018
517
506
396
344
298
97400 (Phosphorylase B)
66200 (Bovine serum albumin)
42699 (Ovalbumin)
31000 (Carbonic anhydrase)
21500 (Soybean trypsin inhibitor)
14400 (Lysozyme)

APPENDIX III

Publications:

1) Colonization of the respiratory tract of lambs by strains of *Mycoplasma ovipneumoniae*.

2) Comparison of *Mycoplasma ovipneumoniae* isolates using bacterial restriction endonuclease DNA analysis and SDS-PAGE.

Veterinary Microbiology, 10 (1985) 533—539
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

COLONISATION OF THE RESPIRATORY TRACT OF LAMBS BY STRAINS OF *MYCOPLASMA OVIPNEUMONIAE*

G. IONAS and ALISON J. MEW

Department of Microbiology and Genetics, Massey University, Palmerston North (New Zealand)

M.R. ALLEY

Department of Veterinary Pathology and Public Health, Massey University, Palmerston North (New Zealand)

J.K. CLARKE*

Department of Microbiology and Genetics, Massey University, Palmerston North (New Zealand)

A.J. ROBINSON** and R.B. MARSHALL

Department of Veterinary Pathology and Public Health, Massey University, Palmerston North (New Zealand)

(Accepted for publication 4 February 1985)

ABSTRACT

Ionas, G., Mew, A.J., Alley, M.R., Clarke, J.K., Robinson, A.J. and Marshall, R.B., 1985. Colonisation of the respiratory tract of lambs by strains of *Mycoplasma ovipneumoniae*. *Vet. Microbiol.*, 10: 533—539.

The age and time of year when colonisation of the nasal cavity of lambs by *Mycoplasma ovipneumoniae* occurs; the persistence of the organism, and its prevalence in the lungs at slaughter were examined in 2 flocks of sheep in New Zealand. No colonisation had occurred at the time of weaning at 6—7 weeks, but *M. ovipneumoniae* was recovered from most lambs on at least one occasion before they were slaughtered when about 8 months old. In most cases, colonisation of the nasal cavity by *M. ovipneumoniae* was a transient phenomenon. At slaughter *M. ovipneumoniae* was recovered from the lungs of 89% of the lambs of one flock and 80% of the other flock.

Bacterial restriction endonuclease DNA analysis (BRENDA) of 34 nasal isolates from one flock showed that it was possible to identify 7 "groups" each with markedly different BRENDA patterns. Lambs initially colonised by one strain, often lost that strain, and if recolonisation occurred it was with a different strain.

M. ovipneumoniae was recovered at slaughter from the lungs of most lambs, both normal and pneumonic. The isolates from one flock were examined by BRENDA, and approximately 90% of them gave similar or identical patterns. The predominant strain isolated from the lungs had been recovered from the nasal cavity of many of the lambs about 3 weeks earlier. This suggests that the nasal and lung isolates do not represent independent populations. However, nasal strains may differ in their ability to colonise the lungs.

*To whom all correspondence should be addressed. **Present address: MRC Virus Research Unit, University of Otago, Dunedin, New Zealand.

0378-1135/85/$03.30

M. arginini colonised the nasal cavity of lambs much later than *M. ovipneumoniae*. At slaughter *M. arginini* was recovered from 12% of the lungs of one flock but was not recovered from the lungs of the other flock.

INTRODUCTION

Mycoplasma ovipneumoniae plays a role in the aetiology of chronic non-progressive pneumonia (CNP) of lambs (Alley et al., 1975; Jones et al., 1976). As little is known about the spread of this organism in sheep we investigated two flocks of lambs to establish the age and time of year at which nasal colonisation occurs, the period for which *M. ovipneumoniae* persists in the nasal cavity and the prevalence of the organisms in the lungs at slaughter.

In an accompanying study (Mew et al., 1985), *M. ovipneumoniae* isolates were examined by bacterial restriction endonuclease DNA analysis (BRENDA), and it was shown that many different "groups" of this organism are present in sheep in New Zealand. Using this approach isolates of *M. ovipneumoniae*, which were recovered from the nasal cavities of one flock over a period of 7 months, were "grouped" and the relative frequency with which these groups could be recovered from the lungs at slaughter was determined.

For completeness all specimens were also tested for the presence of *M. arginini*.

MATERIALS AND METHODS

Experimental animals

Two groups of lambs each from separate flocks were examined: 28 Perendale lambs (Flock 1) located in hill country in the Manawatu and 25 Suffolk lambs (Flock 2) located on the Manawatu plains.

The lambs were first swabbed immediately after weaning when approximately 45—50 days old, and then at 3—4-week intervals until they were slaughtered when 8—9-months old.

Lambs from both flocks were each derived from 20 ewes that were swabbed at weaning. To obtain swabs each animal was firmly restrained in an upright position with its head held by an assistant. A sterile cotton wool swab was inserted into the middle and dorsal meatus of each nostril and rotated. It was then removed and broken off into 3 ml of mycoplasma medium.

Isolation and identification of mycoplasmas

Following slaughter, the lungs were collected in individual plastic bags and taken to the laboratory. The isolation and identification of mycoplasmas

TABLE I

Recovery of *M. ovipneumoniae* and *M. arginini* from Flock 1

	Date of swabbing[a] and approximate age of lambs (days)									
	6 Oct (50)	3 Nov (77)	28 Nov (102)	15 Dec (119)	14 Jan (149)	18 Feb (184)	16 Mar (210)	15 Apr (240)	11 May (266)	12 May[b] (267)
M. ovipneumoniae isolates	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (3.6%)	3 (10.7%)	3 (10.7%)	14 (50.0%)	16 (57.1%)	25 (89.3%)
M. arginini isolates	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (3.6%)	0 (0%)

[a]The nasal cavity of 28 Perendale lambs were swabbed at approximately monthly intervals (6th Oct—11th May). The swabbing began at weaning and ended one day before slaughter.
[b]The lungs at slaughter.

TABLE II

Recovery of *M. ovipneumoniae* and *M. arginini* from Flock 2

	Date of swabbing[a] and approximate age of lambs (days)								
	12 Oct (45)	3 Nov (71)	25 Nov (93)	16 Dec (114)	13 Jan (142)	17 Feb (177)	19 Mar (207)	6 Apr (225)	8 Apr[b] (227)
M. ovipneumoniae isolates	0 (0%)	1 (4%)	5 (20%)	5 (20%)	0 (0%)	3 (12%)	15 (60%)	5 (20%)	20 (80%)
M. arginini isolates	1 (4%)	0 (0%)	1 (4%)	1 (4%)	1 (4%)	1 (4%)	9 (36%)	13 (52%)	3 (12%)

[a]The nasal cavity of 25 Suffolk lambs were swabbed at approximately monthly intervals (12th Oct—6th Apr). The swabbing began at weaning and ended 2 days before slaughter.
[b]The lungs at slaughter.

from the lungs and nasal swabs were performed as described by Clarke et al. (1974).

Bacterial restriction endonuclease DNA analysis

The DNA of *M. ovipneumoniae* isolates was examined by BRENDA as described by Marshall et al. (1981) and Mew et al. (1985).

RESULTS

Recovery of mycoplasmas from the nasal cavity of ewes

M. ovipneumoniae was recovered at the time of weaning from 1 of the 20 ewes (5%) of Flock 1 and 3 of the 20 ewes (15%) of Flock 2.

M. arginini was recovered at the time of weaning from 1 of the 20 ewes (5%) of Flock 1 and 3 of the 20 ewes (15%) in Flock 2. In 2 cases only, *M. arginini* and *M. ovipneumoniae* were both recovered from the same ewe.

Recovery of mycoplasmas from the lambs

The recovery of *M. ovipneumoniae* and *M. arginini* from serial nasal swabs from the 2 flocks and from the lungs at slaughter is recorded in Tables I and II.

The cumulative proportions of lambs from which *M. ovipneumoniae* and *M. arginini* were recovered on at least 1 occasion are recorded in Fig.1.

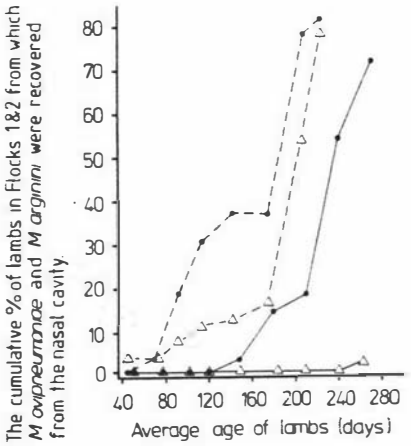


Fig. 1. The cumulative proportions of lambs in Flock 1 from which *M. ovipneumoniae* (●——●) and *M. arginini* (△——△) were recovered and in Flock 2 from which *M. ovipneumoniae* (●---●) and *M. arginini* (△---△) were recovered.

Examination of the lungs of lambs for pathological changes

Moderate to severe consolidation of the anterior lobe was exhibited by 32% (9/28) and 64% (16/25) of lungs from Flocks 1 and 2, respectively. The lesions were typical of CNP (Alley and Clarke 1979).

TABLE III

Classification of *M. ovipneumoniae* isolates from Flock 2

Lamb No.	Isolate[a]								
	1	2	3	4	5	6	7	8	Lungs
	Date of swabbing and approximate age of lambs (days)								
	12/10 (45)	3/11 (71)	25/11 (93)	16/12 (114)	13/1 (142)	17/2 (177)	19/3 (207)	6/4 (225)	8/4 (227)
M 1	—[b]	—	—	—	—	—	4b	—	4a
M 3	—	—	5[c]	1a	—	1b	4b	—	—
M 4	—	—	—	—	—	—	4a	—	4b
M 5	—	—	—	—	—	—	—	—	4b
M 6	—	—	—	—	—	—	—	—	4a
M 7	—	—	—	—	—	—	—	—	4b
M 8	—	—	—	—	—	—	4a	—	4b
M 9	—	—	—	—	—	—	—	—	4b
M 10	—	—	—	—	—	—	2	—	4a
M 11	—	—	—	—	—	—	4b	—	4b
M 12	—	—	—	6	—	3a	3b	3a	7
M 14	—	—	—	1b	—	1a	3c	7	3a
M 15	—	—	2	—	—	—	D		
M 16	—	—	—	—	—	—	4a	—	4a
M 18	—	—	—	—	—	—	4a	—	4a
M 19	—	—	—	—	—	—	4a	—	4a
M 20	—	—	—	—	—	—	—	—	4a
M 21	—	—	2	2	ND[d]	ND	ND	ND	4a
M 25	—	—	—	—	D[e]				
M 26	—	—	2	—	—	—	—	4b	—
M 27	—	—	—	—	—	—	4a	—	—
M 28	—	—	—	1a	—	—	4a	4a	4a
M 29	—	—	—	—	—	—	—	—	4a
M 30	—	—	—	—	—	—	—	—	4b
M 31	—	1a	1a	—	—	—	4b	4a	—
M 32	—	—	—	—	—	—	4a	—	4a

[a]Derived from nasal swabs taken at approximately monthly intervals. The last column refers to isolates derived from the lungs at slaughter.
[b]—, *M. ovipneumoniae* not isolated.
[c]The numbers indicate the BRENDA group and subgroup to which each isolate was assigned for the purpose of this study.
[d]ND = not done.
[e]D = died.

Restriction endonuclease DNA analysis of isolates from Flock 2

Thirty-four of the nasal isolates from Flock 2 were examined. The banding patterns observed were assigned to 7 groups, numbered 1—7, each of which was dissimilar, that is to say, the pattern seen within one group was totally different from that of any other group.

The isolates of 4 of the 7 groups were homogeneous within each group, but in the case of Groups 1, 3 and 4 variations were seen. To accommodate this, isolates within these latter 3 groups were assigned to homogeneous subgroups which were designated by the addition of the letters a, b or c. Two subgroups were recognised in Groups 1 and 4, whereas Group 3 has 3 subgroups.

When isolates of different subgroups within the same group were compared, they all had a definite overall similarity of banding patterns, but nevertheless differed in many bands up to a maximum of about 50%. The classification of the isolates is shown in Table III.

DISCUSSION

The present work shows that neither *M. ovipneumoniae* nor *M. arginini* rapidly colonised the nasal cavity of the lambs. At the time of weaning when our survey began, none of the lambs from either flock showed nasal carriage of *M. ovipneumoniae* and *M. arginini* was recovered from one lamb only. When the rates of colonisation of the 2 flocks by both mycoplasmas are compared it can be seen that *M. ovipneumoniae* spread more rapidly than *M. arginini*, and both mycoplasmas spread more rapidly in Flock 1 than in Flock 2. However, the spread of *M. ovipneumoniae* differed in timing only, because by the late summer or early autumn over 70% of the lambs of both flocks showed nasal carriage of *M. ovipneumoniae* on at least one occasion (Fig.1). Nevertheless, *M. ovipneumoniae* should not be regarded as part of the normal flora of the ovine nasal cavity because many positive lambs were negative when routinely retested and only 5% of the ewes of both flocks carried the organisms in their nasal cavity. Nasal carriage of *M. ovipneumoniae* by ewes is probably important and would provide an obvious source from which lambs could become infected.

The reason why lambs previously positive for *M. ovipneumoniae* should become negative was not investigated, but it is reasonable to suppose that it was due to an immune response. This explanation is apparently inconsistent with the finding that following the loss of *M. ovipneumoniae*, it can recolonise the nasal cavity. However, investigation of the organisms involved in recolonisation showed that in only 2 cases (M28 and M31) did the isolates have the original BRENDA pattern (Table III). Therefore, it would be of interest to know if changes in the BRENDA patterns of groups were correlated with antigenic changes on the mycoplasma membrane.

It is interesting that Jones et al. (1976) have already shown antigenic

differences in strains that may be correlated with pathogenicity, in the sense that when strains isolated from pneumonic lungs were compared serologically with other strains they showed an "apparent polarisation at opposite ends of the antigenic spectrum". Our results show that at least some *M. ovipneumoniae* groups that colonise the nasal cavity can also invade the lungs. However, it is not certain whether or not the groups isolated from pneumonic lungs are more pathogenic than those nasal groups that failed to colonise the lungs, or if they merely happened to be the group present in the nasal cavity at a time when the lamb was stressed, for example by hot, dry weather. Further investigations which attempt to correlate BRENDA patterns, serology and pathogenicity are required to resolve these questions.

Finally, perhaps the most important facet of the present work is that it demonstrates the heterogeneity of *M. ovipneumoniae* isolates even within one flock. The heterogeneity of isolates may be linked to the differences in pathogenicity that have recently been described by Buddle et al. (1982) in New Zealand. This may well explain the difficulties encountered when trying to elucidate the exact role of *M. ovipneumoniae* in CNP.

REFERENCES

Alley, M.R. and Clarke, J.K., 1979. Experimental transmission of ovine chronic non-progressive pneumoniae. N.Z. Vet. J., 27: 217—220.

Alley, M.R., Quinlan, J.R. and Clarke, J.K., 1975. The prevalence of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* in the respiratory tract of sheep. N.Z. Vet. J., 23: 137—141.

Buddle, B.M., Herceg, M. and Davies, D.H., 1982. Ovine Mycoplasma comparative pathogenicity of strains. Annual Report/Agriculture Research Division, New Zealand Ministry of Agriculture and Fisheries, pp. 159—160.

Clarke, J.K., Brown, V.G. and Alley, M.R., 1974. Isolation and identification of mycoplasmas from the respiratory tract of sheep in New Zealand. N.Z. Vet. J., 22: 117—121.

Jones, G.E., Foggie, A., Mould, D.L. and Livitt, S., 1976. The comparison and characterisation of glycolytic mycoplasmas isolated from the respiratory tract of sheep. J. Med. Microbiol., 9: 39—52.

Marshall, R.B., Wilton, B.E. and Robinson, A.J., 1981. Identification of leptospira serovars by restriction endonuclease analysis. J. Med. Microbiol., 14: 163—166.

Mew, A.J., Ionas, G., Clarke, J.K., Robinson, A.J. and Marshall R.B., 1985. Comparison of *Mycoplasma ovipneumoniae* isolates using bacterial restriction endonuclease DNA analysis and SDS-PAGE. Vet. Microbiol., 10: 541—548.

Veterinary Microbiology, 10 (1985) 541—548
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

COMPARISON OF *MYCOPLASMA OVIPNEUMONIAE* ISOLATES USING BACTERIAL RESTRICTION ENDONUCLEASE DNA ANALYSIS AND SDS-PAGE

ALISON J. MEW[1], G. IONAS[1], J.K. CLARKE[1]*, A.J. ROBINSON[2]** and R.B. MARSHALL[2]

[1]*Department of Microbiology and Genetics, and* [2]*Department of Veterinary Pathology and Public Health, Massey University, Palmerston North (New Zealand)*

(Accepted for publication 28 February 1985)

ABSTRACT

Mew, A.J., Ionas, G., Clarke, J.K., Robinson, A.J. and Marshall, R.B., 1985. Comparison of *Mycoplasma ovipneumoniae* isolates using bacterial restriction endonuclease DNA analysis and SDS-PAGE. *Vet. Microbiol.*, 10: 541—548.

Sixteen isolates of *Mycoplasma ovipneumoniae* recovered from the nasal tract or lungs of sheep from different flocks in New Zealand were examined by bacterial restriction endonuclease DNA analysis (BRENDA) using *Eco*R1 and by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). All isolates gave BRENDA patterns which differed entirely from one another. Following 20 serial passages (corresponding to approximately 67 generations) of an isolate, no change was detected in the BRENDA pattern.

When eight isolates were examined by SDS-PAGE most bands were common but, nevertheless, each isolate was unique in the sense that they differed from one another in one or more bands. The marked heterogeneity of patterns observed when strains of *M. ovipneumoniae* are compared by BRENDA, together with the stability of such patterns over many generations, will enable this approach to be used to study the epidemiology of individual strains of *M. ovipneumoniae* within a flock.

INTRODUCTION

Mycoplasma ovipneumoniae can be recovered from the nasal tract of sheep and from both pneumonic and apparently normal lungs. In view of the relationship of this organism to chronic non-progressive pneumonia (CNP) of lambs (Carmichael et al., 1972; Jones et al., 1978; Alley and Clarke, 1979) it was of interest to investigate whether *M. ovipneumoniae* isolated from the nasal cavity and lungs of normal or pneumonic sheep

*To whom requests for reprints should be directed.
**Present address: M.R.C. Virus Research Unit, University of Otago, Dunedin, New Zealand.

0378-1135/85/$03.30

on different farms form a homogeneous population or can be differentiated and perhaps assigned to a limited number of "groups".

Examination of proteins (Razin and Rottem, 1967; Daniels and Meddins, 1973) using polyacrylamide gel electrophoresis, or of DNA fragments (Razin et al., 1983a,b) following digestion with restriction endonucleases are now established methods of distinguishing between different species and strains of microorganisms, including mycoplasmas. This communication investigates the potential of these techniques to differentiate between various isolates of *M. ovipneumoniae*. We report that both approaches, but especially bacterial restriction endonuclease DNA analysis (BRENDA), show that different isolates of this organism are remarkably heterogeneous.

MATERIALS AND METHODS

Mycoplasma *strains*

Eight strains of *M. ovipneumoniae* recovered from the nasal tract or lungs of sheep in New Zealand were examined by BRENDA and by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The site of origin of these strains is shown in Table I. A further eight strains examined by BRENDA were recovered from the lungs of eight sheep, all from different farms. The organisms were isolated and identified as described by Clarke et al. (1974).

TABLE I

The sources from which isolates were recovered. With the exception of isolate 5 all strains were recovered from sheep on different farms in New Zealand

Isolate	Source
1	Pneumonic lung
2	Pneumonic lung
3	Pneumonic lung
4	Normal lung
5	Australian isolate
6	Pneumonic lung
7	Nasal
8	Normal lung

Cloning

All cultures used were cloned at least three times. Broth cultures of *M. ovipneumoniae* were passed through a 0.45 μm filter and the filtrate was used to prepare 10-fold serial dilutions which were spotted onto FM4

agar (Frey et al., 1968). An isolated colony at the limiting dilution was selected and removed with a small plug of agar into broth which was incubated at 37°C until a colour change was observed. Aliquots were stored at -70°C.

Preparation of DNA

M. ovipneumoniae strains were propagated in 300 ml of FM4 medium. The cultures were incubated on a rotary shaker at 37°C until the reaction of the medium was within the pH range 6.5—7.0. At this stage cultures are still in the exponential stage of growth (A.J. Mew, unpublished observations, 1982). The cells were collected by centrifugation at 14 500 *g* for 15 min, washed twice with phosphate buffered saline (0.15 M NaCl, 7.5 mM $Na_2HPO_4 \cdot 12H_2O$, 2.5 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.0; PBS), and resuspended in 1.5 ml of TEB (0.01 M Tris HCl pH 7.5; 0.01 M EDTA, disodium salt, pH 7.2). DNA was extracted from 1.0 ml of this suspension essentially as described by Marshall et al. (1981) except that the lysozyme step was omitted. Briefly, 1.0 ml of the cell suspension in TEB was incubated at 37°C for 15 min and 0.1 ml of a 10% solution of sodium dodecyl sulphate and 0.1 ml of Pronase (10 mg ml^{-1}) was added. Incubation was continued overnight at 50°C. Sodium perchlorate was added to a concentration of 1 M. The mixture was incubated for another hour at 50°C and made up to a volume of 5.0 ml with STE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.5, and 1 mM EDTA); it was then extracted three times with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1). The upper layer was removed on each extraction and then dialysed exhaustively against TEB at 4°C.

Restriction endonuclease digestion of DNA

Digestion with *Eco*R1 and subsequent electrophoresis was also as described by Marshall et al. (1981) i.e. the digestion mix was incubated at 37°C for 45 min and then heated at 65°C for 10 min. To precipitate the DNA, 1/20 volume of 5 M NaCl and 2 volumes of ethanol were added and after reaction at -20°C for 30 min the mixture was centrifuged at 8500 g for 5 min. The supernatant was discarded, the pellet washed with cold 70% ethanol and dried at 37°C, and 45 μl of TEB + 0.05% SDS + 20% glycerol was added to each sample, mixed and left at room temperature overnight. Lambda DNA was included as a control.

Gel electrophoresis and photography

Electrophoresis was carried out in a 0.7% agarose gel and run at 8 V cm^{-1} for 3.5—4 h. The electrophoresis buffer contained 0.5 μg ml^{-1} ethidium bromide.

The gel was photographed on Kodak Tri-X film through a Wratten 23A gelatin filter.

SDS-PAGE of mycoplasma *proteins*

M. ovipneumoniae cultures were propagated in FM4 medium until a pH change was first observed. The cells were then sedimented at 14 500 × *g* for 15 min, washed twice in PBS and the pellet resuspended in PBS to make an approximately 10% suspension. An aliquot was assayed for protein content and 100 µl of the 10% suspension was dissolved in 25 µl of 'final sample buffer' (Laemmli, 1970) to which 12.5 µl of bromophenol blue tracking dye was added. The samples were heated to 100°C for 3 min, left to cool and 60 µg of protein (usually about 10—15 µl of sample) was added to a track on the gel.

The SDS-PAGE system used 10% acrylamide and was as described by Laemmli (1970) but with two modifications: a vertical slab gel 140 mm long (running gel) was used and the gel was stained overnight with a mixture of 25% isopropanol, 10% glacial acetic acid and 0.04% Coomassie brilliant blue R in distilled water. The gel was de-stained in 10% glacial acetic acid.

RESULTS

Digestion of *M. ovipneumoniae* DNA with *Eco*R1 gave rise to a complex of 30—40 bands after electrophoresis in agarose gel and all 16 isolates gave substantially different patterns. The results obtained from the eight isolates recorded in the table are illustrated in Fig. 1. To demonstrate the reproducibility of these patterns, cultures were propagated in duplicate and the DNA was extracted and digested independently. The original BRENDA patterns were reproduced in all cases. In further experiments one isolate was used to prepare 8 replicate cultures which were processed independently; the BRENDA patterns obtained for each of the eight replicate cultures were indistinguishable. This isolate was then passaged 20 times by making a 10-fold dilution as soon as a colour change was observed. Following this serial passage the DNA was extracted and examined by BRENDA. No band changes were detected.

The protein composition of the eight isolates recorded in Table I was examined by SDS-PAGE. Each isolate gave a unique banding pattern (Fig. 2). Gels prepared using protein extracted from eight replicate cultures of one isolate gave indistinguishable tracks for all replicates.

Although each track prepared from the protein of different isolates was unique, when the pattern was considered as an overall entity, all isolates (in contrast to the BRENDA results) had the majority of their bands in common. This similarity is most evident in the lower molecular weight range at the lower part of the gel.

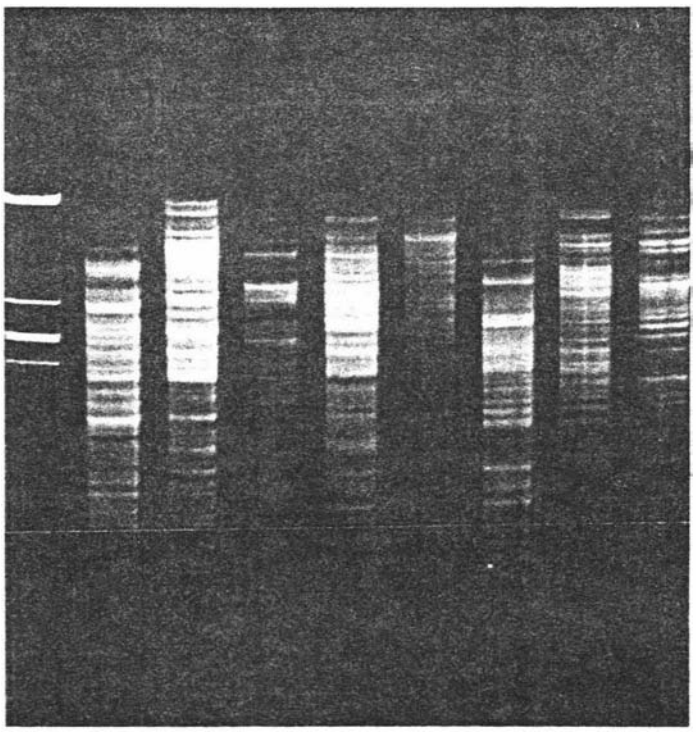


Fig. 1. DNA from bacteriophage λ (left lane) and eight isolates of *M. ovipneumoniae* derived from sheep of different flocks (see Table I) were digested using restriction endonuclease *Eco*R1. The resulting digest was assayed by electrophoresis in an agarose gel, each pattern is unique and there are no obvious common bands.

DISCUSSION

The main finding of this investigation was that, when examined by BRENDA using *Eco*R1 digestion, the patterns obtained from 16 isolates of *M. ovipneumoniae*, all derived from sheep on different farms, were not only distinguishable from one another but substantially different in their overall pattern. This implies that *M. ovipneumoniae* strains are heterogeneous and that BRENDA patterns cannot be used for species identification. Since all the isolates examined were so completely different, we have no evidence that organisms derived from any one site or source, that is from pneumonic lung, normal lung, or the nasal cavity, are more closely related to each other than they are to other isolates. This does not preclude the possibility that inter-relationships may become evident when a much larger number of isolates is examined. The heterogeneity of patterns, together with their stability, allows individual isolates of *M. ovipneumoniae* to be identified. This stability of electrophoretic patterns is demonstrated by the production of a constant BRENDA pattern over about 67 cell divisions. It will now be possible to examine multiple isolates from both nasal passages.

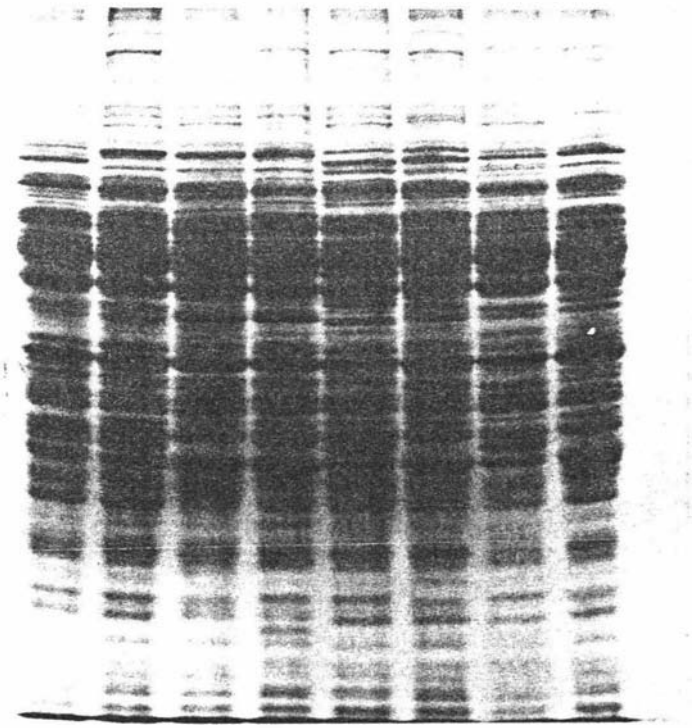


Fig. 2. Proteins from eight isolates of *M. ovipneumoniae* obtained from different sources (see Table I) examined by electrophoresis through a 10% SDS-PAGE gel. Although many common bands are present no two tracks are identical.

trachea and lungs from sheep within a flock to find how many strains of *M. ovipneumoniae* exist within that flock, and to investigate the proportion of these strains which are capable of colonising the lungs. This approach has been applied and is described by Ionas et al. (1985).

Other workers (Razin et al., 1983a,b) have examined mycoplasmas, ureaplasmas and acholeplasmas using DNA cleavage patterns following digestion with a range of restriction enzymes. The patterns obtained with *A. axanthum* isolates also differed markedly from one another and in this respect are similar to *M. ovipneumoniae*. In contrast, the pattern obtained with *M. gallisepticum* and *M. genitalium* showed minimal, or no variation of pattern. Hence Razin et al. (1983b) suggested that mycoplasmas of strict host and tissue specificity exhibit marked homogeneity. If this proves to be the general rule, then it appears that *M. ovipneumoniae* may be an exception. However, further work on many mycoplasmas using many restriction enzymes is required before final conclusions can be drawn.

Although the differences in BRENDA patterns of *M. ovipneumoniae* are of interest because they can be applied to epidemiological studies, it is possible that they represent base changes which are not reflected by

changes in proteins, hence leaving biological properties such as antigenicity or virulence unaltered. However, protein differences clearly exist between the eight isolates examined by SDS-PAGE where no two gave an overall identical pattern.

Jones et al. (1976) examined proteins from 10 isolates of *M. ovipneumoniae* using PAGE and concluded that they showed a 'very close resemblance'. The present investigation used SDS-PAGE gels, so these two studies cannot be directly compared, but interestingly Jones et al. (1976) in the same communication demonstrated by metabolic inhibition (MI) and growth inhibition (GI) tests that their *M. ovipneumoniae* isolates were heterogeneous.

GI and MI tests presumably detect differences in surface antigens and it should be possible, for example, by using reactive fluorescent compounds which cannot cross membranes, to identify those bands on SDS-PAGE gels which represent surface proteins. If these were identified and characterised they could be used individually to produce antisera with a greater potential for discriminating between isolates than is possible when antisera are prepared against whole organisms.

REFERENCES

Alley, M.R. and Clarke, J.K., 1979. The experimental transmission of ovine chronic non-progressive pneumonia. N.Z. Vet. J., 27: 217—220.

Carmichael, L.E., St. George, T.D., Sullivan, N.D. and Horsfall, N., 1972. Isolation, propagation, and characterisation studies of an ovine *Mycoplasma* responsible for proliferative interstitial pneumonia. Cornell. Vet., 62: 654—679.

Clarke, J.K., Brown, V.G. and Alley, M.R., 1974. Isolation and identification of mycoplasmas from the respiratory tract of sheep in New Zealand. N.Z. Vet. J., 22: 117—121.

Daniels, M.J. and Meddins, B.M., 1973. Polyacrylamide gel electrophoresis of mycoplasma proteins in sodium dodecyl sulphate. J. Gen. Microbiol., 76: 239—242.

Frey, M.C., Hanson, R.P. and Anderson, D.P., 1968. A medium for the isolation of avian mycoplasmas. Am. J. Vet. Res., 29: 2163—2179.

Ionas, G., Mew, A.J., Alley, M.R., Clarke. J.K., Robinson, A.J. and Marshall, R.B., 1985. Colonisation of the respiratory tract of lambs by strains of *Mycoplasma ovipneumoniae*. Vet. Microbiol., 10: 533—539.

Jones, G.E., Foggie, A., Mould, D.L. and Livitt, S., 1976. The comparison and characterisation of glycolytic mycoplasmas isolated from the respiratory tract of sheep. J. Med. Microbiol., 9: 39—52.

Jones, G.E., Gilmore, J.S. and Rae, A., 1978. Endobronchial inoculation of sheep with pneumonic lung-tissue suspensions and with bacteria and mycoplasmas isolated from them. J. Comp. Pathol., 88: 85—96.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680—685.

Marshall, R.B., Wilton, B.E. and Robinson, A.J., 1981. Identification of *Leptospira* serovars by restriction endonuclease analysis. J. Med. Microbiol., 14: 163—166.

Razin, S. and Rottem, S., 1967. Identification of *Mycoplasma* and other microorganisms by polyacrylamide gel electrophoresis of cell proteins. J. Bacteriol., 94: 1807—1810.

Razin, S., Harawawa, R. and Barile, M.F., 1983a. Cleavage patterns of the *mycoplasma* chromosome, obtained by using restriction endonucleases, as indicators of genetic relatedness among strains., Int. J. Syst. Bacteriol., 33: 201—206.

Razin, S., Tully, J.G., Rose, D.L. and Barile, M.F., 1983b. DNA cleavage patterns as indicators of genotypic heterogeneity among strains of *Acholeplasma* and *Mycoplasma* species. J. Gen. Microbiol., 129: 1935—1944.

BIBLIOGRAPHY

ALLEY, M.R. (1975a).

The pathogenisis of pneumonia in sheep. *Thesis, Ph.D., Massey University.*

ALLEY, M.R. (1975b).

The bacterial flora of the respiratory tract of normal and pneumonic sheep. *New Zealand Veterinary Journal. 23*: 113-118.

ALLEY, M.R. & CLARKE, J.K. (1977).

The influence of micro-organisms on the severity of lesions in chronic ovine pneumonia. *New Zealand Veterinary Journal. 25*: 200-202.

ALLEY, M.R. & CLARKE, J.K. (1979).

The experimental transmission of chronic non-progressive pneumonia. *New Zealand Veterinary Journal. 27*: 217-220.

ALLEY, M.R. & CLARKE, J.K. (1980).

The effect of chemotherapeutic agents on the transmission of ovine chronic non-progressive pneumonia. *New Zealand Veterinary Journal. 28*: 77-80.

AMAR, A., ROTTEM, S. & RAZIN, S. (1974).

Characterization of the mycoplasma membrane proteins. IV. Disposition of proteins in the membrane. *Biochimica et Biophysica Acta. 352*: 228-244.

ASA, P.B., ACTION, R.T., CASSELL, G.H. & WISE, K.S. (1979).

Identification and characterization of protein antigens of two mycoplasma species. *Journal of Immunology. 124*: 997-999.

ASKAA, G. & ERNO, H. (1976).

Elevation of *Mycoplasma agalactiae* subsp *bovis* to species rank: *Mycoplasma bovis*. (Hale *et al*) *comb. nov. International Journal of Systematic Bacteriology. 26*: 323-325.

BAKKE, T. & NOSTVOLT, S. (1982).

An investigation of ovine pneumonia in four herds from central Norway. I. Prevalence of pneumonia and microbiological findings. *Acta Veternaria Scandinavia. 23*: 248-258.

BALL, H.J. & TODD, D. (1978).

Comparison of antigens of pneumonia-associated mycoplasma species by gel diffusion. *Infection and Immunity. 21*: 954-958.

BARBER, T.L. & FABRICANT, J. (1961).

Primary isolation of mycoplasma organisms (PPLO) from mammalian species. *Journal of Bacteriology. 83*: 1268-1273.

BARBER, T.L. & YEDLOUTSCHNING, R.J. (1970).

Mycoplasma infections of goats. *Cornell Veterinary Journal. 60*: 297-308.

BOIDIN, A.G., CORDY, D.R. & ADLER, H.E. (1958).

A pleuropneumonia-like organism and a virus in ovine pneumonia in California. *Cornell Veterinarian. 48*: 410-430.

BRADFORD, M.M. (1976).

A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry. 72*: 248-254.

BRENNER, D.J., FANNING, G.R., RAKE, A.V. & JOHNSON, K.E. (1969).

Batch procedure for thermal elution of DNA from hydroxyapatite. *Analytical Biochemistry. 28*: 447-459.

BRENNER, D.J. & FALKOW, S. (1971).

Molecular relationships among members of the *Enterobacteriaceae. Advances in Genetics. 16*: 81-118.

BRENNER, D.J., McWHORTER, A.C., KNUTSON, J.K.L. & STEIGERWALT, A.G. (1982).

Escherichia vulneris: A new species of *Enterobacteriaceae* associated with human wounds. *Journal of Clinical Microbiology. 15*: 1133-1140.

BRIAN, P.N. (1980).

A study of mycoplasmas of the ovine lung and their relationship to chronic non-progressive pneumonia of sheep in New Zealand. *Thesis, M.Sc., Massey University.*

BRITTEN, R.J., GRAHAM, D.E. & NEUFELD, B.R. (1974).

Analysis of repeating DNA sequences by reassociation. *Methods in Enzymology. 29*: 363-406.

BROGDEN, K.A., ROSE, D., CUTLIP, R.C., LEHMKUHL, H.D. & TULLY, J.C. (1988).

Isolation and identification of mycoplasmas from the nasal cavity of sheep. *American Journal of Veterinary Research. 49*: 1669-1672.

CARMICHAEL, L.E., St.GEORGE, T.D., SULLIVAN, N.D. & HORSFALL, N. (1972).

Isolation, propagation and characterization studies of an ovine mycoplasma responsible for proliferative interstitial pneumonia. *Cornell Veterinarian. 62*: 654-679.

CHAN, H.W. & ROSS, R.F. (1984).

Restriction endonuclease analysis of two porcine mycoplasma deoxyribonucleic acids: Sequence-specific methylation in the *Mycoplasma hyopneumoniae* genome. *International Journal of Systematic Bacteriology. 34*: 16-20.

CHANDLER, D.K.F., RAZIN, S., STEPHENS, E.B., HARASAWA, R. & BARILE, M.F. (1982).

Genomic and phenotypic analysis of *Mycoplasma pneumonia* strains. *Infection and Immunity. 38*: 604-609.

CHRISTIANSEN, C. (1987).

The mycoplasma genome: Part I. *Microbiological Sciences. 4*: 168-172.

CHRISTIANSEN, C. & ERNO, H. (1982).

Classification of the F38 group of caprine mycoplasma strains by DNA hybridization. *Journal of General Microbiology. 128*: 2523-2526.

CLARKE, J.K., BROWN, V.G. & ALLEY, M.R. (1974).

Isolation and identification of mycoplasmas from the respiratory tract of sheep in New Zealand. *New Zealand Veterinary Journal. 47*: 591-596.

CLAUSEN, T. (1968).

Measurement of ^{32}P activity in a liquid scintillation counter without the use of a scintillator. *Analytical Biochemistry. 22*: 70-73.

COTTEW, G.S. (1971).

Characterization of mycoplasmas isolated from sheep with pneumonia. *Australian Veterinary Journal. 47*: 591-596.

COTTEW, G.S., WATSON, F.A., ERDAG, O. & BUCKLEY, L.S. (1968).

Differentiation of *Mycoplasma agalactiae* from other mycoplasmas of sheep and goats. *Journal of Comparative Pathology. 78*: 275-283.

COTTEW, G.S. & YEATS, F.R. (1981).

Occurrence of mycoplasmas in clinically normal goats. *Australian Veterinary Journal. 57*: 52-53.

DANIELS, M.J. & MEDDINS, B.M. (1973).

Polyacrylamide gel electrophoresis of mycoplasma proteins in sodium dodecyl sulphate. *Journal of General Microbiology. 76*: 239-242.

DE LAY, J. (1970).

Re-examination of the association between melting point, buoyant density and chemical base composition of deoxyribonucleic acid. *Journal of Bacteriology. 101*: 738-754.

DEVEREUX, J., HAEBERLI, P. & SMITHIES, O. (1984).

A comprehensive set of sequence analysis programs for the Vax. *Nucleic Acids Research. 12*: 387-395.

DOCKTER, M.E. (1979).

Fluorescent photochemical surface labelling of intact human erythrocytes. *The Journal of Biological Chemistry. 254*: 2161-2164.

DOWNEY, N.E. (1957).

A preliminary investigation into the aetiology of enzootic pneumonia of sheep ("Southland Pneumonia"). *New Zealand Veterinary Journal. 5*: 128-133.

DUBRAY, G. & BEZARD, G. (1982).

A highly sensitive period acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. *Analytical Biochemistry. 119*: 325-329.

DYSART, T.H. (1976).

Enzootic pneumonia - its effect on the meat industry. *In Proceedings of the New Zealand Veterinary Association Sheep Society: 6th Seminar, Massey University*. 94-100.

ECKHARDT, T. (1978).

A rapid method for the identification of plasmid DNA in bacteria. *Plasmid. 1*: 584-588.

ERNO, H., AL-AUBAIDI, K.M., OJO, M.O., MINGA, U.M. & SIKDAR, A. (1978).

Classification and identification of ovine and caprine mycoplasmas. *Acta Veterinaria Scandinavia. 19*: 392-406.

FOGGIE, A., JONES, G.E. & BUXTON, D. (1976.

The experimental infection of specific pathogen-free lambs with *Mycoplasma ovipneumoniae. Research in Veterinary Science. 21*: 28-35.

FORD, J.P., COCA-PRADOS, M. & HSU, M.T. (1980).

Enzymatic analysis of 5-methylcytosine content in eukaryotic DNA. *The Journal of Biological Chemistry. 255*: 7544-7547.

FORSHAW, K.A. (1972).

Electrophoretic patterns of strains of *Mycoplasma pulmonis. Journal of General Microbiology. 72*: 493-499.

FOWLER, R.C., COBLE, D.W., KRAMER, N.C. & BROWN, T.M. (1963).

Starch gel electrophoresis of a fraction of certain of the pleuropneumonia-like group of micro-organisms. *Journal of Bacteriology. 86*: 1145-1151.

FREY, M.L., HANSON, R.P. & ANDERSON, D.P. (1968).

A medium for the isolation of avian mycoplasmas. *American Journal of Veterinary Research. 29*: 2163-2179.

FRIIS, N.F., PALSSON, P.A. & PETURSSON, G. (1976).

Mycoplasma ovipneumoniae demonstrated in Icelandic sheep. *Acta Veterinaria Scandinavia. 17*: 255-257.

FURLONG, S.L. & COTTEW, G.S. (1973).

Identity of mycoplasmas from sheep in Queensland and Victoria. *Australian Veterinary Journal. 49*: 215-216.

GILMOUR, J.S., JONES, G.E. & RAE, A.G. (1979).

Experimental studies of chronic pneumonia of sheep. *Comparative Immunology and Microbiology of Infectious Diseases. 1*: 285-293.

GOIS, M., KUKSA, F., FRANZ, J. & TAYLOR-ROBINSON, D. (1974).

The antigenic differentiation of seven strains of *Mycoplasma hyorhinis* by growth-inhibition, metabolism-inhibition, latex-agglutination and polyacrylamide-gel-electrophoresis. *Journal of Medical Microbiology. 7*: 105-115.

GOLTZ, J.P., ROSENDAL, S., McCRAW, B.M. & RUHNKE, H.L. (1986).

Experimental studies on the pathogenicity of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* for the respiratory tract of goats. *Canadian Journal of Veterinary Research. 50*: 59-67.

GRACIA, J.L., LEON-VIZCAINO, L., MERCADO, M. & GARRIDO, F. (1988).

Atypical pneumonia associated with *Mycoplasma ovipneumoniae* in housed lambs. *Medicina Veterinaria. 4*: 357-361.

HARASAWA, R. & BARILE, M.F. (1983).

Survey of plasmids in various mycoplasmas. *The Yale Journal of Biology and Medicine. 56*: 783-788.

HARRIS, R.E. & ALLEY, M.R. (1977).

Pneumonia in sheep: does it affect weight gain? *New Zealand Veterinary Journal. 25*: 108.

HOROWITZ, S.A., GARRET, B., DAVIS, J.K. & CASSALL, G.H. (1987).

Isolation of *Mycoplasma pulmonis* and identification of surface antigens. *Infection and Immunity. 55*: 1314-1320.

HU, P.C., COLE, R.M., HUANG, Y.S., GRAHAM, J.A., GARDNER, D.E., COLLIER, A.M. & CLYDE, W.A. (1982).

Mycoplasma pneumoniae infection: Role of a surface protein in the attachment organelle. *Science. 216*: 313-315.

HU, P.C., COLLIER, A.M. & BASEMAN, J.B. (1977).

Surface parasitism by *Mycoplasma pneumoniae* of respiratory epithelium. *The Journal of Experimental Medicine. 145*: 1328-1343.

INTERNATIONAL COMMITTEE ON SYSTEMATIC BACTERIOLOGY: SUBCOMMITTEE ON THE TAXONOMY OF *MOLLICUTES*. (1979).

Proposal of minimum standards for descriptions of new species of the class *Mollicutes. International Journal of Systematic Bacteriology. 29*: 172-180.

IONAS, G. (1983).

Studies on *Mycoplasma ovipneumoniae* in New Zealand sheep: Epidemiology and comparison of isolates. *Thesis, M.Sc. Massey University*.

IONAS, G., MEW, A.J., ALLEY, M.R., CLARKE, J.K., ROBINSON, A.J. & MARSHALL, R.B. (1985).

Colonisation of the respiratory tract of lambs by strains of *Mycoplasma ovipneumoniae. Veterinary Microbiology. 10*: 533-539.

JOHNSON, J.L. (1984).

Nucleic acids in bacterial classification. p 8-11. *Bergeys Manual of Systematic Bacteriology, Vol 1.* Williams and Wilkins Co, Baltimore.

JONES, G.E., FOGGIE, A., MOULD, D.L. & LIVITT, S. (1976).

The comparison and characterisation of glycolytic mycoplasmas isolated from the respiratory tract of sheep. *Journal of Medical Microbiology. 9*: 39-52.

JONES, G.E., GILMOUR, J.S. & RAE, A. (1978).

Endobronchial inoculation of sheep with pneumonic lung-tissue suspensions and with the bacteria and mycoplasmas isolated from them. *Journal of Comparative Pathology. 88*: 85-96.

JONES, G.E., GILMOUR, J.S. & RAE, A. (1982a).

The effect of *Mycoplasma ovipneumoniae* and *Pasteurella haemolytica* on specific pathogen-free lambs. *Journal of Comparative Pathology. 92*: 261-266.

JONES, G.E., GILMOUR, J.S. & RAE, A.G. (1982b).

The effects of different strains of *Mycoplasma ovipneumoniae* on specific pathogen-free and conventionally-reared lambs. *Journal of Comparative Pathology. 92*: 267-272.

JONES, G.E. & WOOD, A.R. (1988).

Microbiological and serological studies on caprine pneumonias in Oman. *Research in Veterinary Science. 44*: 125-131.

KAHANE, I., TUCKER, S. & BASEMAN, J.B. (1985).

Detection of *Mycoplasma pneumoniae* adhesin (P1) in the nonhaemadsorbing population of virulent *Mycoplasma pneumoniae*. *Infection and Immunity. 49*: 457-458.

KIRTON, A.H., O'HARA P.J., STORTRIDGE, E.H. & CORDES, D.O. (1976).

Seasonal incidence of enzootic pneumonia and its effect on the growth of lambs. *New Zealand Veterinary Journal. 24*: 59-64.

KLINKERT, M., HERRMANN, R. & SCHALLER, H. (1985).

Surface proteins of *Mycoplasma hyopneumoniae* identified from an *Escherichia coli* expression plasmid library. *Infection and Immunity. 49*: 329-335.

KONTE, M. & BREARD, A. (1988).

First isolation of *Mycoplasma ovipneumoniae* in Senegal. *Revue d'Elevage et de Medicine Veterinaire des Pays Tropicaux. 40*: 113-115.

KRAUSS, A. & WANDERA, J.G. (1970).

Isolation and properties of mycoplasma from the respiratory tract of sheep with jaagsiekte. *Journal of Comparative Pathology. 80*: 389-396.

LAEMMLI, U.K. (1980).

Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature. 227*: 680-685.

LEACH, R.H., COTTEW, G.S., ANDREWS, B.E. & POWELL, D.G. (1976).

Atypical mycoplasmas from sheep in Great Britain and Australia identified as *Mycoplasma ovipneumoniae*. *The Veterinary Record. 98*: 377-379.

LEITH, D.K., TREVINO, L.B., TULLY, J.G., SENTERFIT, L.B. & BASEMAN, J.B. (1983).

Host discrimination of *Mycoplasma pneumoniae* proteinaceous immunogens. *Journal of Experimental Medicine. 157*: 502-514.

LIVINGSTONE, C.W. & GAUER, B.B. (1979).

Isolation of *Mycoplasma ovipneumoniae* from Spanish and Angora goats. *American Journal of Veterinary Research. 40*: 407-408.

MACKAY, J.M.K. & NISBET, D.I. (1966).

Jaagsiekte - A hazard of intensified sheep husbandry. *The Veterinary Record. 78*: 18-20.

MACKAY, J.M.K., NISBET, D.I. & FOGGIE, A. (1963).

Isolation of PPLO's (Genus *Mycoplasma*) from cases of sheep pulmonary adenomatosis. *Veterinary Record. 75*: 550-551.

MACRINA, F.L., KOPECKO, D.J., JONES, K.R., AYERS, D.J. & M^{C}COWEN, S.M. (1978).

A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid. 1*: 417-420.

MADDY, A.H. (1964).

A fluorescent label for the outer components of the plasma membrane. *Biochemica et Biophysica Acta. 88*: 390-399.

MALONE, F.E., M^{C}CULLOUGH, S.J., M^{C}LOUGHLIN, M.F., BALL, H.J., O'HAGAN, J. & NEILL, S.D. (1988).

Infectious agents in respiratory disease of housed, fattening lambs in Northern Ireland. *The Veterinary Record. 122*: 203-207.

MANDEL, M. & MARMUR, J. (1968).

Use of ultra-violet absorbance temperature profile for determining the guanine plus cytosine content of DNA. *Methods in Enzymology. 12*: 195-206.

MARSHALL, R.B., WILTON, B.E. & ROBINSON, A.J. (1981).

Identification of *Leptospira* serovars by restriction endonuclease analysis. *Journal of Medical Microbiology. 14*: 163-166.

MEW, A.J. (1982).

The characterization of strains of *Mycoplasma ovipneumoniae* by restriction endonuclease analysis. *Thesis, M.Sc. Massey University.*

MEW, A.J., IONAS, G., CLARKE, J.K., ROBINSON, A.J. & MARSHALL, R.B. (1985).

Comparison of *Mycoplasma ovipneumoniae* isolates using bacterial restriction endonuclease DNA analysis and SDS-PAGE. *Veterinary Microbiology. 10*: 541-548.

NASCIMENTO, E.R., NASCIMENTO, M. da G. F., FREUNT, E.A. & ANDERSON, H. (1986).

Isolation of *Mycoplasma mycoides* from outbreaks of caprine mycoplasosis in Brazil. *British Veterinary Journal 142*: 246-257.

NORMAN, N.G. (1985).

An investigation of the heterogeneity of *Mycoplasma ovipneumoniae* using restriction endonuclease analysis. *Thesis, M.Sc. Massey University.*

PETERSON, A.M. & POLLOCK, M.E. (1969).

Deoxyribonucleic acid homology and relative genome size in *Mycoplasma. Journal of Bacteriology. 99*: 639-644.

RAZIN, S. (1968).

Mycoplasma taxonomy studied by electrophoresis of cell proteins. *Journal of Bacteriology. 98*: 687-694.

RAZIN, S. (1985).

Molecular biology and genetics of mycoplasmas (*Mollicutes*). *Microbiological Reviews. 49*: 419-455.

RAZIN, S., BARILE, M.F., HARASAWA, R., AMIKAM, D. & GLASER, G. (1983a).

Characterization of the mycoplasma genome. *The Yale Journal of Biology and Medicine. 56*: 357-366.

RAZIN, S., HARASAWA, R. & BARILE, M.F. (1983b).

Cleavage patterns of the mycoplasma chromosome, obtained by using restriction endonucleases, as indicators of genetic relatedness among strains. *International Journal of Systematic Bacteriology. 33*: 201-206.

RAZIN, A. & RAZIN, S. (1980).

Methylated bases in mycoplasma DNA. *Nucleic Acids Research. 8*: 1383-1390.

RAZIN, S. & ROTTEM, S. (1967).

Identification of *Mycoplasma* and other micro-organisms by polyacrylamide-gel electrophoresis of cell proteins. *Journal of Bacteriology. 94*: 1807-1810.

RAZIN, S., TULLY, J.G., ROSE, D.L. & BARILE, M.F. (1983c).

DNA cleavage patterns as indicators of genotypic heterogeneity among strains of *Acholeplasma* and *Mycoplasma* species. *Journal of General Microbiology. 129*: 1935-1944.

REICH, P.R., SOMERSON, N.L., HYBNER, C.J., CHANOCK, R.M. & WEISSMAN, S.M. (1966).

Genetic differentiation by nucleic anid homology. I. Relationships among *Mycoplasma* species of man. *Journal of Bacteriology. 92*: 302-310.

RIETHMAN, H.C., BOYER, M.J. & WISE, K.S. (1987).

Triton X-114 phase fractionation of an integral membrane surface protein mediating monoclonal antibody killing of *Mycoplasma hyorhinis*. *Infection and Immunity. 55*: 1094-1100.

RIGBY, P.W.J., DIECKMANN, M., RHODES, C. & BERG, P. (1977).

Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology. 113*: 237-251.

SALISBURY, R.M. (1957).

Enzootic pneumonia of sheep in New Zealand. *New Zealand Veterinary Journal. 5*: 124-127.

SCHMIDT-ULLRICH, R., KNUFERMANN, H. & WALLACH, D.F.H. (1973).

The reaction of 1-dimethylaminonaphthalene-5-sulphonyl chloride (DANSCl) with erythrocyte membranes: A new look at "vectoral" membrane probes. *Biochimica et Biophysica Acta. 307* 353-365.

SKARE, J., SUMMERS, W.P. & SUMMERS, W.C. (1975).

Structure and function of herpesvirus genomes. I. Comparisons of 5 HSV-I and 2 HSV-2 strains by cleavage of their DNA with *Eco*RI restriction endonuclease. *Journal of Virology. 15*: 726-732.

SOMERSON, N.L., REICH, P.R., WALLS, B.E., CHANOCK, R.M. & WEISSMAN, S.M. (1966).

Genetic differentiation by nucleic acid homology. II. Genotypic variations within two *Mycoplasma* species. *Journal of Bacteriology. 92*: 311-317.

STAMP, J.T. & NISBET, D.I. (1963).

Pneumonia of sheep. *Journal of Comparative Pathology. 73*: 319-329.

St.GEORGE, T.D. (1972).

Investigations of respiratory disease of sheep in Australia. *Australian Veterinary Journal. 48*: 318-322.

St.GEORGE, T.D. & CARMICHAEL, L.E. (1975).

Isolation of *Mycoplasma ovipneumoniae* from sheep with chronic pneumonia. *The Veterinary Record. 97*: 205-206.

St.GEORGE, T.D., SULLIVAN, N.D., LOVE, J.A. & HORSEFALL, N. (1971).

Experimental transmission of pneumonia in sheep with a mycoplasma isolated from pneumonic sheep lung. *Australian Veterinary Journal. 47*: 282-283.

SUGINO, W.M., WEK, R.C. & KINGSBURY, D.T. (1980).

Partial nucleotide sequence similarity within species of *Mycoplasma* and *Acholeplasma. Journal of General Microbiology. 121*: 333-338.

SULLIVAN, N.D., St.GEORGE, T.D. & HORSEFALL, N. (1973a).

A proliferative interstitial pneumonia of sheep associated with mycoplasma infection: 1. Natural history of the disease in a flock. *Australian Veterinary Journal. 49*: 57-62.

SULLIVAN, N.D., St.GEORGE, T.D. & HORSEFALL, N. (1973b).

A proliferative interstitial pneumonia of sheep associated with mycoplasma infection: 2. The experimental exposure of ypung lambs to infection. *Australian Veterinary Journal. 49*: 63-68.

THE SUBCOMMITTEE ON THE TAXONOMY OF *MYCOPLASMATALES*. (1972).

Proposal for minimal standards for descriptions of new species of the order *Mycoplasmatales. International Journal of Systematic Bacteriology. 22*: 184-188.

THOMAS, G.B. & DIERKS, R.E. (1980).

Relationship between two serotypes of avian mycoplasmas by serological and DNA-DNA hybridization tests. *Avian Diseases. 24*: 939-954.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979).

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Acadame of Science. 76*: 4350-4354.

WANDERA, J.G. (1970).

Clinical pulmonary adenomatosis of sheep produced experimentally. *British Veterinary Journal. 126*: 185-193.

YOGEV, D. & RAZIN, S. (1986).

Common deoxyribonucleic acid sequences in *Mycoplasma genitalium* and *Mycoplasma pneumoniae* genomes. *International Journal of Systematic Bacteriology. 36*: 426-430.

ZOLA, H., BAXENDALE, W. & SAYER, L.J. (1970).

Polyacrylamide gel electrophoresis of lysates of *Mycoplasmas. Research in Veterinary Science. 11*: 397-399.

ZOUZIAS, D., MAZAITIS, A.J., SIMBERKOFF, M. & RUSH, M. (1973).

Extrachromosomal DNA of *Mycoplasma hominis. Biochemica et Biophysica Acta. 312*: 484-491.