THE CHARACTERISATION OF STRAINS OF *MYCOPLASMA OVIPNEUMONIAE* BY RESTRICTION ENDONUCLEASE ANALYSIS

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ABSTRACT

Studies of the pathogenicity of *Mycoplasma ovipneumoniae* would be facilitated by an *in vitro* method of identifying and classifying isolates of this organism, and it is with the development of such a method, and its possible applications, that this thesis is mainly concerned.

The method chosen was Restriction Endonuclease Analysis (REA) which has already been successfully applied to the identification of some viruses and bacteria.

Initially the REA patterns of 2 strains of *M. ovipneumoniae* were observed. They differed markedly in restriction pattern, but the pattern exhibited by any one of them was recognisable and reproducible. Furthermore, patterns did not change significantly after limited passage *in vitro*. An extension of this study to 8 isolates from widely differing sources, showed that all gave markedly different patterns. It was concluded that, unlike the findings for *Leptospira* and *Rhizobium*, REA could not be used for the identification of species, but is an extremely powerful method for identifying strains of *M. ovipneumoniae*.

Despite the marked heterogeneity of isolates from different sources, the relative stability of pattern of an individual isolate, suggested that REA could be used to examine the epidemiology of individual strains of *M. ovipneumoniae* within a flock of sheep. Hence, we undertook a study of *M. ovipneumoniae* isolates obtained by serial swabbing of the nasal cavities of a flock of lambs over a 6-month period, and from the lungs of the same lambs at slaughter. It was shown that 54 isolates from the nasal cavities fill into 7 major groups with respect to restriction pattern (although minor differences were detected within a group). There was a tendency for these groups to occur sequentially. None of the isolates were shown to persist for long periods, but a later strain could replace an earlier one. The isolates from the lungs were more homogeneous and the predominant strain fell within one of the 7 "nasal" groups. This suggests that nasal isolates

may vary in their pathogenicity for the lungs, although other explanations are possible (see General Discussion).

Notwithstanding the apparent stability of mass cultures of M. ovipneumoniae following limited passage in vitro, the unexpectedly large number of restriction patterns found with field strains, led us to re-examine, in more detail, the stability of cloned isolates. A multiply-cloned isolate was propagated in vitro and 8 sub-clones selected before and, a further 8 sub-clones after 20 passages. Some limited heterogeneity was detected among the 8 sub-clones selected before passage, and a somewhat greater degree of heterogeneity was detected among sub-clones selected after passage. It should, however, be emphasised that these differences were small compared to the total lack of similarities seen when isolates from different sources were examined. Limited passage in the presence of sub-lethal concentrations of antibody did not increase the heterogeneity of patterns - if anything, the reverse is true.

Explanations for these findings and future experiments to confirm or deny these possibilities are discussed.

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INTRODUCTION

Chronic, Non-Progressive Pneumonia (CNP) is a common disease of sheep in New Zealand, and typically affects lambs in late summer and early autumn when they are 6-8 months old.

Although workers differ in the designation used, similar, if not identical lesions have been described and studied in sheep in Australia (St George et al, 1971); Iceland (Friis et al., 1976); Scotland (Foggie et al., 1976); Iraq (Al-Sultan and Zubaidy, 1978) as well as other countries. Despite these studies, the cause of CNP has not been unequivocally established. Mycoplasma ovipneumoniae has been consistently isolated from a high proportion of CNP lesions (Cottew 1971; Carmichael et al., 1971; Clarke et al., 1974; Leach et al., 1976; Friis et al., 1976), but has also been isolated from the lungs of normal sheep, though with decreased frequency and in lower titres (Alley et al., 1975).

M. ovipneumoniae is not the only organism which has been detected in CNP lesions: thus, bacteria, especially Pasteurella haemolytica, are usually present and Mycoplasma arginini is present in some batches of lesions but not in others. Viruses, especially Parainfluenza virus type III (PI3) have been detected in ovine pneumonic lesions, and this organism is undoubtedly, pathogenic. However, those lesions it produces in transmission experiments are not typical of CNP and, conversely, PI3 cannot be consistently isolated from CNP lesions.

The problem of the aetiology of CNP should be resolved by transmission experiments, but these have given variable results in the hands of different workers. Sullivan et al., (1973a, 1973b) (using conventionally reared lambs) transmitted a disease, which they claim was indistinguishable from the field disease. The lambs were inoculated with either lung homogenate or a culture of M. ovipneumoniae. St George et al., (1971) produced pneumonic lesions in newborn lambs with a culture of M. ovipneumoniae, however, they found older lambs (the normal age group for CNP) were not as susceptible. Similarly Jones et al., (1979),

produced pneumonic lesions in sheep using either a combination of M. ovipneumoniae, P. haemolytica and M. arginini, or lung homogenate. Other workers (Alley et al., 1979; Davies et al., 1981; Foggie et al., 1976; Gilmour et al., 1979), have obtained widely differing or erratic results with attempts to reproduce CNP in sheep with pure cultures of M. ovipneumoniae.

The consistent presence of M. ovipneumoniae in CNP lesions seems to indicate that it plays a role in the pathogenesis of the disease, and the apparent success of some transmission experiments supports the concept that it is the primary pathogen. However, before a final conclusion is reached, the variability of results must be explained. Possible explanations are listed below:

1. Dosage variability.
2. Variability in administration of dose.
3. The disease may have a complex aetiology, i.e. require two or more organisms to cause lesions, or
4. Different strains of M. ovipneumoniae may vary in their virulence.

It is with the latter possibility that this thesis is mainly (if indirectly) concerned.

For practical reasons, large scale transmission experiments with many, randomly selected, fresh isolates of M. ovipneumoniae could not be undertaken in this study. Therefore, it seemed desirable to develop a method of classifying and identifying M. ovipneumoniae strains so that ultimately the virulence of a limited number of selected strains could be examined. This could be done both directly, by transmission experiments, and, indirectly by looking for a correlation between the severity of CNP lesions and the presence of particular strains of M. ovipneumoniae.

The following methods have been used to detect strain difference within various mycoplasma species.

1. Strain differences have typically been shown by serological methods. This approach depends on the ability of antibody to interfere with the growth, or ability to metabolise, of intact mycoplasmas, i.e. detects surface antigens.
2. Differences in the virulence of strains have been demonstrated using transmission experiments. For example, Kume et al., (1976) showed that strains of Mycoplasma synoviae exhibited varying degrees of pathogenicity; and, similarly Rodriguez and Kleven (1980) showed that two strains of Mycoplasma gallisepticum differed markedly in the incidence and severity of the disease they caused.
3. Strain differences have been established using Polyacrylamide Gel Electrophoresis (PAGE) on mycoplasma proteins
4. Serological strain differences have been reinforced using DNA-DNA hybridisation techniques (Thomas and Dierkes, 1980).

Some work has already been done on the serology of M. ovipneumoniae by Jones et al. (1976), who concluded that quantitative differences were detectable between strains, but these differences were not adequate to allow serotypes to be established. Nevertheless, the well-established link between surface antigens, immunity and virulence, in other organisms, led us to conclude that a serological study of New Zealand isolates could be valuable - particularly if it could be undertaken in conjunction with another method of classification. Unfortunately, none of the other methods of classification seemed attractive in our circumstances, e.g. PAGE seemed open to different interpretations: thus, Jones et al. (1976) stated that the gel banding patterns 'demonstrated a very close resemblance' between strains of M. ovipneumoniae - although the casual observer might find this subjective judgement less than obvious from the data shown. For this reason we were attracted to the possibility

of using restriction endonucleases for examining mycoplasmas. This technique is highly successful in distinguishing strains of organisms, e.g. viruses which have a limited size of genome (Esposito et al., 1978; Witteck et al., 1980; Lonsdale et al., 1979a). Although the number of bands would increase with genome size and thus give less readily-interpretable results, it nevertheless seemed possible that mycoplasmas - though larger than viruses, being the smallest living cells (which must limit genome size) - would be amenable to this approach.

At an early stage of this investigation, it bacame clear that Restriction Endonuclease Analysis (REA) had a greater potential for unequivocally distinguishing strains than had serology. Therefore, following a preliminary study of serological differences between strains (not recorded here), this present study was undertaken to answer the following initial questions:

1. Does REA give a recognisable and reproducible pattern with one isolate? If so,
2. Are isolates heterogeneous with respect to banding pattern? If so,
3. Is there a very large number or only a limited number of such patterns?

While there would be some merit in investigating flocks of sheep from a wide geographical area, it was decided to concentrate this investigation on one flock of sheep. Hence, isolates were obtained by serially swabbing the nasal cavity of a flock of lambs over a six-month period. Further isolates were obtained from the lungs at slaughter, and all were examined using REA.

This approach, combined with the conclusion (see Chapter 2.6) that REA readily distinguished several isolates of M. ovipneumoniae, allowed us to direct our attention to the following further questions, viz.:-

4. Regardless of differences between flocks, are there only a limited number of strains of M. ovipneumoniae found within a flock?

5. Can the nasal cavity of sheep be sequentially colonised by different strains?
6. Do strains isolated from lungs (at slaughter) represent a homogeneous group, and are they distinguishable or indistinguishable from nasal isolates.

As will be seen later in this thesis, unexpectedly high variation was found, so, at the conclusion of this work, we initiated a preliminary study to investigate the possible source of the observed heterogeneity of pattern.

CHAPTER 1 Historical Review

1.1 Discovery and characterisation of Restriction Endonucleases

Thirty years ago, Luria et al., (1952) showed that bacteriophage grown on one strain of a species of bacteria, displayed a wide variation in its ability to propagate on other strains. It was shown in this case (Luria and Human, 1952), that the host range of the bacteriophage was determined by the bacterial cells, in which it was propagated, rather than by the bacteriophage. Studies on Escherichia coli strains B and K (Arber and Dussoix, 1962; Dussoix and Arber, 1962) led to a model, now called "Restriction and Modification" to account for this phenomenon.

It was proposed that the bacterial cells have an enzyme with two distinct and paradoxical functions: one (an endonuclease) recognises and cleaves DNA at a specific sequence of bases, whereas the second modifies the same base sequence, so that it is no longer a substrate for the endonuclease. Normally, the DNA of the bacterial cell is rapidly modified following synthesis, and hence is protected from digestion. The presence of the restriction endonuclease provides a degree of protection for the bacterium against invasion by foreign DNA - be it plasmid, bacterial or bacteriophage DNA - always provided that the extraneous DNA was "normal" and unmodified.

In 1968, Meselson and Yuan (1968) and Linn and Arber (1968) reported the isolation of the first restriction endonucleases from E. coli K and E. coli B. These enzymes recognise a specific nucleotide sequence but cut at non-specific sites away from the recognition sequence, and are now referred to as "Class I" enzymes. In 1970 (Smith and Wilcox, 1970; Kelly and Smith, 1970), the first Class II enzyme, i.e. one that both recognises and cleaves a specific nucleotide sequence, was isolated from Haemophilus influenzae serotype 'd'. Since the discovery of

this endonuclease and its use to fragment DNA from Simian Virus - 40, more than 80 restriction endonucleases have been discovered and many have been characterised.

Some enzymes have been shown to carry out both restriction and modification of DNA. This dual function occurs with the enzymes from E. coli K and B, and has its basis in the presence of three different subunits. The effects of mutations in the respective genes have shown that each subunit performs a different function: one is responsible for recognising the DNA sequence; the second for the cleaving activity; and the third for modifying the bases (which, in this case, is by methylation). More rarely restriction may occur without modification: thus E. coli strain W is lysogenic for a temperate phage, WØ, which controls a host-restriction mechanism but not a host-modification mechanism (Kerszman et al., 1967). In this case, a W-specific host modification is controlled by the genome of strain W.

The most important feature of restriction endonucleases is their ability to recognise specific target sequences, which in most cases are palindromes, 4 to 6 base pairs long. The symmetry of the target is probably related to the symmetry of the enzyme. Recognition of a sequence of bases as a target is destroyed by mutations that change a base pair within the target (or delete it altogether), or by modification by methylation or glucosylation of target bases. Class II enzymes produce 2 symmetric cuts, one in each strand, within the target, and these cuts are either coincidental or 'staggered'. Staggered cuts leave ends that are terminated by short "sticky ends" (i.e. complementary single strands). At physiological temperatures these ends are too short for stable pairing, so a pair of staggered cuts results in a complete break in the DNA duplex. However, these "sticky ends" are useful in the formation of DNA recombinants in vitro - which uses the property of the sticky ends to form fairly stable duplexes at low temperature, which can then be sealed covalently by a ligase.

Although these enzymes are known only in bacteria, they are not necessarily a product of bacterial genes per se: thus, some plasmids and phages also code for restriction endonucleases. Therefore, these enzymes may also be involved in competition among sub-bacterial particles.

Nomenclature for restriction endonucleases isolated from bacteria was suggested by Smith and Nathans (1973). The enzymes are given a 3-letter stem name, which abbreviates the genus and species of the organism from which they were isolated. Where necessary, a fourth letter is added to designate the strain. Roman numerals following the system name are assigned to differentiate multiple enzymes from the same source. For example, Hind III is one of multiple restriction enzymes from Haemophilus influenzae, serotype d.

Restriction enzymes from different sources can have the same recognition sites and if so, they are called "isochizomers". While isochizomers recognise the same nucleotide sequence, they may or may not cleave at the same site. However, their existence means that a given restriction enzyme may occur in several different bacterial strains, and so it may be possible to avoid working with highly pathogenic bacteria as the same enzyme is likely to be found elsewhere. Similarly, although one strain may be a poor source of a given enzyme, it is likely that an alternative and more productive source will exist.

Most Class II restriction enzymes have not been characterised to any great extent because interest in them has centred around their uses as biochemical reagents rather than their inherent properties as nucleases. One exception is EcoRI, which has been purified and examined extensively. It has been revealed, using the oligonucleotide TGAATTCA, that although the recognition site for EcoRI - GAATTC - is necessary and sufficient for cleavage to occur, sequences outside this hexanucleotide do influence the rate of cleavage (Thomas and Davis, 1975). Furthermore, the specificity for this hexanucleotide can be relaxed by changing the pH and ionic conditions of the reaction (Polisky et al., 1975).

Purification to homogeneity has not been reported for any other restriction enzyme. It has been noted that most enzymes show a broad pH optimum, a broad magnesium ion optimum, and are inhibited by high concentrations of Sodium Chloride (Roberts, R.J. 1976). Characterisation has usually involved a description of the cleavage patterns on different DNA samples and the determination of recognition sequences.

The discovery, isolation and characterisation of restriction endonucleases from a variety of sources has led to their application for a range of purposes. The principle uses of restriction endonucleases are set out below:

1. Mapping of DNA
2. Cloning of genes
3. Classification and identification of microorganisms
4. Epidemiological studies

1.2 Applications of Restriction Endonucleases

1.2.1 Mapping of DNA

Restriction endonucleases have been used - in conjunction with other methods, e.g. electron microscopy, DNA hybridisation, etc. - to produce maps of the genomes of many viruses.

The restriction endonuclease digestion products of the DNA are separated into bands containing fragments of the same molecular weight, by gel electrophoresis. The molecular weight of each fragment (i.e. one band) is estimated from a comparison of its electrophoretic mobility with that of DNA fragments whose molecular weight has been measured with the electron microscope.

The locations of restriction enzyme cleavage sites within a genome can be determined from the overlaps of fragments produced by different enzymes. That is, a specific restriction fragment may be recovered from the gel - produced by electrophoresis digested DNA - by several means,

e.g. electrophoresis, chemically, etc., and incubated with a second endonuclease which will result in either no change or in further fragmentation. The electrophoretic pattern of such digests show the location of the cleavage sites of the second enzyme in the original DNA fragment. For example, EcoRI fragment A (13.7 megadaltons) is cleaved, by Hind III, into fragments of 6.0, 4.1 and 3.6 megadaltons. Since there are two Hind III cleavage sites in EcoRI A, at least one of the three secondary products is a Hind III fragment. The only secondary digestion product with a molecular weight equal to that of a Hind III fragment, is the 6.0 megadalton product. Therefore, the linkage map of the three products made by Hind III cleavage of EcoRI A is 4.1 - 6.0 - 3.6. Determination of the structure of fragments which give rise to more than three secondary digestion products, requires additional information, either from incompletely digested molecules or from secondary digestion with a third endonuclease.

In this way, Skare and Summers (1977), proved that the Herpes Simplex Virus type 1 (HSV-1) genome is composed of 4 classes of molecules with large (50 megadalton) and small (16 megadalton) segments joined end to end in each of the four possible orientations.

The presence of a gene on a particular restriction fragment can be established by various procedures: e.g. "Marker rescue", where a single-strand nick is produced in the DNA of a mutant, the other strand recovered as an intact circle and annealed to a denatured fragment of wild-type DNA produced by a restriction endonuclease. This technique was first used for ØX174 (Edgell et al., 1972) where a mutant single-stranded circle was annealed to a denatured restriction fragment of the replicative form DNA, and the resulting partial heteroduplex molecule used to infect E. coli. By selecting for wild-type phage, growth will only occur if the fragment used to make the heteroduplex, spans the portion of the genome containing the mutation, thus allowing it to be corrected.

In an alternative approach to mapping, although still using restriction fragments, a detailed fragment map of lambda DNA was obtained. This was made possible by comparing the enzyme digestion products from the large number of physically mapped deletion mutants with the pattern obtained by restriction enzyme digestion of wild-type DNA.

Apart from the examples cited above, restriction endonucleases have been used to assist in the mapping of genomes of Adenoviruses (Tibbetts, 1977); SV-40 (Winocour et al A.N.Y.A.S.,1980) and Orthopoxviruses (Mackett and Archard, 1979).

1.2.2 Cloning of genes

The ability of some restriction endonucleases to cleave DNA into fragments with complementary "sticky ends", has been used to open up a whole new field of Recombinant DNA Technology.

These single-stranded "sticky ends" are used to splice restriction fragments - usually separated by gel electrophoresis - into a replicon that has been cut with the same endonuclease. However, this approach is not confined only to restriction endonucleases that give staggered cuts. Therefore, restriction endonucleases that produce flush, double-stranded ends can also be used to produce recombinant replicons by using an enzyme to add single-stranded complementary homopolymer tails, e.g. poly(A) and poly(T), to the termini of strands of opposite polarity.

Following annealing of the cut replicon with the restriction fragment, a polynucleotide ligase can be used to covalently close the molecule, producing a recombinant replicon.

The recombinant replicon is typically introduced into E. coli cells made competent by incubating in warm $CaCl_2$. Once inside a susceptible cell, the replicon (including the introduced genes) will be reproduced along with the cell.

Two kinds of bacterial replicon, each with its own advantages and disadvantages, have been used to clone foreign DNA - plasmids and temperate phages.

Phages can be introduced into bacteria by infection at an efficiency of around 10^6 times that of transfection of naked DNA, e.g. plasmids. However, plasmids can carry properties which greatly enhance selection of the recombinant replicon. For example, a plasmid which carries 2 different antibiotic resistence genes, one of which contains a restriction endonuclease site. After exposure to splicing conditions, followed by transfection, three classes of cells are distinguishable:

a) those without a plasmid are sensitive to both antibiotics;
b) those with the original plasmid are resistant to both antibiotics;
and c) those with the recombinant plasmid are resistant to only one antibiotic - since the splicing of the gene has inactivated the other resistance gene.

Both phages, e.g. lambda , and plasmids, e.g. pSC101 have been improved as vectors by reduction in size (through fragmentation of a naturally-occurring replicon), so that a particular restriction endonuclease will cut at only one site in the circular DNA of the replicon; and only 2 sites in the recombinant replicon, i.e. on either side of the inserted fragment. However, these reduced replicons must still carry the genes necessary for autonomous replication. It is also, clearly, an advantage to select multiple-copy plasmids or temperate phages that can be induced to multiply vegetatively, when choosing a vector for molecular cloning.

Following lysis of the selected cells, the circular recombinant replicons can be separated from the fragmented chromosomal DNA. In addition, a recombinant replicon can be cleaved by the same endonuclease used to create the "sticky ends", and the components separated. In this way,

large amounts of a specific fragment of DNA, can be readily obtained in a relatively pure form.

This approach to molecular cloning has also been extended to eukaryotic host cells (instead of prokaryotic host cells). For example, DNA from tumour viruses can transduce spliced DNA into cultured animal cells. In this way, genes from a variety of eukaryotic sources can be expressed in standard cell cultures.

There are 2 basic uses for molecular cloning procedures:

a) For basic research, molecular cloning can be used to expand our knowledge of the structure and function of genes. It provides a simpler environment for the study of gene regulation and function, so that even genes from complex eukaryotic organisms can be isolated and studied in the simpler prokaryotes. For example, nuclear DNA from Dictyostelium discoideum (a slime mould), has been cleaved with EcoRI, and one of the bands cloned in E. coli using plasmid pSC101, carrying tetracycline restistance (Firtel et al., 1976) and the DNA analysed by renaturation kinetics.

b) Molecular cloning has also had practical applications. For example, the use of bacteria to manufacture desired proteins, e.g. human hormones or interferon. While differences in regulatory elements generally prevent expression of eukaryotic genes in prokaryotes, this problem can be overcome by fusing bacterial regulatory elements with the mammalian sequence before splicing into a replicon. In this way, to increase bacterial synthesis of insulin by bringing about its secretion from the cell, the gene for this mammalian protein has been fused with the leader sequence of the gene for a secreted bacterial protein, i.e. penicillinase.

While molecular cloning is probably the most important practical application of restriction endonucleases, with

its potential for far-reaching effects, a detailed consideration lies outside the scope of this thesis.

1.2.3 Classification and identification of microorganisms

The classification and identification of microorganisms using Restriction Endonuclease Analysis (REA), is a relatively new field, but the technique seems to have potential in 3 areas:

a) The validation (or otherwise) of established taxonomic methods to distinguish species within a genus. This technique also allows an assessment of genetic relatedness between each species of a genus.

b) To allow the species identification of an individual isolate: this requires (as does (a) above) that the variation within a species is less than the variation between species.

c) The discrimination between individual strains within a species.

The justification for these tentative conclusions is considered below, but it should be noted here that different approaches require a range of restriction enzymes.

a) Genetic relatedness between species

The comparison of restriction profiles of species within the same genus can give an indication as to the genetic relatedness, if any, between those species. For example, Muller et al.,(1978), compared the genomes of 5 Orthopox-viruses, i.e. rabbitpox, vaccinia, cowpox, ectromelia and fowlpox viruses, and concluded that the profiles from viruses of each group were markedly different (note: lesser variation occurred with a group). Taking similarities of restriction patterns to represent genetic relatedness, they found that vaccinia and rabbitpox virus were the most closely related of the 5 virus groups studied. In general, it was concluded that the phenotypic relatedness between these poxviruses was well-reflected by the similarities and

dissimilarities of restriction pattern, i.e. REA. in general, supports and augments classical taxonomic methods.

In addition, Mackett and Archard (1979), also investigating Orthopoxviruses, i.e. rabbitpox, vaccinia, monkeypox, variola, cowpox and ectromelia virus observed that the distribution of Hind III sites suggested that an internal region of molecular weight approximately 30 x 10^6 daltons. is highly conserved between Orthopoxvirus genomes. although some type-specific differences can occur within this region.

b) Species identification of isolates

Muller et al., (1978) found that the restriction patterns produced on digestion, by the endonucleases Hind III, EcoRI and BamHI, of the genomes of representative strains of 6 Orthopoxviruses,i.e. rabbitpox, vaccinia, cowpox, ectromelia and fowlpox viruses, were clearly distinguishable. Similarly, Esposito et al.,(1978) examined 12 Orthopoxviruses by REA and found they could be placed into 4 groups on the basis of their Hind III profiles. These groups corresponded to already established species, i.e. cowpox, vaccinia. variola and monkeypox viruses. Although 4 major groups were defined, the profiles were especially useful in differentiating variola minor virus from variola major virus. which together constituted one of the groups.

Mackett and Archard (1979) also examined the Hind III profiles of Orthopoxviruses - rabbitpox, vaccinia, monkeypox, variola, cowpox and ectromelia viruses - and found that the profiles were similar and characteristic for strains of the same Orthopoxvirus species.

Less clear-cut results were obtained in a study of 9 Parapoxviruses - 6 Stomatitis Papulosa (SP) and 3 Orf virus strains. Witteck et al.,(1980) showed that REA of the viral genomes divided the SP strains into 2 distinct groups. and the Orf virus strains into a single, more heterogeneous group (irrespective of the endonuclease used). Almost no resemblance existed between the restriction patterns of the 2 SP virus groups, and the Orf virus isolates, and this heterogeneity of restriction patterns contrasted with the

close serological and biological relatedness exhibited by these Parapoxviruses. Therefore, it can be concluded that, while REA with Parapoxviruses is less useful for species identification, it is still useful for strain identification (see below), and further results may require a reassessment of present taxonomic systems as they apply to Parapoxviruses.

It has also been shown that Herpes Simplex Virus Type I (HSV-1) can be distinguished from HSV-2 by REA. Thus, Skare et al., (1975) compared 2 HSV-2 strains and 6 HSV-1 strains, and found that strains of HSV-1 differ from strains of HSV-2 at most of their cleavage sites. Therefore, even allowing for intraspecies differences, a new isolate of HSV could be assigned as either type 1 or type 2 on the basis of its restriction pattern.

REA has been extended to include bacteria in a few instances. For example, Marshall et al., (1981) were able to distinguish serovars of Leptospira interrogans by REA with EcoRI. Serovars hardjo and balcanica gave patterns that differed from each other, and from other members of the Hebdomadis subgroup.

Mielenz et al., (1979) compared the restriction patterns of 3 species of Rhizobium, i.e. R. trifolii; R. japonicum and R. meliloti, and found that these species could be distinguished on the basis of their restriction pattern. Indeed, they showed that what they had previously called "R. trifolii mutant strains" were, in fact, derivatives of R. japonicum and not R. trifolii.

REA has also been used to identify a species of mycoplasma contaminating virus stocks and causing a cytopathic effect in cell culture. Thus, Darai et al., (1981) isolated a mycoplasma from a cell culture, digested the DNA with the restriction endonuclease, BstEII, and showed that the pattern produced was identical to that produced after digestion with BstEII of the DNA of the type strain of M. hyorhinis. However, the surprisingly few bands obtained on electrophoresis of the digested DNA, leave these results open to question and will be discussed later in this thesis.

c) Identification of strains

Individual strains within a species of microorganism can be identified by their characteristic restriction patterns. That is, when 2 strains exhibit identical restriction patterns, they can be considered to be identical or very closely related.

Esposito et al., (1978) found that, while Hind III profiles were useful in grouping the Orthopoxviruses into species, Sal I endonuclease profiles also showed intra-species, i.e. strain differences. This observation of strain differences within Orthopoxvirus species was confirmed in the findings of Mackett and Archard (1979); while Witteck et al., (1980) reported the existence of strain differences within species of Parapoxvirus i.e. Stomatitis Papulosa and Orf virus.

Whether or not differences in restriction pattern between strains reflect biological differences or not is another matter. For example, Skare et al (1975) observed that 2 strains of HSV-1 that showed slightly different patterns (one from the other) were shown to be functionaly similar to one another, yet different from other HSV-1 strains, e.g. in their ability to transform hamster embryo fibroblast cells.

However, examination of strains within one species of virus has yielded new information on the existence of a new type of virus in the case of study of Human Papillama Virus (HPV) by Gissman et al., (1977). These workers examined 36 isolates of HPV from individual human warts by REA (using EcoRI, BamHI, Hind II, Hind III, Hpa II and Hae III). Four different cleavage patterns were observed, and while three isolates (HPV1, HPV2, HPV3) had many cleavage sites in common, a fourth one (HVP4) was found to be entirely different. They concluded, after further examination of these isolates by other methods, e.g. serologically, that while HPV1-3 are closely related, HPV-4 represents a new human papilloma virus.

In the light of indications of quite extensive strain variation within a virus species by REA, the homogeneity of isolates of Leptospira interrogans serovar hardjo (Marshall et al., 1981) and Mycoplasma hyorhinis (Darai et al., 1981), from widely differing sources, provides an interesting contrast.

1.2.4 Epidemiological studies

The ability of REA to unequivocally identify strains within a species, has been used in epidemiological studies of viruses - principally Herpesviruses. The technique has, so far, proved most useful in the study of the infections of Herpes Simplex Virus (HSV) Types 1 and 2. To illustrate this, several epidemiological studies of HSV are considered below.

HSV Types 1 and 2 are widespread within the human population, and can cause serious, and sometimes fatal, diseases. The control of spread of infection requires an understanding of the epidemiology of the organism, and Restriction Endonuclease Analysis (REA) has provided a useful tool for this purpose. The use of REA in epidemiological studies is based on the assumption that epidemiologially unrelated strains of virus show different restriction patterns.

Linneman et al. (1978) reported the unusual occurrence of HSV-1 infection of 2 neonates within the same nursery (most neonatal herpetic infections are caused by HSV-2). REA on the two isolates from the nursery, together with two unrelated isolates, was performed in a "blind" study, i.e. all 4 isolates were coded, REA performed and the results obtained without the investigators' knowledge as to the origin of the isolates. The results showed that all were HSV-1 and that 2 were indistinguishable one from the other (regardless of restriction enzyme used) while the other two isolates differed from each other as well as from the two identical isolates. Upon breaking the code, it was apparent that the indistinguishable pair were isolated from the neonates under study. It was concluded, therefore, that

either the neonates had been infected by a common source, or the infection had been transmitted from one neonate to the other by nursery staff. Chronological data supported the latter hypothesis.

A similar application was used in a study reported by Buchman et al.,(1978) in which an outbreak of herpetic disease was experienced in association with a Paediatric Intensive Care Unit. Nine isolates from this outbreak, as well as 5 unrelated isolates were coded and a "blind" study performed by REA using 4 restriction endonucleases. It became clear on breaking the code, that analyses of the isolates readily discriminated between those belonging to the temporal cluster of hospital infections and the unrelated strains. They also showed that there were 2 independent introductions of HSV-1, resulting in 2 clusters of epidemiologically unrelated infections.

In this way, REA has been useful in "fingerprinting" strains of herpesvirus isolated from different sources to provide information on source and spread of infection when an outbreak has occurred.

An important feature of Herpes Simplex Virus is its ability to become latent in the ganglia after primary infection of epithelial cells, then, following a stimulus, to cause recrudescence of the disease. Lonsdale et al. (1979a) analysed the restriction profiles of 31 HSV-1 isolates from the trigeminal, superior cervical and vagus ganglia from 17 individuals (12 from USA; 2 from Japan; 3 from Norway). With the exception of the 3 Norwegian isolates which gave identical restriction profiles, virus isolates from the ganglia of different individuals could all be distinguished one from another. In contrast, virus isolates from the trigeminal, superior cervical and vagus ganglia of the same individual, or virus isolates from explants of a single ganglia were indistinguishable. On the basis of these results, it was concluded that a single virus strain infects each individual initially, and virus descended from this event subsequently infects and becomes latent in different cells of the same ganglion as well as

in different ganglia.

Therefore, the use of REA on isolates of HSV-1 has provided further evidence of latency of a primary infecting strain in the ganglia, which may cause recrudescence of the disease following a stimulus.

Rescrudescences do not necessarily only occur following stimulation of the latent virus. Another possible source of recrudescent lesions is reinfection with exogenous virus. Buchman et al., (1979), analysed 17 coded isolates of HSV-2 from genital and perigenital lesions of 8 patients by REA with 4 restriction enzymes. The results showed that, in 2 of the patients, infection with HSV-2 in the same or nearby site occurred in the face of a prior infection with a genetically different strain of the same serotype.

REA has not been confined solely to human herpesviruses. Sabine et al.,(1981) developed a rapid technique that distinguished 3 abortigenic strains of Equine Herpesvirus-1 from 3 non-abortigenic strains (by REA using EcoRI, BglII and Bam HI). These workers showed that significant differences in restriction pattern exist between abortigenic strains (which were identical to each other), and non-abortigenic strains (which showed minor differences from each other). These results correspond to differences between strains as shown by their behaviour in cell culture and by serological tests. Therefore, REA has been used to distinguish pathogenic from relatively non-pathogenic strains.

This technique has also been used in examination of DNA viruses other than Herpesviruses. Gissman et al., (1977) examined 36 isolates of Human Papilloma Virus from individual human warts (Verrucae vulgares and Verrucae plantares). They reported the discovery of a new Human Papilloma Virus (HPV-4), which was markedly different from the previous ones - HPV1, HPV2, and HPV3 - which were closely related, having many cleavage sites in common. In addition, they reported preliminary data that suggested that HPV-4 occurs in approximately 20% of Verrucae vulgares with low virus

production, whereas HPV1 occurs in approximately 70% of these warts with high virus yields. Therefore, REA has not only led to the discovery of a new strain of virus, but provided information on a possible association of virus type with particular lesions produced.

Similarly, Ostrow et al., (1981) showed that the papillomavirus DNA of isolates from 4 meathandlers to be distinct from that of known HPV, indicating the existence of a 'new' HPV. While REA has been used in the study of the epidemiology of DNA viruses such as Human Papilloma Virus (see above); Orf virus (Robinson et al., in press); and Adenovirus (Wadell et al., Part I), the most extensive use of the technique in epidemiological studies has been with Herpes Simplex Virus.

So far, we have considered the power of REA to distinguish isolates within a species. However, it can also be used as an unequivocal method to distinguish between 2 related species, especially HSV1 and 2. Thus, while different strains can be distinguished within both HSV-1 and HSV-2 by differences exhibited in their restriction profiles, these differences are minor compared with those that enable HSV-1 to be distinguished from HSV-2. HSV types 1 and 2 have been differentiated by a number of methods, e.g. kinetic neutralisation, indirect immuno-peroxidase tests, which tend to be time-consuming. Lonsdale et al., (1979b) developed a rapid, simplified technique based on the REA of radioactivity-labelled DNA of HSV. These workers stated that this technique distinguishes HSV-1 strains from HSV-2 strains unambiguously and that large numbers of isolates can be screened in 3 to 4 days. Consequently, this could be particularly valuable in large-scale epidemiological studies, e.g. in examining the prevalence of a particular virus within a population over a period of time.

1.3 Effect of genome size on Restriction Endonuclease Analysis

Most restriction endonucleases can be considered in 2 general groups: those that recognise and cleave at 4 base pair sequences, and those that recognise and cleave at 6 base pair

sequences. Therefore, assuming a G+C content of 50%, and random bases (perhaps a somewhat naive theoretical approach!), the average size of DNA fragments would be 1.8×10^5 daltons, and 2.9×10^6 daltons for endonucleases that recognise 4 and 6 base pair sequences respectively. This implies that a "medium-sized" virus with DNA of molecular weight 10^8 daltons, e.g. Herpesvirus, should generate 556 fragments with an endonuclease that recognises and cleaves at 4 base pairs, and 35 fragments with a 6 base pair endonuclease. Such a calculation is only a rough guide, since base pairs will not be random, and G+C content will not be 50%. Indeed, the number of fragments generated in Herpesvirus DNA with EcoRI (a 6 base-pair endonuclease) is only 14.

Current technology of gel electrophoresis sets a limit on the number of bands that can be recognised. While the upper limit is, to some extent subjective, probably most observers would agree, the number would be between 30 and 100. Taking the actual figure of 14 bands for Herpesvirus, which has DNA of molecular weight 10^5 daltons, and ignoring G+C content, one would expect Mycoplasma DNA which has a molecular weight of approximately 5×10^8 daltons (Bergey's Manual of Determinative Bacteriology, 8th Ed.) would give 5 times the number of bands, i.e. 70. This speculative estimate may be too low because the G+C content of M. ovipneumoniae is low (28%), and EcoRI recognises a 6 base pair sequence that is A-T-rich. While this might be too high a figure for unequivocal identification of individual bands, it does give some grounds to suppose that it might be possible to distinguish strains of M. ovipneumoniae by REA. It was such a calculation that encouraged us to embark on the project of examining strains of M. ovipneumoniae by REA.

In the course of this study, it became clear from the literature, that microorganisms larger than viruses could indeed be examined and identified by REA. These microorganisms, included bacteria, e.g. Leptospira (Marshall et al., 1981); Rhizobium (Mielenz et al., 1979) and Mycoplasma hyorhinis (Darai et al., 1981). Results recently

obtained with M. hvorhinis (Darai et al., 1981) seem to be in contrast with the present work, however, detailed consideration of this work in relation to our own results is dealt with in the General Discussion.

CHAPTER 2 Development of Restriction Endonuclease Analysis for Mycoplasma ovipneumoniae

2.1 Growth studies of two strains of M. ovipneumoniae in vitro

2.1.1 Introduction

Growth studies of M. ovipneumoniae in vitro (Major et al., 1979) revealed an exponential phase which progressed immediately into the death phase, i.e. no stationary phase was observed. Spontaneous lysis of mycoplasma cells which occurs in the death phase, might release the DNA into the surrounding medium. Since nucleases are present in the serum (which constitutes 10% of the FM4 medium), the exposure of the DNA to these could alter the restriction enzyme cleavage pattern of mycoplasmal DNA.

Therefore, in an attempt to ensure all cultures were in the exponential phase of growth, when harvested for DNA extraction, it was decided to investigate a method of estimating growth in cultures propagated under different conditions, i.e. shaking and stationary.

Growth studies (Major et al., 1979) showed that cultures remain in the exponential phase of growth until the turbidity stops increasing, at which point the death phase rapidly ensues. However, preliminary observations revealed that some batches of FM4 media formed precipitates to varying extents on incubation at 37^{o}C, which would interfere with the interpretation of turbidity readings of a culture. Consequently, it was decided to use measurements of pH change of the medium to indicate growth, and compare these with final turbidity readings, in order to find the best times to harvest the cultures. That is, the range of pH readings, within which, one could be reasonably certain that cultures were still within the exponential phase.

Therefore, this section investigates the growth of cultures of 2 strains of M. ovipneumoniae, shaking and stationary, using turbidity and pH readings of the medium as indicators of growth.

2.1.2 Materials and Methods

a) FM4 medium - See Appendix

b) Source of M. ovipneumoniae strains

Strain 2 was isolated from the lungs of a New Zealand sheep in 1973 by Dr J.K. Clarke. Strain 10 was isolated from the lungs of an Australian sheep, and was kindly supplied by Dr T.D. St. George.

c) Propagation of M. ovipneumoniae strains

Duplicate cultures of each of two M. ovipneumoniae strains (2 and 10) were prepared as follows:

9ml of FM4, in a 25ml universal bottle, was inoculated with 1ml of a stock culture, which was stored at -70°C, and incubated at 37°C until a colour change of the medium was first observed (to approximately pH 7.2). At this stage, the culture was added to approximately 300ml of pre-warmed FM4 in a 1 litre conical flask. Strains 2 and 10 were propagated in duplicate - one 300ml culture was placed on a rotary shaker (125rpm); and the other was left stationary, at 37°C. Approximately 5ml samples were removed at intervals and the pH reading and turbidity* measurement was taken. This was continued until no further pH change or increase in turbidity occurred.

2.1.3 Results

Duplicate cultures of strains 2 and 10 of M. ovipneumoniae were measured for pH change and turbidity after incubation - one shaking, and one stationary - at 37°C.

The final turbidity readings of all 4 cultures are shown in Table I:

*Turbidity measurements were taken using a Bausch and Lomb Spectronic 20 Spectrophotometer at 640nm.

Strain	Final Turbidity Readings	
	Stationary	Shaking
2	0.125	0.50
10	0.14	0.54

TABLE I : Final Turbidity readings of 4 cultures of M. ovipneumoniae

Growth curves of strains 2 and 10, shaking and stationary, using measurement of pH of the medium as a growth indicator, are shown in Figure 1.

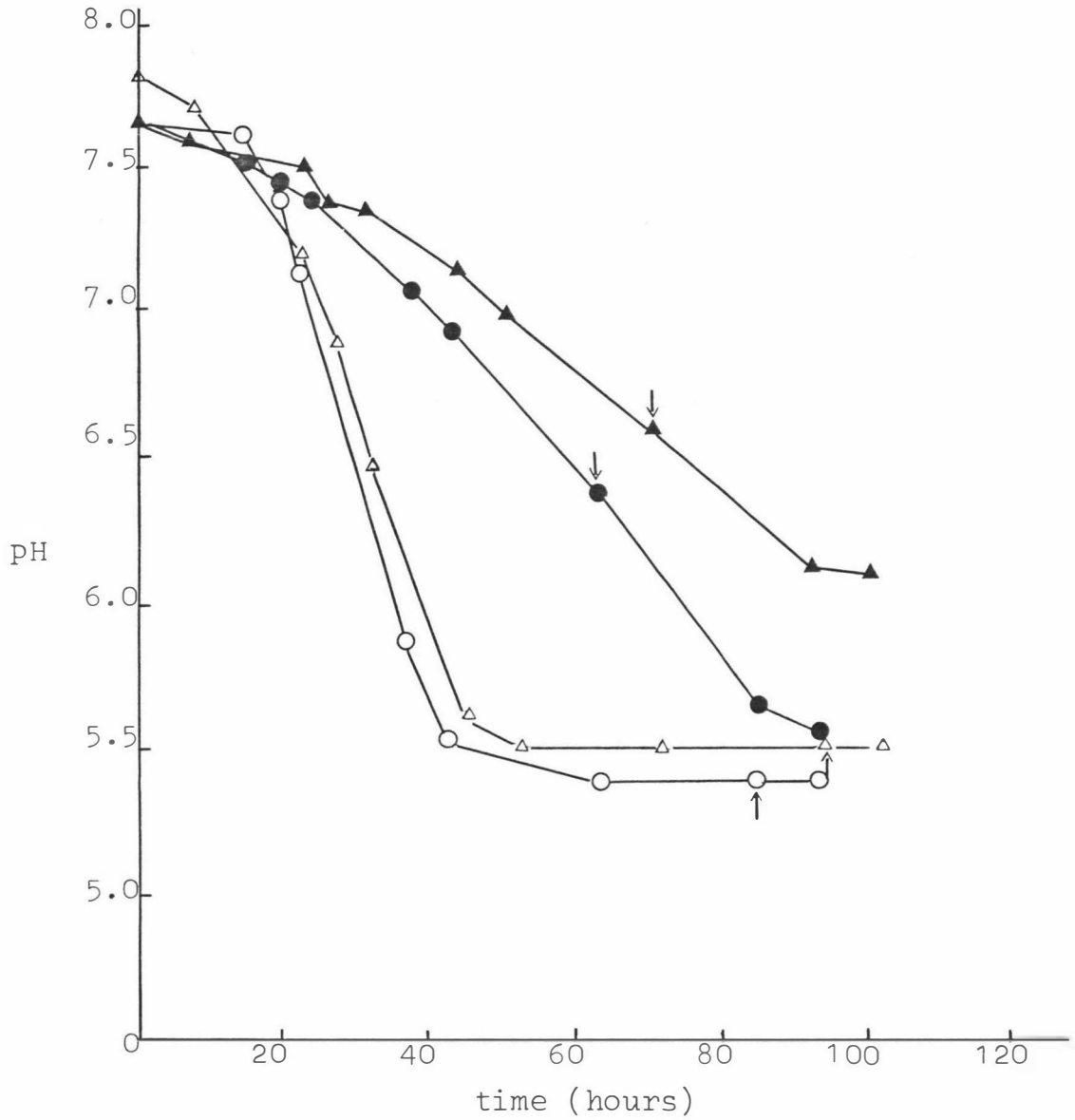

Figure 1 : Monitoring pH of stationary and shaking cultures of two strains of M. ovipneumoniae (strains 2 and 10). △ = strain 2 shaking; ▲ = strain 2 stationary; o = strain 10 shaking; ● = strain 10 stationary. Arrows indicate at which point maximum turbidity occurred.

2.1.4 Conclusion

Several conclusions were drawn from the results of this experiment:

1. Cultures that were incubated while shaking grew more rapidly than did those incubated while stationary (Fig. 1).
2. Cultures that were incubated while shaking gave a greater yield of cells than did those incubated while stationary (Table I).
3. As a consequence of the previous two points, it was concluded that, for DNA extraction, strains should be propagated in a shaking culture.
4. Although the cultures remained in the exponential phase until the reaction of the medium reached approximately pH 6.0; nevertheless, it was decided to harvest cultures while the reaction of the medium was in the range pH 6.5 - 7.0. In this way, even allowing for strain variability, it was ensured that cultures had not yet reached the death phase, i.e. were still within the exponential phase of growth, when harvested for DNA extraction.

2.2 REA of cultures harvested in the death phase

2.2.1 Introduction

It was decided to attempt to ensure that all cultures of M. ovipneumoniae, propagated for DNA extraction, were harvested while still within the exponential phase of growth, to avoid the lysis of cells which occurs in the death phase (Major et al., 1979) and so prevent possible exposure of the DNA to serum nucleases present in the medium, which would alter the restriction enzyme cleavage pattern. However, technical errors (i.e. inadvertant harvesting of cells during the death phase) or possible premature lysis of cells within exponential cultures, could result in inadvertant exposure of the DNA to serum nucleases. This section examines the effect (if any) on the restriction patterns of one isolate when harvested in the death phase as compared to standard conditions.

2.2.2 Materials and Methods

This section validates the biological aspects of the techniques for DNA analysis. The processing of cultures for DNA extraction, digestion and electrophoresis is described in a subsequent section (Chapter 2.4).

FM4 Medium - See Appendix.

Strain of M. ovipneumoniae

M14-7 was isolated from the nasal cavity of a lamb in 1981 and cloned, as described in Chapter 3.1.2(a).

Production of exponential and death phase cultures of M. ovipneumoniae

Duplicate 9.0ml aliquots of FM4 in a 50ml conical flask, were each inoculated with 1.0ml of stock culture from -70°C freezer, and incubated at 37°C on a rotary shaker at 125rpm. When a colour change was observed, indicating the culture had reached approximately pH 7.0, the duplicate 10ml cultures were each added to 300ml of prewarmed FM4 in a 1 litre conical flask. Both cultures were incubated

shaking at 37°C – cells were collected from one culture when the pH of the medium reached pH 6.9 and from the other after a further 24 hour period of incubation, when the reaction of the medium was pH 5.8.

Cells were collected, the DNA extracted, analysed, digested and electrophoresed in an agarose gel which was then photographed (see Chapter 2.4).

2.2.3 Results

A strain of M. ovipneumoniae (M14-7) was grown in duplicate cultures, and harvested respectively in the exponential and death phases. The DNA was extracted and processed independently for each culture (see Chapter 2.4) and the restriction enzyme patterns compared.

The results are shown in Fig. 2.

The spectrophotometry profiles for both nucleic acid extracts is shown in Table II.

Wavelength (nm)	230	240	250	260	270	280	Ratio $\frac{260}{280}$
Nucleic acid extracted from exponential culture	0.06	0.07	0.115	0.13	0.10	0.06	2.17
Nucleic acid extracted from death phase culture	0.65	0.51	0.495	0.48	0.42	0.34	1.4

Table II : Spectrophotometry profiles of nucleic acid extracts from exponential and death phase cultures

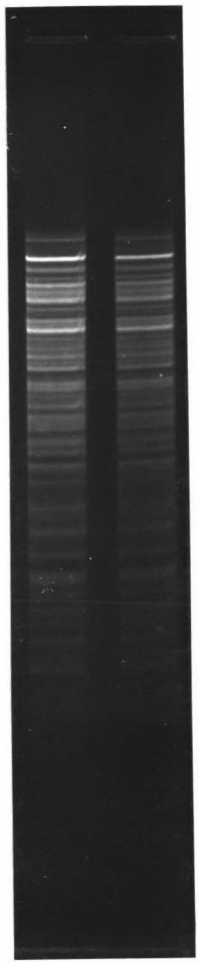

Figure 2 : Comparison of REA patterns of DNA from 2 cultures of a strain of M. ovipneumoniae (M14-7), harvested in the log phase (lane 1), and the death phase (lane 2). The DNA was digested with EcoRI, electrophoresed through a 0.7% agarose gel into which was incorporated Ethidium Bromide (0.5ug/ml). The gel was illuminated by ultraviolet light, and photographed through a Wratten 23Å filter. No differences in pattern were detected.

2.2.4 Conclusion

It can be seen in Figure 2, that the restriction pattern of DNA extracted from cultures in the death phase does not differ from that of DNA extracted from culture in the exponential phase. This implies that it may be acceptable to harvest cells in the death phase. It was, nevertheless, decided, on first principles, to routinely harvest cells in the exponential phase although this may be unnecessary.

Despite the unchanged restriction pattern of DNA from death phase cells, it was observed that there was a difference between the DNA, from the two cultures, in their spectrophotometry profiles. The DNA from the exponential phase culture exhibited a standard profile, i.e. the 230nm reading was equal to the 280nm reading; the ratio of 280nm reading to the 280nm reading was within the range 1.8 to 2.4.

In interesting contrast, the DNA from the death phase culture exhibited an unusual profile, i.e. the 230nm reading was not equal to the 280nm reading; and the $\frac{260}{280}$ ratio was below the range 1.8 - 2.4.

The reasons for this variation in spectrophotometry profile were not investigated, since death phase cultures were not used in further experiments.

2.3 Effect on restriction pattern of freezing cell suspensions prior to DNA extraction

2.3.1 Introduction

Preliminary observations indicated that different isolates of M. ovipneumoniae showed markedly varying growth rates in vitro. Estimated division times varied from less than 5 hours to 12 hours, for both nasal and lung isolates, therefore cultures of isolates (propagated in FM4 for DNA extraction) reached the exponential phase at varying (and often inconvenient) times which were difficult to predict. Since it was decided that cultures were to be harvested while in the exponential phase, it became necessary to halt the procedure at some point, preferably before lysis of the cells with Pronase and SDS. It was therefore decided to harvest the cultures, resuspend the cells in Tris-EDTA, then freeze the cell suspension at -70^{o}C until required. However, it was not known if freezing the cell suspensions, then thawing them before lysis and extraction of the DNA, would damage the DNA in a way that would affect the restriction pattern. Therefore, this section investigates the effects, if any, of freezing and thawing cell suspensions, on the pattern of bands produced after digestion of the DNA with a restriction endonuclease.

2.3.2 Materials and Methods

PBS - See Appendix

FM4 Medium - See Appendix

Tris-EDTA Solution

1.0M Tris-HCl pH 7.5	1.0ml
0.2M EDTA disodium salt pH 7.2	5.0ml
Distilled water	4.0ml

Make up fresh from stock solutions before use

Strain of M. ovipneumoniae

Strain 10 was isolated fron the lungs of an Australian sheep and kindly supplied by Dr T.D. St George, in 1973.

Propagation of strain of M. ovipneumoniae

One 9.0ml aliquot of FM4 medium in a 50ml conical flask, was inoculated with 1.0ml of stock culture from -70°C freezer, and incubated at 37°C on a rotary shaker at 125rpm. When a colour change, indicating the culture had reached approximately pH 7.0, 1.0ml was inoculated into 9.0ml of fresh FM4 medium, while the remaining 9.0ml was inoculated into approximately 300ml of FM4 medium in a 1 litre conical flask. Both cultures were incubated at 37°C on a rotary shaker at 125rpm.

Cells were collected from the 300ml culture by centrifugation when the reaction of the medium reached pH 6.9, washed twice with 2 x 20ml of PBS, resuspended in 1.0ml of Tris-EDTA and frozen at -70°C.

When the 10ml culture had reached pH 7.0, it was inoculated into 300ml of FM4 in a 1 litre conical flask, and the culture incubated at 37°C on a rotary shaker (125rpm). Cells were collected by centrifugation when the reaction of the medium reached pH 6.95, washed twice with 2 x 20ml of PBS and resuspended in 1.0ml of Tris-EDTA. At this stage, the first cell suspension was thawed, and both cell suspensions were lysed, the DNA extracted, analysed, digested and electrophoresed in an agarose gel which was then illuminated with ultraviolet light and photographed (see Chapter 2.4).

2.3.3 Results

The DNA of M. ovipneumoniae strain 10 was extracted: a) immediately following the propagation and harvest of one culture; and b) after the cell suspension had been frozen at -70°C. The DNA was processed independently for each culture (see Chapter 2.4), and the restriction enzyme patterns compared.

The results are shown in Fig. 3.

The spectrophotometry profiles were standard for both extracts, i.e. the 230nm readings were approximately equal

to the 280nm readings; and both $\frac{260}{280}$ ratios were within the range 1.8 - 2.4.

Figure 3 : Comparison of REA patterns from 2 cultures of a strain of M. ovipneumoniae (strain 10): the DNA immediately extracted from the cell suspension (lane 1), and following thawing of the cell suspension that had been stored at -70°C. No differences in pattern were detected.

2.3.4 Conclusion

Figure 3 shows that no change in restriction pattern occurs when the cell suspensions are frozen at - 70°C prior to the extraction of DNA. This meant that the cell suspensions could be stored at -70°C until required.

2.4 Restriction Endonuclease Analysis of *Mycoplasma ovipneumoniae* : Materials and Methods

2.4.1 Propagation of mycoplasmas for DNA extraction

1. FM4 Medium - See Appendix
2. Tris-EDTA Solution

1.0M Tris-Hcl pH 7.5	1.0ml
0.2M EDTA disodium salt pH 7.2	5.0ml
Distilled water	4.0ml

Made up fresh from stock solutions before use.

3. Phosphate Buffered Saline (PBS) - See Appendix.

Propagation of *Mycoplasma ovipneumoniae*

To prepare stock inocula of *M. ovipneumoniae*, isolates were propagated at 37°C in FM4 medium until the medium reached pH 6.5 - 7.0 (at this stage cultures are still in the exponential phase). Then 1.5ml aliquots were frozen at -70°C until required.

To prepare a culture for DNA extraction, 13.5ml of FM4 in a 50ml conical flask, was inoculated with 1.5ml of stock culture from -70°C freezer, and incubated at 37°C on a rotary shaker at 125 rpm. When a colour change was seen, indicating the culture had reached approximately pH 7.0, the 15ml was added to approximately 300ml of prewarmed FM4 in a 1 litre conical flask. The cells were harvested when a colour change indicating a reaction of pH 6.5 - 7.0 was observed.

Harvesting *M. ovipneumoniae* cells

The *M. ovipneumoniae* cells were collected by centrifugation at 14,500g for 30 minutes, and washed twice with 2 x 20ml of sterile PBS. The cells were then resuspended in 1.5ml of Tris-EDTA solution : 1ml was stored at - 70°C for DNA extraction, and 0.5ml stored at 4°C for gel precipitin tests.

2.4.2 Extraction of DNA from mycoplasmas

1. 10% SDS – 10% w/v Sodium Dodecyl Sulphate (BDH – specially pure). Stored at room temperature without autoclaving.

2. Pronase – 10mg/ml in distilled water (Calbiochem B grade). Initially preincubated at 37°C for 2 hours to remove DNAase activity. Stored at -20°C.

3. Saline Tris-EDTA (STE) Buffer (10X)–

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

Diluted 1/10 before use. Stored at room temperature.

4. Phenol – Redistilled under Nitrogen and stored in the dark at -20°C under Nitrogen.

5. Chloroform – Reagent Grade.

6. Iso-Amyl Alcohol – Reagent Grade.

7. Dialysis Tubing (10mm flat width – Processed as follows: Suitable lengths were cut and boiled in an aqueous solution of 5% Sodium Carbonate solution. The solution was changed until no odour or colour could be detected. The dialysis tubing was then boiled in distilled water once; in .001M EDTA once; washed with distilled water; boiled again in distilled water and allowed to cool. The processed dialysis tubing in distilled water was stored at 4°C.

8. Tris-EDTA (TE) Buffer – 1M Tris-HCl
pH 7.5 – 40ml
0.2 EDTA (disodium
salt) pH 7.2 – 20ml
Distilled water to 4000ml

DNA Extraction

M. ovipneumoniae isolates, propagated in FM4 and harvested in the exponential phase were centrifuged, resuspended and frozen in 1.0ml of Tris-EDTA solution and stored at -70°C until required (see Chapter 2.4.1). Cell suspensions were then thawed, and to each isolate, 100ul of 10% SDS and 100ul of 10mg/ml of Pronase was added.
Note: Experiments (see Ch. 2.3) were undertaken to show the storage of all suspensions at -70°C did not affect the exponential results of REA.

The cell suspensions were incubated overnight at 50°C, then 0.17g of Sodium Perchlorate (BDH analar) was added to each (i.e. to approximately 1M concentration) and the lysate was then incubated at 50°C for 1 hour.

Further pipetting was done using 5.0ml serological pipettes with the tips cut off to avoid shearing the DNA. A Phenol/Chloroform/Iso-Amyl Alcohol solution was made up in the proportions 25:24:1, and equilibrated by the addition of one tenth of a volume of working strength STE buffer. This solution was cooled to room temperature in the dark to avoid oxidation of the phenol.

The lysates were incubated for 1 hour with Sodium Perchlorate; 4.0ml of STE buffer (working strength) was then added to each and the solutions slowly poured into screw-capped, glass centrifuge tubes. 5.0ml of Phenol/Chloroform/Iso-Amyl Alcohol mix was then added to each and the tubes were rocked gently back and forth to mix the two layers. After 5 minutes, the tubes were centrifuged at 1,000 - 2,000 rpm for 5 minutes at room temperature.

The upper aqueous layer was gently removed into another screw-capped centrifuge tube and re-extracted twice with Phenol/Chloroform/Iso-Amyl Alcohol. After the second re-

extraction, the aqueous layer was gently added to dialysis tubing which was then sealed by at least two knots and colour coded. The extracted DNA was then dialysed four times with 1 litre of TE buffer.

2.4.3 Analysis of nucleic acid extract

1. Saline Tris-EDTA (STE) Buffer (10X) -

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

Stored at room temperature.

2. Ethidium Bromide Solution

5.0ug/ml in diltilled water.
Stored in the dark at 4°C.

3. Calf Thymus DNA

50.0ug/ml in distilled water.
Stored at 4°C.

Estimation of concentration of DNA in extract

The nucleic acid extract was removed aseptically from the dialysis tubing using the following procedure:

The multiply knotted dialysis tubing was held at one end with forceps, and all but one knot cut off from the lower end, which was then placed in a sterile bijou. Cuts were made in the upper and lower ends of the tubing and the nucleic acid extract was allowed to drain into the bijou.

100ul of the extract was added to 900ul of TE buffer (used for the final dialysis), and this dilution was used for the Spectrophotometry and Fluorometry. The extract was pipetted using disposable micropipette tips, with the ends cut off to avoid shearing of the DNA.

Spectrophotometry

0.4ml of the 1:10 dilution of each sample was loaded into a quartz-glass cuvette and the optical density measured using a Unicam Spectrophotometer SP500, with a dialysis buffer blank. Readings were taken at 230nm; 260nm; 270nm and 280nm for each sample. The 260:280 ratio was checked to be within the range 1.8 - 2.5, showing minimal contamination with protein.
Note: Phenol absorbs strongly at 270nm, so a peak reading at 270nm would indicate phenol contamination, suggesting the sample needed to be re-dialysed.

An estimate of the nucleic acid concentration was made from the 260nm reading, on the assumption that an optical density reading of 1.0 was equivalent to 50ug/ml of nucleic acid. A typical profile is shown below:

Sample	230nm	260nm	270nm	280nm	$\frac{260}{280}$	Nucleic Acid ug/ml
M31-7	0.17	0.27	0.215	0.13	2.1	13.5

Fluorometry

Measurements were taken using a Fluorometer with a 365mu filter, as described by Le Pecq and Paoletti (1966). The volume of extract to be used in the fluorometry assay mix was estimated as follows:

0.5 ÷ Nucleic acid concentration x 2000

e.g. Using the sample shown above (i.e. M31-7)

0.5 ÷ 13.5 x 2000 = 75ul Nucleic acid extract.

Fluorometry Assay Mix:

Nucleic Acid Extract (as calculated)	- 75ul
10 x STE Buffer	- 200ul
Ethidium Bromide (5ug/ml)	- 800ul
Distilled water to	-2000ul

A standard fluorometry curve, using Calf Thymus DNA was prepared using the following protocol:

Final concentration of DNA	Calf Thymus DNA (50ul/ml)	H_2O	10 X STE Buffer	Ethidium Bromide (5ug/ml)
0+	0*	1000*	200*	800*
0.2	8	992	200	800
0.4	16	984	200	800
0.6	24	976	200	800
0.8	32	968	200	800
1.0	40	960	200	800

TABLE III : Protocol for preparation of Standard Fluorometry Curve. This curve (a linear relationship was prepared for each batch of assays.

\+ Concentrations of DNA in ug/ml

* Volumes expressed as ul

2.4.4 Digestion of DNA with EcoRI

1. Lambda DNA - 325ug/ml
   Stored at 4°C

2. EcoRI - 4 units*3/microlitre
   Stored in 50% Glycerol at -20°C

3. EcoRI Buffer (5X)

1M Tris-HCl pH 7.5	50.0ml
5M NaCl	5.0ml
1M $MgCl_2$	2.5ml
Bovine Serum Albumin (FrV Sigma)	50.0mg
Distilled water to	100 ml

   Stored at 4°C

4. Mycoplasma DNA - See Chapter 2.4.2 (b).

5. Tris-EDTA (TE) Buffer + 0.05% SDS + 20% Glycerol

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

   Stored at room temperature

6. RNAase[*1] - (Sigma IA) 5mg/ml in distilled water.
Initially boiled for 5 minutes before use to destroy any DNAase.
Stored at -20°C.

Digestion of DNA with EcoR

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

2 X 1000 X 10(dilution factor) ÷ DNA concentration (fluorometric)

This volume of nucleic acid extract was added to a 1500ul Microfuge Tube (Eppendorf) containing 20ul of 5 X EcoRI. This mixture was diluted to 100ul with distilled water[*2]. 2ul (8 units) of EcoRI was then added, and the microfuge tube was gently tapped to mix the contents.

The digestion mix was incubated at 37°C for 45 minutes, then heated at 65°C for 10 minutes (to destroy the enzyme activity). The fragmented DNA was subsequently precipitated from solution by the addition of 1/20 volume of 5M Sodium Chloride, and 2 volumes of absolute ethanol. The tubes were then inverted to mix the contents, and placed at -20°C for a minimum of 30 minutes, after which they were spun in a microfuge at 8,500g for 5 minutes. The supernatant was discarded and the pellet was washed gently with cold 70% ethanol, then dried at 37°C for 10 minutes. 45ul of TE buffer + 0.05% SDS + 20% glycerol was added to each tube, which was tapped gently, then placed at room temperature overnight, to resuspend the pellet.

For each digest of 8 isolates prepared, 0.325 ug of lambda DNA was digested with 8 units of EcoRI. This acted both as a control to ensure that correct conditions had been used, and as a molecular weight maker.

Technical Footnotes:

1. RNAase -
   Initially, 10 ug of RNAase was routinely added to the digestion mix to destroy the RNA present in the nucleic acid extract. However, during the course of this investigation, the RNAase began to interfere with either the digestion of the DNA or the migration of DNA fragments through the gel, resulting in unclear restriction patterns.
   On investigation, it was discovered that the restriction pattern was not affected by the presence of intact RNA in the digestion mix (i.e. by omission of the RNAase). The RNA migrated more rapidly than the DNA and appeared as a large band at the end of the gel (see Fig. 6). Consequently, in all subsequent studies, the RNAase was omitted from the digestion mix.

2. Occasionally, the concentration of DNA in the nucleic acid extract was such that more than 80ul of the extract was required to give 2.0ug of DNA. In this case, 200ul of digestion mix was prepared, using 40ul of 5 X EcoRI buffer.

3. 1 unit - Complete digestion of 1ug of lambda DNA in 15 minutes at $37^{o}C$ in a reaction volume of 50ul.

2.4.5 Gel electrophoresis and photography

1. Electrophoresis (E) Buffer (10X)

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

Stored at 4°C without autoclaving. Diluted with distilled water when required.

2. Ethidium Bromide Solution

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

3. Perspex Gel Electrophoresis Tank - See Figure 4.

Gel electrophoresis of DNA samples

To 1.5 litres of E buffer (working strength), 300ul of Ethidium Bromide Solution was added (final concentration 0.5ug/ml). This was used to prepare 100ml of 0.7% Agarose (Biorad. Electrophoresis Grade). The agarose was dissolved by boiling in a flask fitted with a reflux condenser, and cooled to approximately 40°C before pouring.

A glass plate (20cm x 15cm) was cleaned and cellotape was stuck around the edge, partially projecting vertically to prevent spillage of the agarose. The plate was placed on a level surface, the 100ml of cooled agarose was poured on, and a perspex 'comb' was applied to one end to form wells. The agarose was allowed to set for 20 minutes, whereupon the 'comb' and cellotape were removed. The glass plate containing the agarose was placed in an electrophoresis tank (see Fig. 4), the compartments filled with E buffer (containing 0.5ug/ml Ethidium Bromide) to just below the gel top.

The wells in the agarose were then washed out with E buffer prior to loading the samples. The microfuge tubes containing the samples were gently agitated, to ensure the DNA was resuspended in the buffer, and 2.5ul of Bromophenol Blue in 50% Glycerol was added to the lambda sample to act as an electrophoresis marker. The samples were loaded into the wells which were then topped up with E buffer. The

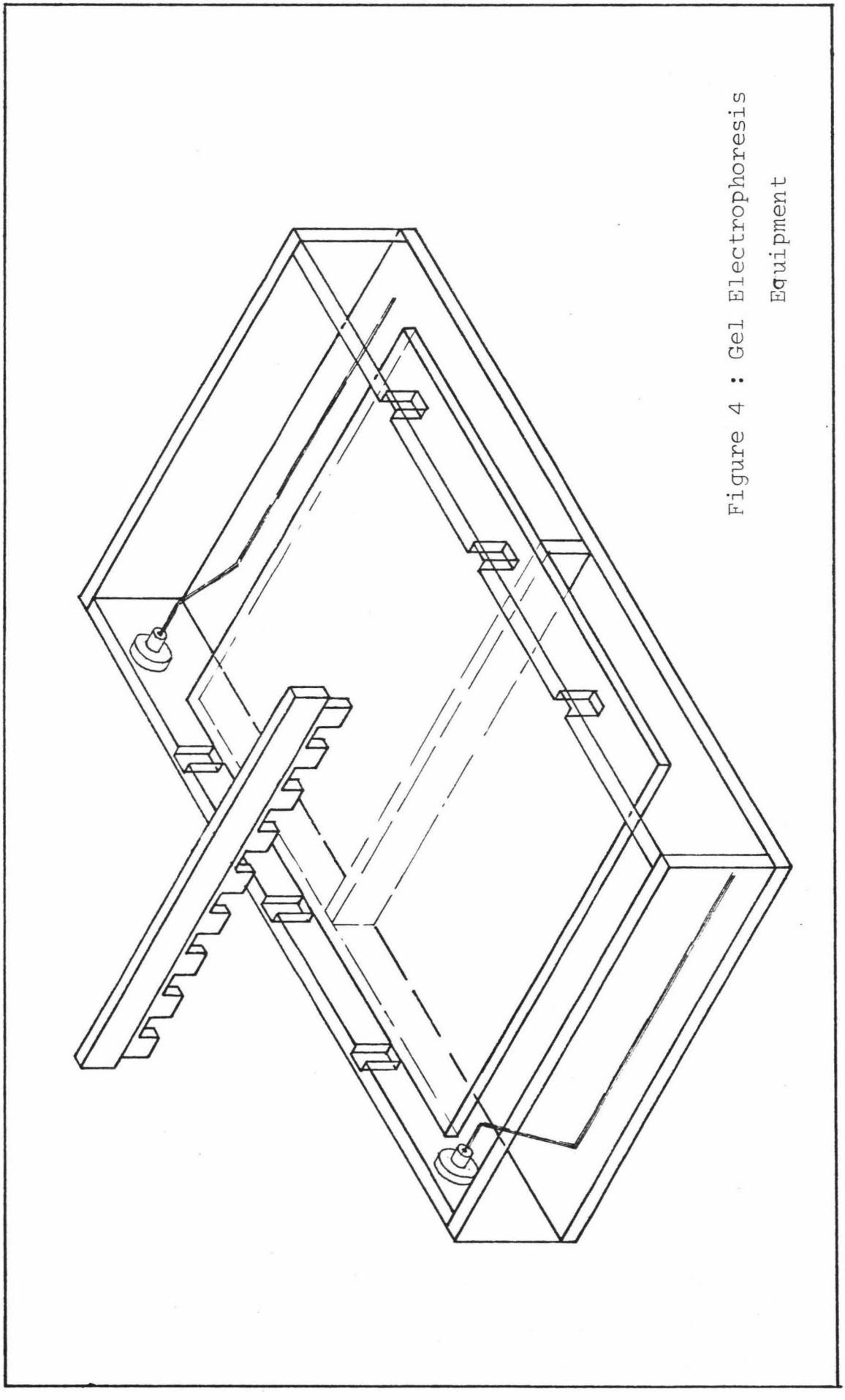

Figure 4 : Gel Electrophoresis Equipment

electrophoresis was then performed at 8 V/cm for 3.5 - 4 hours.

Photography of gel

When the Bromophenol Blue marker had travelled 12cm, the current was switched off, the glass plate containing the gel removed, and the gel rinsed with the remaining E buffer to remove any dust. The gel was then removed from the glass plate and placed onto a filter plate measuring 15cm x 15cm, in a box illuminated from below by four 15 W germicidal ultraviolet lamps.

The gel was photographed on Kodak Tri-X film by means of a 120 format plate camera through a Wratten 23 Å gelatin filter.

The film was developed for 5 minutes, fixed for 5 minutes, then washed for 10 minutes and suspended from a clip to dry.

2.5 Reproducibility of Restriction Endonuclease Analysis of M. ovipneumoniae

2.5.1 Introduction

Restriction Endonuclease Analysis (REA) of viruses, e.g. Herpesviruses (Skare et al., 1978), Orthopoxiviruses (Esposito et al., 1978) indicated marked heterogeneity of strains within a species, so strains could be "fingerprinted" by this technique and followed in epidemiological studies (Buchman et al., 1978; Lonsdale et al., 1979a; Gissman et al., 1977). In marked contrast, strains of Leptospira interrogans serovar hardjo were remarkably homogeneous (Marshall et al., 1981), although bacteria have a larger genome than viruses and thus would seem to have a greater scope for variation. If REA is to be useful in epidemiological studies of isolates of M. ovipneumoniae, a prerequisite is the ability of the technique to unequivocally distinguish strains of M. ovipneumoniae. A further requirement would be the ability of REA to give a reproducible pattern - not only, when the same DNA preparation was digested in duplicate, but also when the same strain was propagated in duplicate cultures, and the DNA extracted and digested independently.

This section investigates the ability of REA to fulfill both requirements. The two strains selected for this purpose were isolated from different sheep, one in New Zealand, and one in Australia. Therefore, if any strain differences occurred in M. ovipneumoniae that were detectable by this technique, they were likely to be demonstrated by examining these two strains.

2.5.2 Materials and Methods

Origin of strains

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

Strain 10 was isolated from the lungs of an Australian sheep and kindly supplied by Dr T.D. St. George in 1973.

Both strains had been cloned at least three times before the commencement of this study. Cultures were propagated in standard FM4 medium (see Appendix), and frozen in 1.5ml aliquots at -70°C, while still in the exponential phase of growth.

DNA extraction and Restriction Endonuclease Analysis

See Chapter 2.4.

2.5.3 Results

Strains 1 and 10 were each grown in duplicate, and the DNA was extracted independently for all 4 cultures. Each DNA extract was digested, in duplicate, with the restriction endonuclease, EcoRI, and electrophoresed through a 0.7% agarose gel within which was incorporated Ethidium Bromide (0.5mg/ml). Lambda DNA was included as a control (Lane 1). The gel was illuminated with ultraviolet light, and photographed through a Wratten 23 Å gelatin filter.

The results are shown in Figure 5.

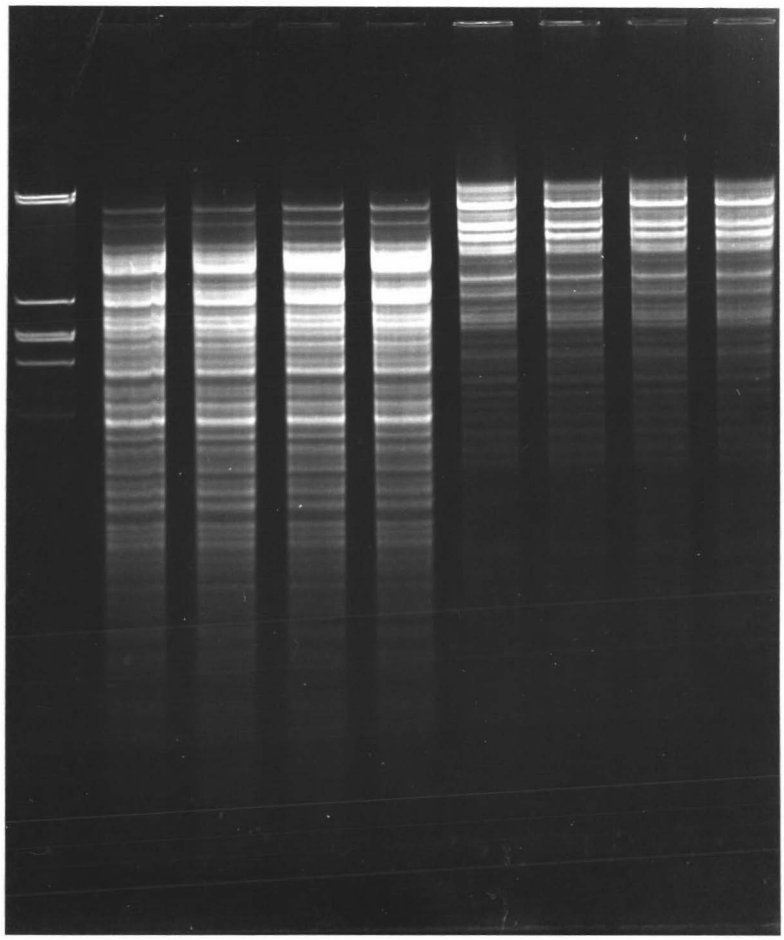

Figure 5 : REA of DNA from 2 strains of M. ovipneumoniae (strains 1 and 10). Each strain was propagated in duplicate ('a' and 'b') and the DNA extracted from each of the 4 cell suspensions. Each DNA extract was digested in duplicate. Lane 1: Lambda; Lanes 2 and 3: Strain 1(a); Lanes 4 and 5: Strain 1(b); Lanes 6 and 7: Strain 10(a); Lanes 8 and 9: Strain 10(b). No differences in pattern were detected between DNA digested in duplicate, e.g. lanes 2 and 3, or between duplicate DNA extracts, e.g. lanes 3 and 4.

2.5.4 Conclusion

Several conclusions can be drawn from the results of this experiment:-

a) Restriction Enzyme Analysis (REA) gives a recognisable and reproducible pattern of bands with one strain of M. ovipneumoniae not only when the DNA is digested in duplicate (e.g. lanes 2 and 3; lanes 4 and 5) but also when the DNA is extracted and digested from separate cultures of the same strain (e.g. lanes 3 and 4; lanes 7 and 8);

b) REA can unequivocally distinguish between 2 strains of M. ovipneumoniae (lanes 5 and 6).

It should, perhaps be noted here, that lambda DNA was digested under the same conditions as M. ovipneumoniae DNA (for each experiment) and run in lane 1.

Bacteriophage Lambda Marker

The pattern produced served as both a control for the technique and, as a molecular weight marker. The molecular weights of the fragments produced on digestion of lambda DNA with EcoRI have been estimated (Thomas and Davis, 1975).

Fragment	Pattern	Molecular Weight (megadaltons)
A	———	13.7
D	———	4.74
E	———	3.73
C	———	3.48
B	———	3.02
F	———	2.13

Often, an extra band appears above, but close to, band 'A' while band 'F' is reduced in intensity (see Fig. 5). This is a consequence of a relatively large proportion of the lambda molecules becoming circularised or forming concatamers through hydrogen bonding of their cohesive ends. In this way, the 'F' fragment is joined to the 'A' fragment, and,

because there is no recognition site for EcoRI between fragments 'A' and 'F', a fragment with the combined molecular weights of 'A' and 'F' is produced.

2.6 Restriction Endonuclease Analysis of 8 strains of M. ovipneumoniae

2.6.1 Introduction

While it was established that REA could give a reproducible pattern with one strain of M. ovipneumoniae and also unequivocally distinguish two strains from widely differing sources (Ch. 2.5), there was no indication as to the extent of heterogeneity that exists among strains of M. ovipneumoniae isolated from different sources in New Zealand.

Therefore, this section examines 8 different isolates of M. ovipneumoniae by REA. These strains included both nasal and lung isolates, obtained from sheep on different farms, at different times, and included the Australian and New Zealand strains examined in the previous section.

2.6.2 Materials and Methods

Origin of Strains

Lane Number	Strain	Origin of Strain (Year)	Country of Origin
1	(Lambda)	-	-
2	1	Lung (1973)	New Zealand
3	2	Lung (1973)	New Zealand
4	4	Lung (1973)	New Zealand
5	5	Lung (1973)	New Zealand
6	10	Lung (1973)	Australia
7	37E	Nasal cavity (1980)	New Zealand
8	L3/C3	Lung (1981)	New Zealand
9	MPP74	Nasal cavity (1980)	New Zealand

TABLE IV : Origin of M. ovipneumoniae strains shown in Fig. 6

All isolates of M. ovipneumoniae, were propagated in standard FM4 medium (see Appendix), and frozen in 1.5ml aliquots at -70°C, while still in the exponential phase of growth.

DNA Extraction and Restriction Enzyme Analysis

See Chapter 2.4.

2.6.3 Results

The 8 strains of M. ovipneumoniae were propagated in FM4, the cells collected, DNA extracted and digested with EcoRI. The resultant fragmented DNA was electrophoresed through a 0.7% Agarose gel (within which was incorporated 0.5ug/ml Ethidium Bromide), illuminated with ultraviolet light, and photographed through a Wratten 23 Å gelatin filter.

The results are shown in Figure 6.

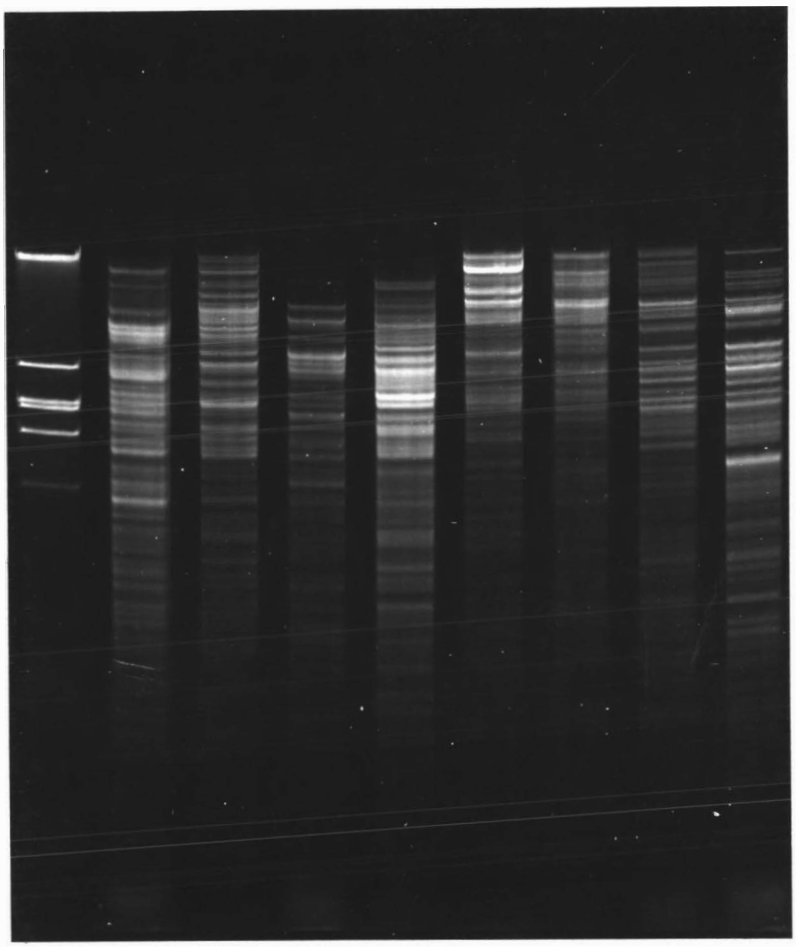

Figure 6 : REA of 8 strains of M. ovipneumoniae from widely different sources. Lane 1: Lambda; Lane 2: strain 1; Lane 3: strain 2; Lane 4: strain 4; Lane 5: strain 5; Lane 6: strain 10; Lane 7: 37E; Lane 8: L3/C3; Lane 9: MPP74. None of the strains show any significant similarity.

2.6.4 Conclusion

It can be seen from Fig. 6 that marked heterogeneity, with respect to restriction pattern, exists among strains of M. ovipneumoniae, isolated from different sources. Note that no similarity in pattern exists between any of the 8 strains, i.e. the pattern differs aling its whole length.

This heterogeneity among different strains of M. ovipneumoniae led to concern as to the value of REA in epidemiological studies. That is, if all isolates of M. ovipneumoniae exhibited different restriction patterns, REA would not be of any use in the study of the prevalence and spread of strains of M. ovipneumoniae through a flock of sheep.

However, it must be stressed that the 8 isolates studied in this section were obtained from widely differing sources.

In order for REA to be useful as an epidemiological tool, 2 criteria must be satisfied:-

a) isolates from distinctly different sources must, in general, be different (as regards restriction pattern);

b) not all isolates from similar sources, e.g. sheep from within a flock, must differ.

The first criterion is clearly satisfied (see above), and at this point, the assumption is made that isolates from within one flock will not be totally heterogeneous. If the latter assumption is correct (as will be shown later), this allows us to use REA as an epidemiological tool - see subsequent chapters.

CHAPTER 3 Isolation and Identification of Mycoplasma ovipneumoniae from a Flock of Lambs

3.1 Isolation of M. ovipneumoniae from a flock of lambs

3.1.1 Introduction

Several authors (St George et al., 1971; Alley et al., 1971; Carmichael et al., 1971; Friis et al., 1976) claim that M. ovipneumoniae plays an important role (whether as a primary pathogen or as a secondary invader) in the pathogenesis of Chronic, Non-Progressive Pneumonia (CNP). These workers assume that M. ovipneumoniae first colonises the nose and subsequently invades the lungs. CNP has a well-established seasonal peak in New Zealand, in March/April, and this requires that M. ovipneumoniae must (if it is to play a major role in the pathogenesis of CNP) become disseminated in the flock before or during March.

However, no data to validate this has been published, so this section investigates the following:

a) The time of colonisation of the nasal cavity of sheep with M. ovipneumoniae.

b) The persistence of M. ovipneumoniae in the nasal cavity of lambs following colonisation.

c) The prevalence of M. ovipneumoniae in the lungs of lambs at salughter.

Practical considerations necessarily limit the scope of this investigation, so this section reports the isolation and identification of M. ovipneumoniae from the upper respiratory tract of one flock of lambs, taken at 3-week intervals over a period of 6 months; also the isolation and identification of M. ovipneumoniae from the lungs of the lambs at slaughter.

Acknowledgment: This aspect of work was undertaken jointly with Mr G. Ionas who required isolates for further study by serology and PAGE.

3.1.2 Materials and Methods

FM4 Broth and FM4 Agar - See Appendix

Source of lambs

26 Suffolk lambs from a farm on the Manawatu Plains, viz. Massey University Sheep Unit No. 1, were used in this study. These lambs were approximately 6-weeks old at the commencement of this investigation, and, in principle, the same 26 lambs were sampled each month. However, on some occasions up to three of these lambs were not available for technical reasons.

Isolation of *Mycoplasma ovipneumoniae* from nasal cavity

Nasal swabs were broken off into 3ml aliquots of FM4 broth in bijoux bottles. These were then shaken vigorously and transported to the laboratory, where they were shaken again before 0.3ml aliquots were inoculated into 3.0ml aliquots of FM4 broth. The original specimen bottle was discarded.

Inoculated bijoux bottles were incubated at 37°C for 7 days or until growth occurred as indicated by a colour change in the pH indicator in the medium. If no pH change occurred during the 7-day incubation period, 0.3ml of the broth was transferred to 3.0ml of fresh FM4 liquid medium, which was then incubated at 37°C for a further 7 days. If a colour change was still not observed after 7 days, 0.05ml of the medium was spotted onto FM4 agar plates which were incubated at 37°C for 4-5 days. A swab was considered negative if no pH change occurred on the first or second incubation, and no colonies were observed on plates examined using a plate microscope.

If a pH change occurred in liquid FM4 medium, 0.3ml of the culture was transferred to 3.0ml of fresh FM4 broth and incubated at 37°C until a pH change occurred or for 7 days. 0.05ml of the culture was spotted on FM4 agar, the plates were incubated at 37°C for 4 to 5 days and examined for colonies using a plate microscope.

b) Isolation of M. ovipneumoniae from lung tissue

The lambs were slaughtered in April 1981, and the lungs were collected from the abbatoir and placed in individual plastic bags to prevent cross-contamination. Due to hygiene regulations, all lungs were handled by a meat inspector prior to collection. The lungs were labelled and transported to the laboratory where they were examined for pneumonic lesions. Where there were macroscopic lesions, a specimen was cut aseptically from the periphery of the lesion and from within the lung tissue (to avoid contamination from organisms present on the surface of the lung.

Where there were no macroscopic lesions, a specimen was cut from within the tissue of the right apical lobe.

All specimens were cut into small pieces (using sterile scissors) and placed in 3.0ml of FM4 broth, to give an approximate 20% suspension. The lung suspension was shaken at intervals for 15 minutes at room temperature, and after the solid material had settled, 0.3ml of the supernatant was transferred to 3.0ml of FM4 broth. The lung suspension was discarded and the inoculated medium was incubated at 37°C for 7 days or until a pH change - indicating growth - occurred.

If no pH change occurred after 7 days, 0.3ml of broth was transferred to 3.0ml of fresh FM4 medium and incubated at 37°C for a further 7 days, when 0.05ml of the broth was spotted onto FM4 agar and the plates incubated at 37°C for 4 to 5 days. If no pH change occurred during the first or second incubations, and no colonies were observed on the plates (using a plate microscope), the specimen was considered negative.

If a pH change occurred in liquid FM4 medium, 0.3ml of the culture was transferred to 3.0ml of fresh FM4 broth and incubated at 37°C until a pH change occurred or for 7 days, at which time, 0.05ml of the culture was spotted onto FM4 agar. The plates were incubated at 37°C for 4 to 5 days and examined for colonies using a plate microscope.

Isolates, from both nasal swabs and lung specimens, were passaged in FM4 medium - in some cases up to 30 times - as individual colonies at low density on agar were too small to be clearly identified without serial passage *in vitro*. It should be noted that *M. ovipneumoniae* isolates which failed to produce individual colonies at low density showed confluent growth of microcolonies when inoculated at high density.

c) Cloning of isolates

Tenfold serial dilutions of *M. ovipneumoniae* isolates prepared in FM4 broth, and 0.05ml of each dilution was spotted onto FM4 agar. Plates were then incubated at 37°C for 4-5 days when they were examined for the presence of colonies, using a plate microscope. Isolated colonies near the limiting dilution were removed along with a plug of agar, using a sterile pasteur pipette, and transferred to 3.0ml of FM4 broth. The suspension was vigorously agitated and incubated at 37°C until a pH change occurred (3 to 5 days), 1.0ml of culture was then inoculated into 9.0ml of FM4 broth and incubated at 37°C until a pH change to approximately pH 7.0 was observed. Aliquots of 1.5ml were then frozen at -70°C until required.

3.2.3 Results

The isolation of *M. ovipneumoniae* from the nasal cavity over a six month period and from the lungs at slaughter of a flock of 26 Suffolk lambs is shown in Table V.

The cumulative proportion of the 26 lambs from which *M. ovipneumoniae* was recovered at least once from the nasal cavity by 3-weekly swabbing; also the proportion of these lambs from which *M. ovipneumoniae* was recovered from the lungs at slaughter, is shown in Figure 7. It should be noted here that not all mycoplasmas recovered in this study were *M. ovipneumoniae*. Of those few that were not, all were identified serologically as *M. arginini*, but are not further considered in this study.

TABLE V : Isolation of M. ovipneumoniae from a) the nasal cavities of a flock of lambs swabbed at approximately 3-week intervals, and b) from the lungs at slaughter

Swab series	1	2	3	4	5	6	7	8	Lung
Date	7/10 80	3/11 80	25/11 80	16/12 80	13/1 81	17/2 81	19/3 81	6/4 81	8/4 81
Approx. age lambs(days)	45	71	93	114	142	177	207	225	227
Lamb No. M1	-	-	-	-	-	-	+	-	+
M3	-	-	+	+	-	+	+	-	-
M4	-	-	-	-	-	-	+	-	+
M5	-	-	-	-	-	-	-	-	+
M6	-	-	-	-	-	-	-	-	+
M7	-	-	-	-	-	-	-	-	+
M8	-	-	-	-	-	-	+	-	+
M9	-	-	-	-	-	-	-	-	+
M10	-	-	-	-	-	-	+	-	+
M11	-	-	-	-	-	-	+	-	+
M12	-	-	-	+	-	+	+	+	+
M14	-	-	-	+	-	+	+	+	+
M15	-	-	+	-	-	-	ND	ND	ND
M16	-	-	-	-	-	-	+	-	+
M18	-	-	-	-	-	-	+	-	+
M19	-	-	-	-	-	-	+	-	+
M20	-	-	-	-	-	-	-	-	+
M21	-	-	+	+	ND	ND	ND	ND	+
M23	-	-	-	-	ND	ND	ND	ND	ND
M26	-	-	+	-	-	-	-	+	-
M27	-	-	-	-	-	-	+	-	-
M28	-	-	-	+	-	-	+	+	+
M29	-	-	-	-	-	-	-	-	+
M30	-	-	-	-	-	-	-	-	+
M31	-	+	+	-	-	-	+	+	-
M32	-	-	-	-	-	-	+	-	+
	0	1	5	5	0	3	15	5	20

- = M. ovipneumoniae not isolated

ND = not done

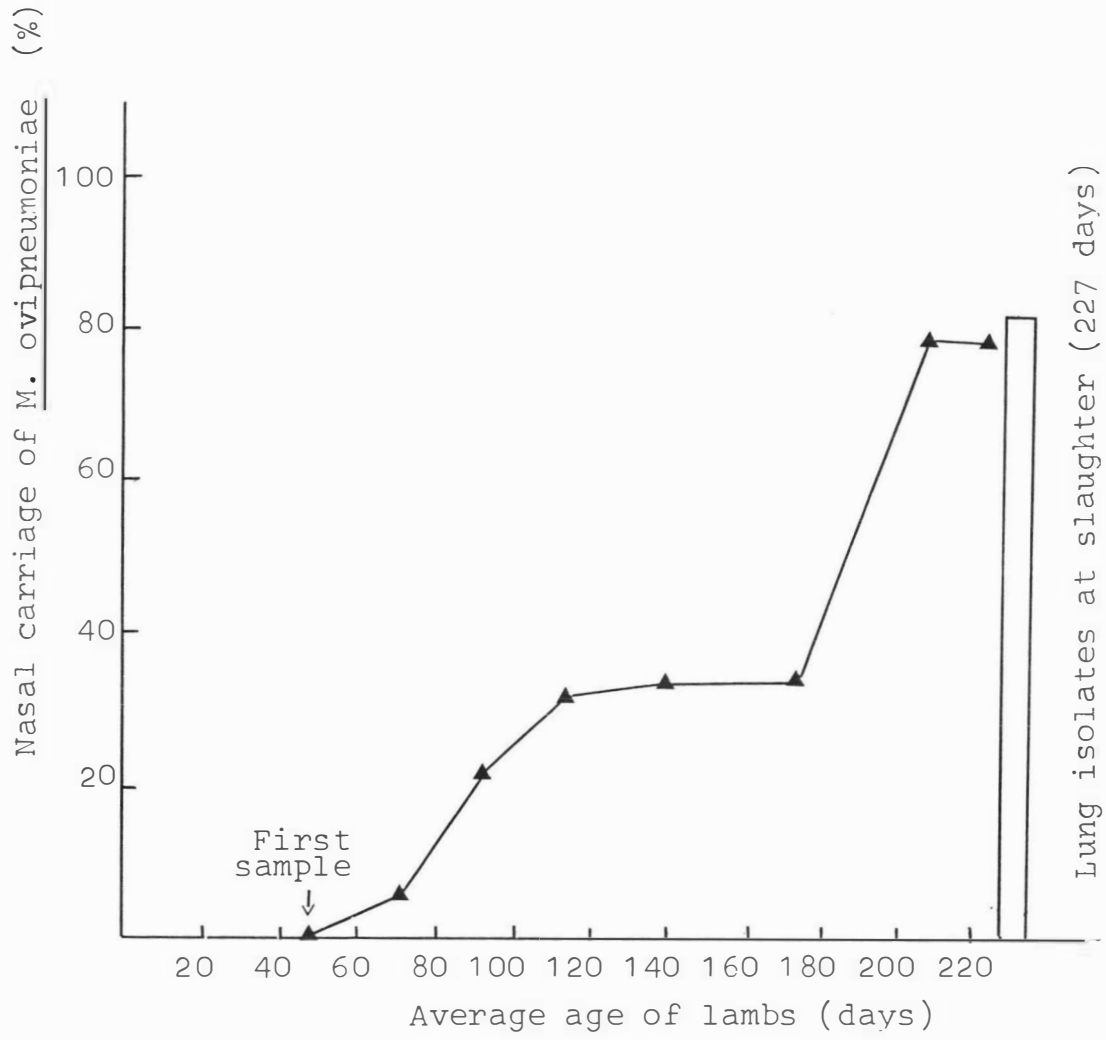

Figure 7 : a) Cumulative proportion of 26 lambs from which M. ovipneumoniae was recovered from the nasal cavity by monthly swabbing.

b) The proportion of these lambs from which M. ovipneumoniae was recovered from the lungs at slaughter.

3.1.4 Discussion

The results of this section will be considered more fully in the General Discussion (Chapter 6). However, several conclusions drawn from Table V and Figure 7 are stated below.

a) Colonisation of the nasal cavity of the lambs in this flock by M. ovipneumoniae, commenced when the lambs were approximately 2 months old.

b) By 6 months the nasal cavity of 90% of the lambs had been colonised at least once by M. ovipneumoniae.

c) This organism does not appear to persist for a long period in the nasal cavity. The longest persistence detected was 48 days.

d) A peak incidence of M. ovipneumoniae in the nasal cavity occurred when the lambs were approximately 6 months old. This decreased markedly when the lambs were examined 3 weeks later, prior to slaughter.

e) The isolation rate of M. ovipneumoniae from the lungs was much higher than that from the nasal cavity (even at its peak). That is, the isolation rate from the lungs at slaughter was 77%, as compared with a peak prevalence of 58% in the nasal cavity.

3.2 Identification of isolates by gel precipitin test

3.2.1 Introduction

Presumptive identification of mycoplasmas isolated from the sheep respiratory tract as M. ovipneumoniae, was based on several observations:

a) All were sensitive to digitonin.
Note: In this test, discs soaked in 2% Digotonin in Ethanol) were applied to lawns of isolated on FM4 agar and unequivocal zones of inhibition were observed.

b) All were glycolytic, i.e. utilised glucose as the major energy source.

c) All gave "vacuolated", centreless colonies on agar.

d) All were isolated from the respiratory tract of sheep.

Previous workers (Clarke, Brown and Alley, 1974) examined many glycolytic mycoplasma isolates from the respiratory tract of sheep in New Zealand and showed, using the Immuno-fluorescence Test, that all were M. ovipneumoniae.

However, it was recognised that different restriction patterns exhibited by different isolates of M. ovipneumoniae (see Ch. 2.6) might be attributed to the existence of different species of mycoplasma among the so-called "M. ovipneumoniae" isolates. Therefore, it was decided that each isolate must be unequivocally identified as M. ovipneumoniae, before any investigation into the restriction pattern was to proceed.

M. ovipneumoniae is antigenically unrelated to other mycoplasma species derived from sheep (Bergey's Manual of Determinative Bacteriology, 8th Edition). Hence, isolates of M. ovipneumoniae can be identified by any convenient serological test. In the present work, the gel precipitin test was chosen.

In this test, a standard antigen (M. ovipneumoniae

strain 5) and antiserum (anti-strain 5) was used, and antigens prepared from isolates were examined for their ability to give precipitation lines of identity with the test antigen.

3.2.2 Materials and Methods

1. Agar for gel precipitin tests

Special Noble Agar	3.0g
NaCl	16.0g

Distilled water to 200ml. Sterilised by autoclaving. Dispensed in 20ml aliquots per 85mm plastic petri dish.

2. Antiserum against M. ovipneumoniae strain 5

Antigen was prepared by propagating M. ovipneumoniae strain 5 in Modified FM4 medium which contains neither swine serum nor animal peptone (see Appendix), centrifuged, washed twice with sterile PBS (see Appendix), and resuspended in 0.85% saline to give an approximate 10% solution.

0.5ml of the antigen with an equal amount of Freund's Complete Adjuvant, was injected intramuscularly into rabbits. After one month, intramuscular booster inoculations of 0.5ml of antigen were given at 14 day intervals. Rabbits were bled at intervals and the titre of antibody estimated by gel precipitin tests. Once the titre of 1:16 or greater was reached, further serial bleedings at intervals were pooled, heated at 56°C for 30 minutes, and stored at -20°C.

3. Source of M. ovipneumoniae strain 5

M. ovipneumoniae strain 5 was isolated from a New Zealand sheep by Dr J.K. Clarke in 1973, and has been subsequently cloned a minimum of 10 times.

Gel Precipitin Tests

Each isolate, (propagated in FM4 medium, centrifuged and resuspended in 0.5ml of Tris-EDTA solution - See Chapter 2.4), was diluted 1:2 with Tris EDTA solution; sonicated for 2 x 10 seconds, and Triton X-100 was added to a final concentration of approximately 1%.

Wells were cut using a template. The central well was filled with 50ul of antiserum prepared against M. ovipneumoniae strain 5, and the six peripheral wells were filled with 50ul of different antigen suspensions including one well with M. ovipneumoniae strain 5 antigen as a positive control.

The plate was left overnight at room temperature and examined through a plate microscope. Each batch of antiserum was tested with FM4 medium as a control.

3.2.3 Results

A typical result with 5 isolates plus M. ovipneumoniae strain 5 is shown in Figure 8. All isolates, obtained for this investigation, were identified as M. ovipneumoniae using a gel precipitin test. That is, all gave lines of identity with a standard test system - M. ovipneumoniae strain 5 as test antigen and antiserum raised against M. ovipneumoniae strain 5 as test antiserum.

None of the batches of antiserum gave a line of precipitation when tested against FM4 medium.

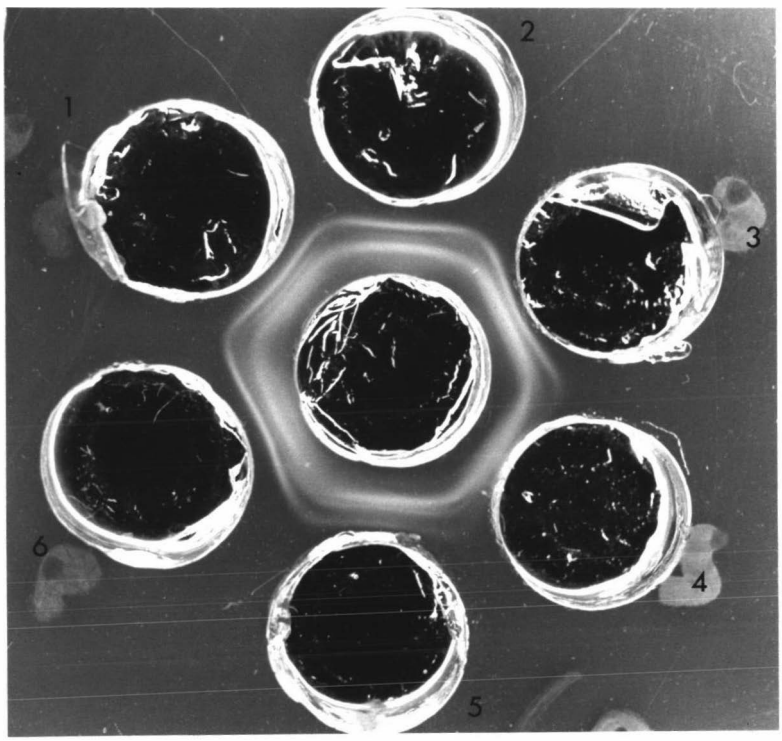

Figure 8 : The Gel Precipitin Test for the identification of isolates. The central well contains antiserum to M. ovipneumoniae strain 5. Well 1 contains M. ovipneumoniae strain 5 antigen; wells 2-6 contain antigens from each of 5 isolates. Note: all isolates showed lines of identity with each other and with the standard antigen (i.e. strain 5).

3.2.4 Conclusion

All glycolytic isolates were confirmed to be *M. ovipneumoniae* by the gel precipitin test.

Note: Isolates that gave centred colonies on agar, produced an alkaline change in medium supplement with 1% arginine, were shown to be *M. arginini* by a gel precipitin test, using a standard strain of *M. arginini* as the immunising and test antigen. This organism was not investigated further in the present work.

CHAPTER 4 Examination of Isolates From a Flock of Lambs By Restriction Endonuclease Analysis

Note: Designation of isolates

Isolates were recovered from lambs on a Massey farm, designated 'M'. The sheep were numbered 1 to 32, but for technical reasons, several were not available and a total of 26 were swabbed.

The isolates were designated by the lamb number, and the lambs were swabbed 8 times at approximately 3-week intervals. The final number indicates the swabbing series number, an 'L' for an isolate obtained from the lungs. For example, an isolate from lamb M14, at the seventh swabbing is named 'M14-7'.

With reference to Table V, P.62, the swabbing series number gives the time of swabbing, and approximate age of lambs at each swabbing.

4.1 Comparison of REA patterns of four isolates following single-cloning and multiple-cloning

4.1.1 Introduction

Work described in subsequent sections requires that the mycoplasma cultures are derived from a single clone. Mycoplasmas were isolated in liquid culture, and passaged serially to obtain more rapid growth. Serial, ten-fold dilutions of such cultures were then spotted on agar, and a single, isolated colony was selected from the limiting dilution. A culture derived in this way would be a clone, unless the original sheep was colonised by 2 strains and both were represented in the final selected colony.

The possibility of a mixed culture, however small, can be further decreased by serial cloning. Unfortunately mycoplasmas grow much more slowly than bacteria, so multiple cloning is exceptionally laborious, and would limit the number of isolates which could be examined.

It is reasonable to suppose that an isolation technique involving a single cloning would be adequate. To test this assumption, four randomly selected singly-cloned isolates were re-cloned a further two times. The REA patterns, before and after serial cloning, are examined and compared in this section.

4.1.2 Materials and Methods

FM4 Medium - See Appendix

FM4 Agar - See Appendix

Source of isolates

Lane Number	Isolate	Number of Clonings	Source
1	(Lambda)	-	-
2	M8-7	1	Nasal cavity
3	M8-7	3	Nasal cavity
4	M12-6	1	Nasal cavity
5	M12-6	3	Nasal cavity
6	M14-L	1	Lungs
7	M14-L	3	Lungs
8	M21-4	1	Nasal cavity
9	M21-4	3	Nasal cavity

TABLE VI : Source of M. ovipneumoniae isolates shown in Fig. 9.

Serial cloning of isolates

All four isolates were cloned once as described in Chapter 3.1.2 (C). A pool of each cloned isolate was prepared by propagating each in FM4 medium, and 1.5ml aliquots stored at -70°C until required.

Serial ten-fold dilutions of an aliquot was made in 3.0ml FM4 broth, 0.05ml was spotted onto FM4 agar, and incubated at 37°C for 4 days. A single colony, selected from a plate at the limiting dilution, was then transferred to FM4 broth, and incubated at 37°C. When a colour change in

the medium (indicating growth) occurred, the cloning procedure was repeated, resulting in an isolate that had been cloned 3 times. A pool of each multiply-cloned isolate was prepared, and 1.5ml aliquots stored at -70°C.

Restriction Endonuclease Analysis of singly- and multiply-cloned isolates

Aliquots of each of the singly- and multiply-cloned isolates were thawed, and inoculated into liquid FN4 medium, and incubated at 37°C. When sufficient growth had occurred, cells were collected for DNA extraction and Restriction Endonuclease Analysis (see Ch. 2.4).

4.1.3 Results

The DNA from each isolate, singly- and multiply-cloned, was extracted, digested and run in adjacent lanes of a 0.7% agarose gel. The result is shown in Figure 9. No changes were observed in the restriction patterns of 4 singly-cloned isolates following triple-cloning.

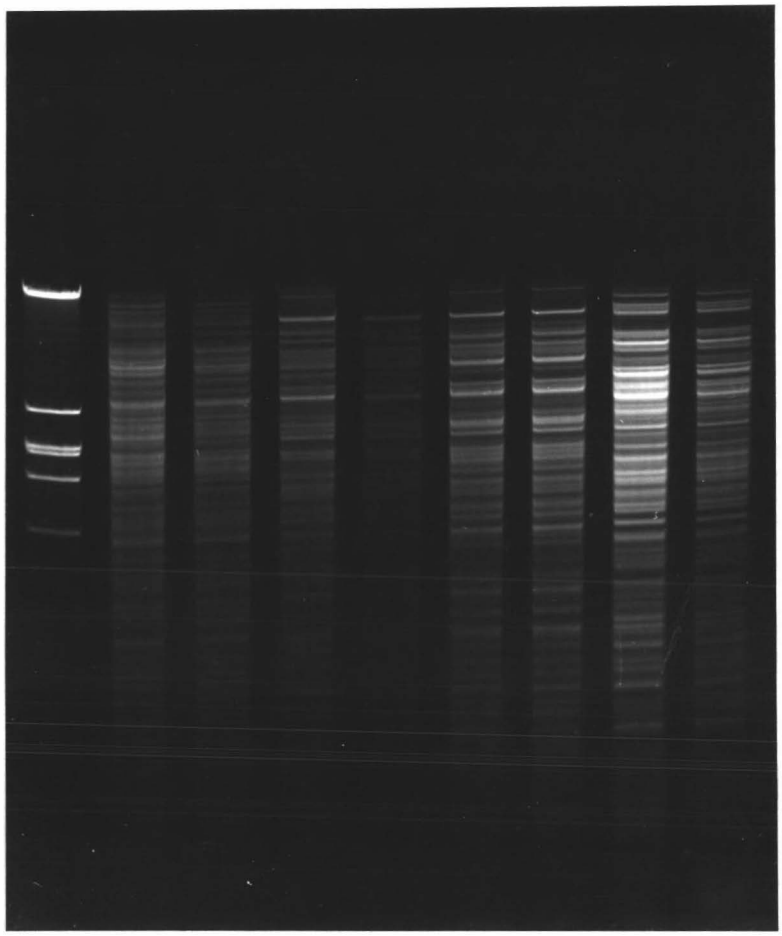

Figure 9 : REA of 4 isolates singly -(C1) and triply - (C3)-cloned. Lane 1: Lambda; Lanes 2 and 3: M8-7 (C1 and C3); Lanes 4 and 5: M12-6 (C1 and C3); Lanes 6 and 7: M14-L (C1 and C3); Lanes 8 and 9: M21-4:(C1 and C3). No differences were detected between singly- and triply-cloned isolates.

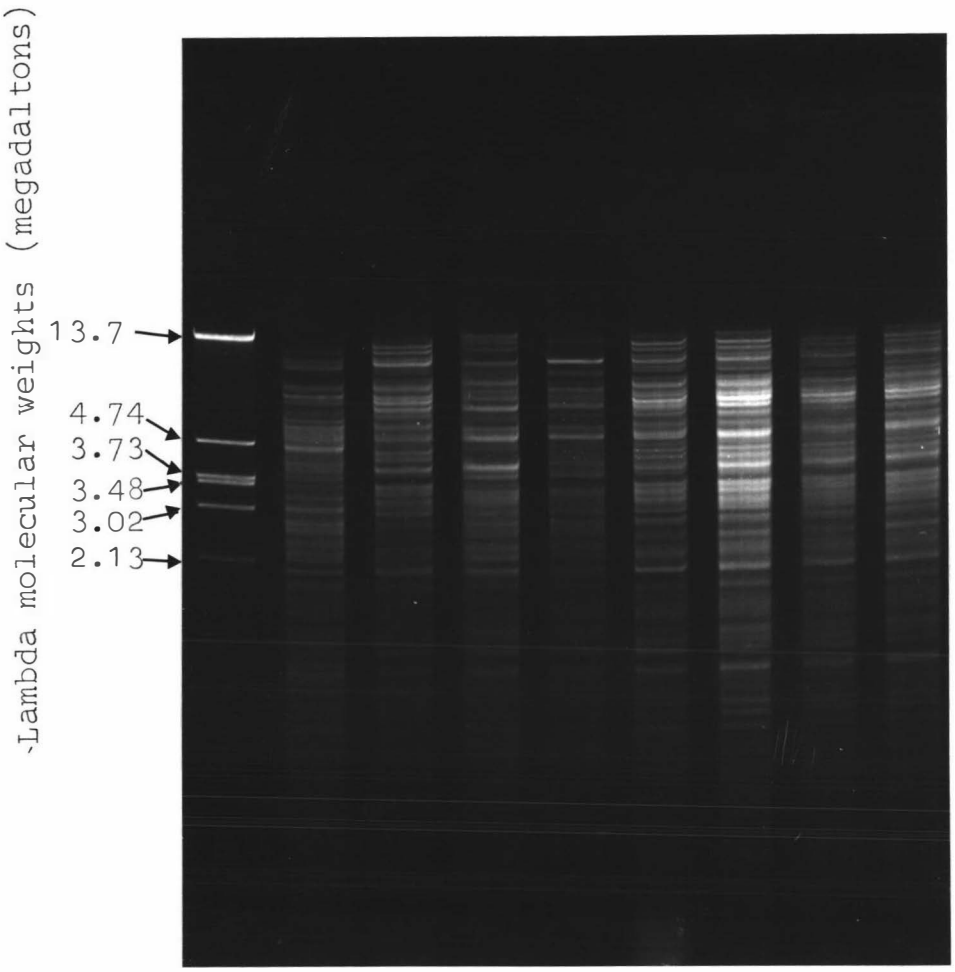

Figure 10 : REA of 8 nasal isolates from a flock of lambs at the 7th swabbing (i.e. age of lambs = 207 days). Lane 1: Lambda; Lane 2:M10-7; Lane 3: M31-7; Lane 4: M12-7; Lane 5: M14-7; Lane 6: M4-7; Lane 7: M8-7; Lane 8: M18-7; Lane 9: M19-7.

Patterns fell into 3 groups:

Group 1: Lanes with closely similar, if not identical, patterns: Lanes 3, 6, 7, 8 and 9

Group 2: Lanes with similar, but not identical, patterns: Lanes 4 and 5.

Group 3: Lane 2

Little or no similarity is evident between the patterns of different groups.

4.1.4 Conclusion

It was concluded that, in the case of these 4 isolates, a single-cloning produced a homogeneous culture. In practice, the possibility of mixed cultures being derived from a single colony is small, so it was decided to proceed with the project on the basis that singlv-cloned isolates could be examined.

4.2 REA of nasal isolates obtained from a flock of lambs at one time

4.2.1 Introduction

A preliminary investigation into restriction patterns of isolates of M. ovipneumoniae, obtained from widely differing sources indicated a high degree of heterogeneity (Ch. 2.6). However, within a limited number of passages in vitro (Ch. 4.1), the restriction pattern of an isolate remains substantially unchanged, and presumably, this stability can be extrapolated to in vivo conditions. This implies that it is unlikely that every isolate is different (with respect to restriction pattern), however, the degree of heterogeneity, if any, within a flock of sheep, remains to be established.

This section investigates whether more than one strain, as defined by its restriction pattern, can exist in one flock at any one time.

For this study, a time was chosen when the nasal cavities of many lambs were colonised by M. ovipneumoniae. That is, at the seventh swabbing (see Ch. 3.1.3, Table V), when 23 lambs were swabbed and M. ovipneumoniae was isolated from 15 of them, one clone was taken from each isolate and examined by REA.

4.2.2 Materials and Methods

Isolation of M. ovipneumoniae from the nasal cavity of lambs

See Ch. 3.1.2

Origin of isolates

Lane Number	Isolate
1	(Lambda)
2	M10-7
3	M31-7
4	M12-7
5	M14-7
6	M4-7
7	M8-7
8	M18-7
9	M19-7

Table VII : Eight isolates from the seventh swabbing as shown in Figure 10

DNA Extraction and Restriction Enzyme Analysis

See Ch. 2.4

4.2.3 Results

15 of the 23 lambs, that were swabbed when they were approximately 207 days old, yielded M. ovipneumoniae from the nasal cavity. REA revealed 3 different patterns: 12 isolates showed 1 pattern; 2 isolates showed a second distinct pattern; and 1 isolate showed a unique pattern.

Figure 10 shows the restriction patterns exhibited by 8 of the 15 isolates.

4.2.4 Discussion

Of the 8 isolates shown in Figure 10, isolates in lanes 3, 6, 7, 8 and 9 (i.e. M31-7; M4-7; M8-7; M18-7; M19-7) show the same pattern, although detailed comparison reveals a minor difference in the region corresponding to approximately 4.74 megadaltons, of M31-7.

The isolates in lanes 4 and 5 (i.e. M12-7 and M14-7 respectively) show the same basic pattern but minor differences occur in 3 separate regions of the pattern - that is, those regions corresponding to approximately 13, 10 and 4 megadaltons. Despite these minor differences the patterns exhibited by M12-7 and M14-7 were considered to be basically the same.

The isolate in lane 2 (i.e. M10-7), however, shows a pattern that differs markedly from the other 7 isolates.

While the 3 different patterns shown in Fig. 10 clearly differ in some respects, it is not implied that they differ in all respects. Thus, a comparison of faster-moving bands, i.e. lower molecular weight bands, shows a considerable degree of similarity among all 3 patterns. It is interesting to compare Fig. 10 above (i.e. differing patterns from the same flock) with Fig. 6 (p.56) which shows differing patterns from isolates obtained from separate flocks. Clearly, the degree of variability (as might be expected) is less in those isolates obtained from the same flock at the same time, than that shown by isolates obtained from different flocks at different times. The significance of this is considered later in the General Discussion.

4.3 REA of serial nasal isolates

4.3.1 Introduction

It was established in the previous section, that some heterogeneity (with respect to restriction pattern) occurs between isolates of M. ovipneumoniae obtained from different lambs of a flock at one time.

This section investigates whether isolates obtained from individual lambs are homogeneous or heterogeneous.

For this study, several lambs that yielded more than one isolate from the nasal cavity when serially swabbed at approximately 3-week intervals were noted, and the isolates from these animals were examined by REA.

4.3.2 Materials and Methods

Isolation and cloning of M. ovipneumoniae from nasal cavities

See Chapter 3.1.2

Origin of isolates

Lane No.	Isolate	Swabbing No.	Approximate Age of lambs (days)
1	(Lambda)	-	-
2	M28	4	114
3	M28	7	207
4	M28	8	225
5	M21	3	93
6	M21	4	114
7	M14	6	177
8	M14	7	207
9	M14	8	225

Table VIII : 8 serial nasal isolates from 3 lambs as shown in Fig. 11

DNA Extraction and REA

See Chapter 2.4.

4.3.3 Results

Several serial nasal isolates of M. ovipneumoniae from 3 lambs were cloned, propagated in FM4 medium, the DNA extracted and REA performed on each. The isolates were grouped according to which lamb they were isolated from, and the restriction patterns compared.

Figure 11 shows the results of comparing serial nasal isolates obtained from 3 lambs: 2 isolates from Lamb M21, and 3 from each from lambs M28 and M14.

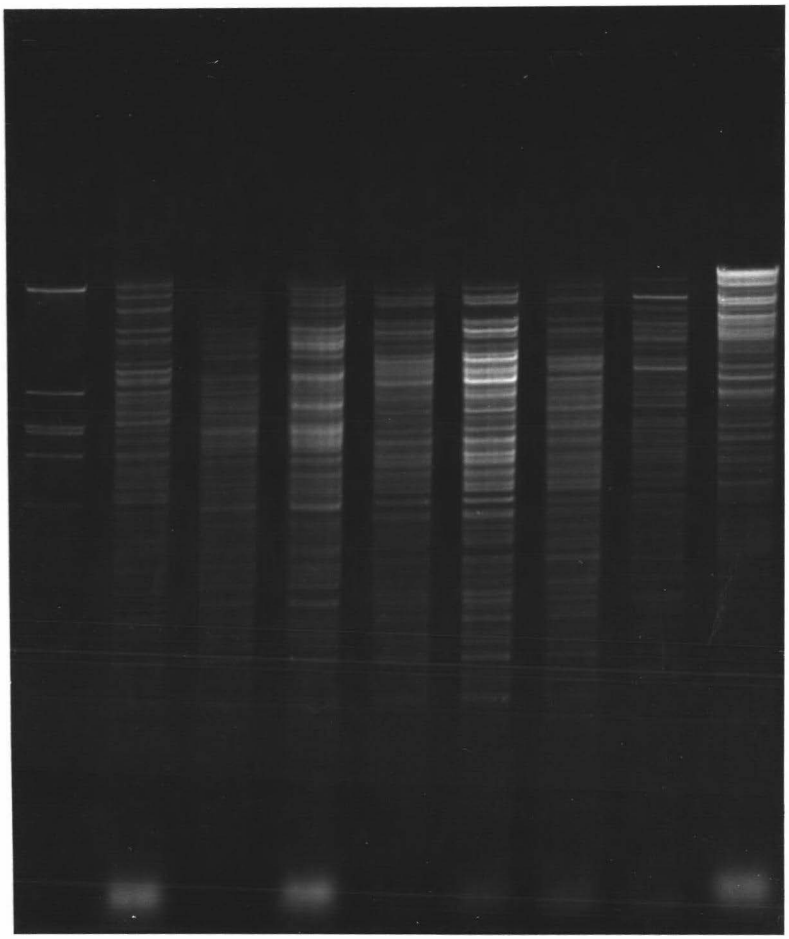

Figure 11 : REA of 8 serial nasal isolates obtained from 3 lambs of one flock. Lane 1: Lambda; Lane 2: M28-4; Lane 3: M28-7; Lane 4: M28-8; Lane 5: M21-3; Lane 6: M21-4; Lane 7: M14-4; Lane 8: M14-7; Lane 9: M14-8. Patterns fell into 5 groups:

Groups 1,2 and 3: Lanes with closely similar, if not identical, patterns: Lanes 2 and 7; Lanes 3 and 4; Lanes 5 and 6

Groups 4 and 5: Lane 8; Lane 9

Little or no similarity is evident between the patterns of different groups.

4.3.4 Discussion

Figure 11 can be considered in 3 sections:

1) Lamb M28. The isolate from the 4th swabbing (lane 2) differs from the isolate obtained from the 7th swabbing (lane 3). However, the isolate obtained from the 8th swabbing (lane 4) is the same as the 7th swabbing isolate.

2) Lamb M21. Isolates from the 3rd and 4th swabbings (lanes 5 and 6, respectively) are identical.

3) Lamb M14. Isolates from the 6th, 7th and 8th swabbings (lanes 7, 8 and 9 respectively) all differ from each other.

Note: M28-4 (lane 2) and M14-6 (lane 7) show the same pattern.

Therefore, although serial nasal isolates from one lamb can be homogeneous, in many cases the isolates exhibit heterogeneity.

For this investigation, a distinct restriction pattern is assumed to correspond to a strain of M. ovipneumoniae.

It is concluded that the colonisation of the nasal cavity of lambs, by a particular strain of M. ovipneumoniae, is usually only transitory. That is, one strain persists for a short time only (e.g. 21 days in the case of lamb M21) and may be supplanted by another strain). The significance of this will be discussed later in the General Discussion.

4.4 REA of lung isolates

4.4.1 Introduction

The previous 2 sections provided information on the colonisation of the nasal cavity by strains of M. ovipneumoniae. However, with respect to CNP, an examination of isolates from the lungs is of particular interest.

The isolation technique (which used post-mortem material) precluded serial isolations which limited the investigation to those organisms present in the lungs at the time of slaughter.

This section investigates whether isolates obtained from the lungs of a flock of lambs slaughtered simultaneously, exhibit one, or more than one, restriction pattern.

4.4.2 Materials and Methods

Isolation and cloning of M. ovipneumoniae from lungs

See Chapter 3.1.2.

Origin of isolates

Lane Number	Isolate
1	(Lambda)
2	M14
3	M5
4	M21
5	M7
6	M9
7	M20
8	M29
9	M12

Table IX : Lung isolates as shown in Fig. 12

DNA extraction and REA

See Chapter 2.4.

4.4.3 Results

M. ovipneumoniae was isolated from 20 lungs out of a total of 24 tested. All 20 isolates were cloned, propagated in FM4 medium, the DNA extracted and REA performed on each.

Three different patterns were seen. 18 isolates showed 1 of these patterns, the other 2 isolates gave unique patterns.

Figure 12 shows the restriction patterns exhibited by 8 of the 20 lung isolates studied.

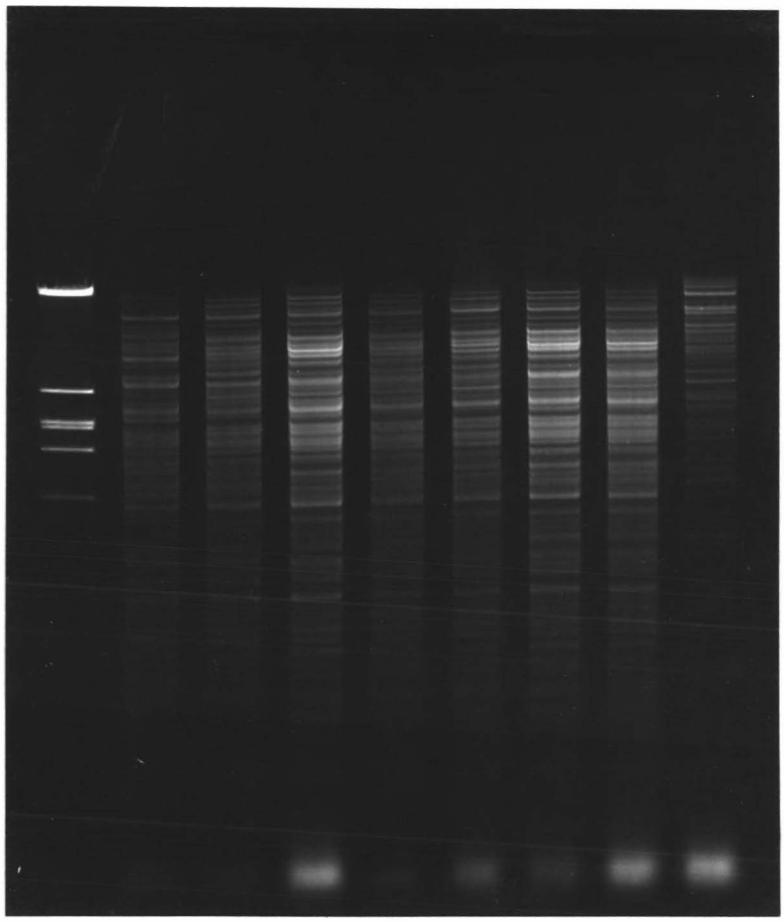

Figure 12 : REA of 8 lung isolates from a flock of lambs at slaughter. Lane 1: Lambda; Lane 2: M14-L; Lane 3: M5-L; Lane 4: M21-L; Lane 5: M7-L; Lane 6: M9-L; Lane 7: M20-L; Lane 8: M29-L; Lane 9: M12-L.
Patterns fell into 3 groups:
Group 1: Lanes with closely similar, if not identical, patterns: Lanes 3, 4, 5, 6, 7 and 8
Groups 2 and 3: Lane 2; Lane 9.
Little or no similarity is evident between the patterns of different groups.

4.4.4 Conclusion

Isolates in lanes 3, 4, 5, 6, 7 and 8 (Fig. 12) all show the same restriction pattern (albeit with minor 1 or 2 band differences), while isolates in lanes 2 and 9 are different from each other as well as the previous 6.

It is concluded, that the lung isolates (in this limited study), were less heterogeneous than the nasal isolates.

4.5 Comparison of nasal and lung isolates by REA

4.5.1 Introduction

Two potential sources (not mutually exclusive) of M. ovipneumoniae in the lung of sheep exist. They are:

1. The strain (or strains) are transferred directly lung to lung.
2. The pneumonic strains could represent nasal strains invading the lower respiratory tract.

If the former is true, then the nasal and lung isolates from an individual animal should differ, and if the latter is correct, then they should be identical.

This section compares nasal and lung isolates from individual sheep, by REA.

4.5.2 Materials and Methods

Isolation and cloning of M. ovipneumoniae

See Ch. 3.1.2

Origin of isolates

Lane No.	Isolate	Source	Swabbing No.	Approx. age of lambs (days)
1	(Lambda)	-	-	-
2	M28	Nasal cavity	4	114
3	M28	Nasal cavity	7	207
4	M28	Lungs	-	227
5	M14	Nasal cavity	7	207
6	M14	Nasal cavity	8	225
7	M14	Lungs	-	227
8	M12	Nasal cavity	7	207
9	M12	Lungs	-	227

Table X : Eight nasal and lung isolates as shown in Fig. 13

DNA extraction and REA

See Ch. 2.4.

4.5.3 Results

Several nasal isolates plus the corresponding lung isolate of *M. ovipneumoniae* from 3 lambs, were cloned, propagated in FM4 medium, the DNA extracted and REA performed on each. The isolates were grouped according to which lamb they were isolated from, and the restriction patterns compared.

Figure 13 shows the results obtained with 8 nasal and lung isolates from 3 lambs: 3 isolates each from lambs M14 and M28, and 2 isolates from lamb M12.

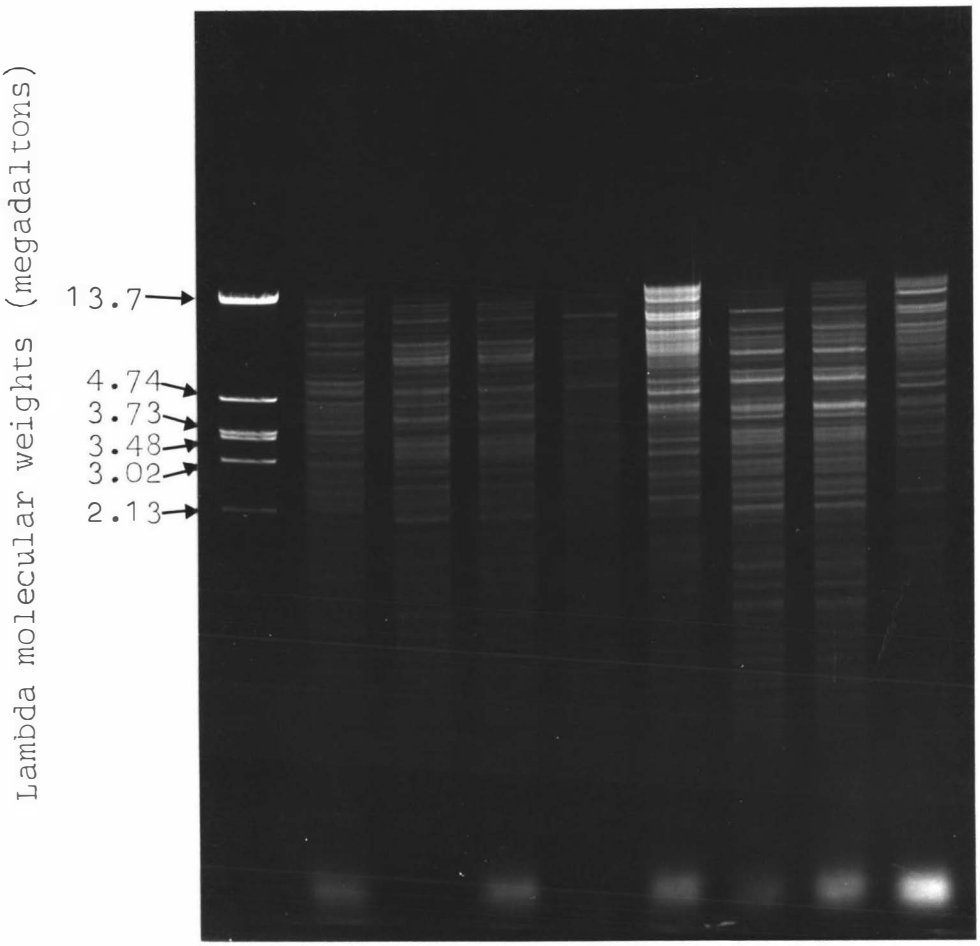

Figure 13 : REA of 8 nasal and lung isolates obtained from 3 lambs of one flock. Lane 1: Lambda; Lane 2: M28-4; Lane 3: M28-7; Lane 4: M28-L; Lane 5: M14-7; Lane 6: M14-8; Lane 7:M14-L; Lane 8: M12-7; Lane 9: M12-L. Patterns fell into 4 groups:
Groups 1 and 2: Lanes with closely similar, if not identical, patterns: Lanes 3 and 4; Lanes 6 and 9
Group 3: Lanes with similar but not identical patterns: Lanes 5, 7 and 8.
Group 4: Lane 2.
Little or no similarity is evident between the patterns of different groups.

4.5.4 Discussion

Figure 13 can be considered in 3 sections:

1. Lamb M28. The isolate from the 4th swabbing (lane 2) differs from that obtained from the 7th swabbing (lane 3). However, the lung isolate (lane 4) is the same as the isolate from the 7th swabbing.

2. Lamb M14. Isolates from the 7th swabbing (lane 5) differ from that obtained, 3 weeks later, at the 8th swabbing (lane 6). However, the lung isolate (lane 7) is identical to the isolate obtained from the 7th swabbing.

3. Lamb M12. Isolates from the 7th swabbing and the lungs (lanes 8 and 9 respectively) are different.

Note: The lung isolate from lamb M12 (lane 6) is identical to the nasal isolate from lamb M14 obtained from the 8th swabbing (lane 9).

It has been noted previously (see Ch. 4.2.4), that while isolates may show the same overall pattern, minor differences between the patterns may occur. This can also be seen in Figure 13, where the nasal isolate from the 7th swabbing of lamb M12 (Fig 13, lane 8) is extremely similar to the isolates from the nasal cavity at the 7th swabbing, and the lungs of lamb M14 (Fig. 13, lanes 5 and 7). That is, the pattern of isolate M12-7 appears to have 2 extra bands in the region of the pattern corresponding to approximately 13 megadaltons, but appears to lack a band at approximately 4.5 megadaltons, when compared with the pattern of M14-L. The pattern shown by isolate M14-7 has already been shown to exhibit minor differences in pattern when compared with M12-7 (see Ch. 4.2.4). Notwithstanding these minor differences, the patterns exhibited by isolates M12-7, M14-7 and M14-L were considered to be basically the same.

It can be concluded that some nasal isolates (and probably all of them), have the ability to invade the lower respiratory tract.

4.6 REA of isolates of M. ovipneumoniae obtained from a flock of lambs: consolidated results

4.6.1 Introduction

The previous 4 sections have considered the following relationships separately:

1. Nasal isolates taken from different lambs of a flock at one time.

2. Nasal isolates taken from the same lamb at different times.

3. Nasal and lung isolates taken from the same lamb.

However, an overall view is required in order to gain information as to the distribution, prevalence and possible spread of particular strains of M. ovipneumoniae within a flock of lambs.

For this purpose, isolates were grouped according to their restriction pattern, and each group was labelled with a number to differentiate it from all other groups.

4.6.2 Materials and Methods

Origin of isolates: their isolation and cloning

See Ch. 3.1.2

DNA Extraction and REA

See Ch. 2.4

Comparison of restriction patterns

Photographs of the restriction patterns shown by all lung and nasal isolates were obtained, then 'contact' prints taken from each. These contact prints were cut into strips - each of which contained the restriction pattern of one isolate, and was labelled on the reverse side. Each strip was subsequently aligned against every other strip in order

to ascertain whether they showed the same, or a different pattern.

Thus, isolates were placed in groups, within which, all members showed the same restriction pattern and this pattern was different from that shown by all other groups.

In this way, isolates were classified according to which restriction pattern they exhibited, and each group was assigned a number, from 1 to 7. If there was any doubt as to the placing of an isolate within any particular group, another gel was run with the isolate in question, and a member of the group, in adjacent lanes.

Occasionally, within a group, there occurred a pattern that was fundamentally the same as those of other members of the group, but nonetheless showed minor differences. These differences occurred in only one or two small regions of the pattern, and involved only 1 or 2 bands. Hence, the isolate was still assigned to the group, but a lower-case letter was placed after the group number, e.g. 3a.

4.6.3 Results

The results are shown in Table XI.

Note: This table represents an extension of Table V (page62) but the restriction classification number of each isolate has now been added.

Swabbing series	1	2	3	4	5	6	7	8	Lungs
Date	7/10 80	3/11 80	25/11 80	16/12 80	13/1 81	17/2 81	19/3 81	6/4 81	8/4 81
Approximate age of lambs (days)	45	71	93	114	142	177	207	225	227
Lamb No. M1	–	–	–	–	–	–	4a	–	4
M3	–	–	5	1	–	1a	4a	–	–
M4	–	–	–	–	–	–	4	–	4a
M5	–	–	–	–	–	–	–	–	4a
M6	–	–	–	–	–	–	–	–	4
M7	–	–	–	–	–	–	–	–	4a
M8	–	–	–	–	–	–	4	–	4a
M9	–	–	–	–	–	–	–	–	4a
M10	–	–	–	–	–	–	2	–	4
M11	–	–	–	–	–	–	4a	–	4a
M12	–	–	–	6	–	3	3a	3	7
M14	–	–	–	1a	–	1	3b	7	3
M15	–	–	2	–	–	–	ND	ND	ND
M16	–	–	–	–	–	–	4	–	4
M18	–	–	–	–	–	–	4	–	4
M19	–	–	–	–	–	–	4	–	4
M20	–	–	–	–	–	–	–	–	4
M21	–	–	2	2	ND	ND	ND	ND	4
M25	–	–	–	–	ND	ND	ND	ND	ND
M26	–	–	2	–	–	–	–	4a	–
M27	–	–	–	–	–	–	4	–	–
M28	–	–	–	1	–	–	4	4	4
M29	–	–	–	–	–	–	–	–	4
M30	–	–	–	–	–	–	–	–	4a
M31	–	1	1	–	–	–	4a	4	–
M32	–	–	–	–	–	–	4	–	4

ND = Not done

– = *M. ovipneumoniae* not isolated

Table XI : Classification of *M. ovipneumoniae* from a flock of lambs a) from the nasal cavities swabbed at 3-week intervals, and b) from the lungs at slaughter. Numbers indicate the REA group to which each isolate belongs.

4.6.4 Discussion

The conclusions from this table, some of which were drawn in earlier sections (4.2 - 4.5) can be summarised as follows.

1. Most individual strains of M. ovipneumoniae were isolated from the nasal cavity of a lamb only once, i.e. did not persist for a further 3 weeks. Some were detected twice (i.e. persisted for at least 3 weeks), and only one was detected thrice, i.e. persisted for not less than 6 weeks.

2. Two strains (strains 1 and 2) predominated in the flock up to the 6th swabbing, i.e. when the lambs were approximately 177 days old; they were then rapidly replaced by another predominant strain (strain 4).

3. Isolates from the lung (taken at one time only) were relatively homogeneous, and in the main, corresponded to those strains present in the nost 20 days earlier (i.e. at the 7th swabbing).

The significance of these conclusions as they relate to the epidemiology of M. ovipneumoniae within a flock of lambs, and the pathogenesis of CNP, will be more fully discussed in the General Discussion.

4.7 REA of multiple isolates of M. ovipneumoniae obtained from one pneumonic lung

4.7.1 Introduction

Practical considerations led us to examine one isolate from a sheep at a time. However, the marked heterogeneity of patterns obtained raised the possibility that multiple isolates obtained from one lamb at one time, might not be identical. This question is most easily answered in circumstances where multiple isolates are assured. Hence, a sample from a pneumonic lung selected randomly at slaughter from the local abbatoir, was divided into 10, an isolate was made from each, and REA patterns compared.

4.7.2 Materials and Methods

Multiple isolation of M. ovipneumoniae from lung tissue

A lung showing definite pneumonic lesions was obtained from the local abbatoir, and a specimen was cut aseptically from the periphery of the lesion. This specimen was divided into 10 segments and M. ovipneumoniae was isolated from each, using standard isolation and cloning techniques - see Chapter 3.1.2.

DNA extraction and REA

See Chapter 2.4.

4.7.3 Results

Ten cloned isolates obtained from one pneumonic lung, were propagated in FM4 medium, the DNA extracted and REA performed on each.

Figure 14 shows the restriction patterns exhibited by all 10 isolates.

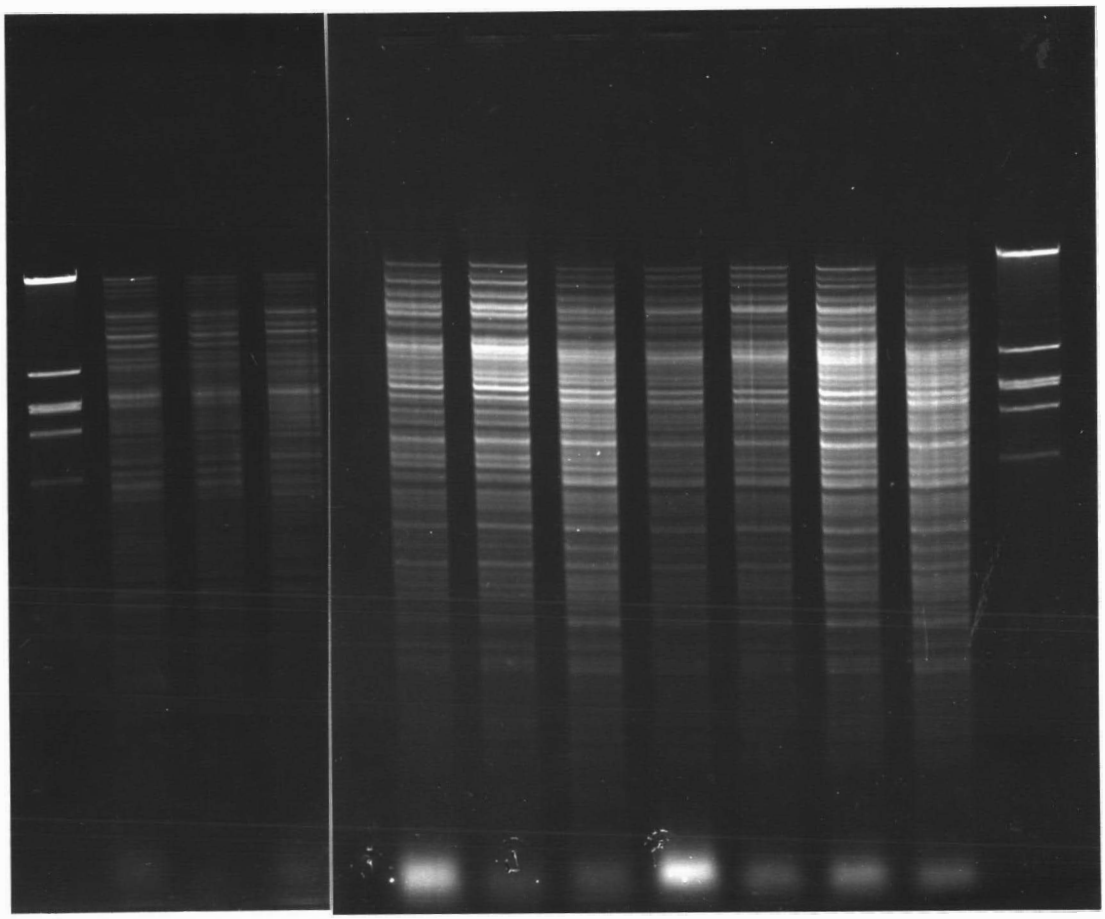

Figure 14 : REA of 10 isolates of M. ovipneumoniae (a-j) obtained from one pneumonic lung (103).
Lanes 1 and 12: Lambda; Lanes 2 to 11: 103 a-j.
Patterns fell into 2 groups:
Groups 1 and 2: Lanes with closely similar, if not identical patterns: Lanes 2, 3 and 4; Lanes 5, 6, 7, 8, 9, 10 and 11.
Little or no similarity is evident between the patterns of different groups.

4.7.4 Conclusion

Two basic patterns are exhibited by the 10 isolates shown in Fig. 14, i.e. 7 of one pattern and 3 of the other.

Note: Within the 2 groups minor variations involving 1 or 2 bands do occur.

It is concluded that more than one strain can be present in the lungs of one sheep. The significance of this will be considered in the General Discussion.

CHAPTER 5 An Investigation into the source of heterogeneity of strains of M. ovipneumoniae

5.1 Effect on restriction patterns of M. ovipneumoniae of passage in vitro

5.1.1 Introduction

The heterogeneity observed among the restriction patterns of strains of M. ovipneumoniae in this study was surprising in view of the results obtained by other workers with Leptospira interrogans serovar hardjo (Marshall et al., 1981) and with Mycoplasma hyorhinus (Darai et al., 1981). These workers reported a high degree of homogeneity among strains of these organisms obtained from widely differing sources. Therefore, it was decided to investigate the possible source of the heterogeneity observed with strains of M. ovipneumoniae.

Three possibilities exist:

1. A large number of stable strains may be independently maintained in the environment. That is, every isolate that shows a different restriction pattern, represents a different strain that remains relatively unchanged over a long period of time.
2. The organism may undergo alterations (e.g. a high mutation rate) or rearrangement (e.g. due to transposons) in its genetic material.
3. Genetic interaction between strains, e.g. by transduction, conjugation, plasmids, etc.

To investigate the long-term stability of strains of M. ovipneumoniae one cloned strain was passaged in vitro, and several sub-clones derived from the culture before and after passage were examined by REA.

In another experiment, the cloned culture of M. ovipneumoniae was passaged serially in the presence of sub-inhibitory concentrations of a specific antiserum. Sub-clones were re-isolated following passage and examined by REA.

5.1.2 Materials and Methods

FM4 Broth and FM4 Agar - See Appendix

Cloning of M. ovipneumoniae strain 5

Previous workers in this laboratory had cloned M. ovipneumoniae strain 5 a minimum of 10 times, and immediately prior to this investigation, it was cloned once more.

Before passage in vitro, 8 clones were obtained from the multiply-cloned M. ovipneumoniae strain 5, by standard methods (see Ch. 3.1.2). These clones were propagated in FM4 broth and frozen in 1.5ml aliquots, while still in the exponential phase, at -70°C.

Passage of M. ovipneumoniae in FM4 broth

M. ovipneumoniae strain 5 was serially passaged by transferring 0.3ml of a growing culture into 3.0ml of fresh FM4 broth, and incubated at 37°C, until a colour change (indicating growth) occurred. This was repeated until the organism had undergone 20 serial passages.

At each passage, 1.5ml of the culture was frozen at -70°C.

After 20 serial passages, 8 clones were taken by standard methods (see Ch. 3.1.2), these clones were propagated in FM4 broth, and while still in the exponential phase, were frozen at -70°C.

Passage of M. ovipneumoniae in the presence of sub-inhibitory concentrations of antiserum

Source of antiserum - See Ch. 3.2.

M. ovipneumoniae strain 5 was passaged in the presence of sub-inhibitory concentrations of antiserum as follows:

Serial 2-fold dilutions of antiserum were made in 50ul of FM4 medium in 8 wells of a sterile disposable plastic microtitre plate. A culture of M. ovipneumoniae strain 5 was diluted 1:2.5 in FM4 broth, and 50ul of this diluted culture was added to each well (final dilution of 1:10). Guinea pig serum (i.e. complement) was diluted 1:5 in FM4 broth, and 100ul was added to each well (i.e. final concentration of complement = 10%). Plates were then covered with a sterile plastic lid, placed in a humidified box, and incubated at 37°C.

Note: A control well was set up containing mycoplasma organisms and complement, but no antiserum.

Plates were checked every 24 hours. When a colour change occurred in the control well, the presence or absence of a colour change in the wells containing antiserum was noted. The plates were then incubated for a further 24-48 hours, i.e. until 2 wells that were previously negative (no colour change) turned positive. When this occurred, the contents of these 2 wells were used to provide the culture of M. ovipneumoniae required for the subsequent passage. This procedure was repeated twice, then 8 clones were obtained from the culture (that had undergone 3 passages in the presence of antiserum), using standard methods (see Ch. 3.1.2).

The clones were propagated in FM4 broth, and while still in the exponential phase, were frozen at -70°C.

DNA extraction and REA

All 24 clones were propagated in FM4, the DNA extracted and REA performed on each - see Ch. 2.4.

5.1.3 Results

The restriction patterns of 8 sub-clones taken prior to passage in vitro, are shown in Figure 15.

The restriction patterns of 8 sub-clones taken following 20 passages in vitro, are shown in Figure 16.

The restriction patterns of 8 sub-clones taken following 3 passages in vitro, in the presence of sub-inhibitory concentrations of antiserum, are shown in Fig. 17.

The patterns were coded with a letter, e.g. "a". Any pattern that differed from "a" by only a few bands (up to 3) was designated "a'" (a-prime). A pattern that differed by 4 or more bands, but still showed the same overall pattern was given a different letter, e.g. "b".

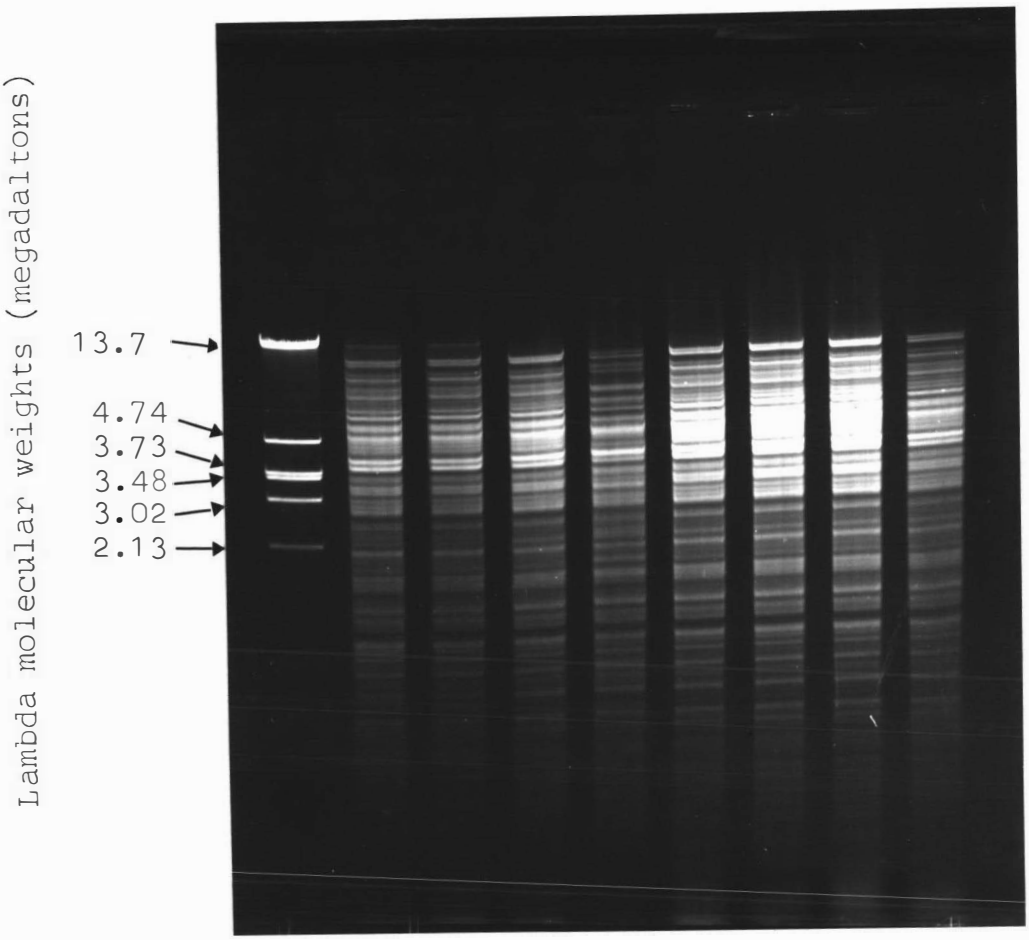

Figure 15 : REA of 8 sub-clones of M. ovipneumoniae strain 5, prior to the in vitro passage experiment.
Lane 1: Lambda; Lanes 2 to 9: strain 5, sub-clones 1-8. Patterns fell into 3 sub-groups:
Sub-group a - Lanes 4, 6, 7, 8 and 9
Sub-group a' - Lanes 2 and 3
Sub-group a" - Lane 5.
Note: Differences exhibited between sub-groups a, a' and a" are considered minor compared with differences exhibited by isolates from different sources.

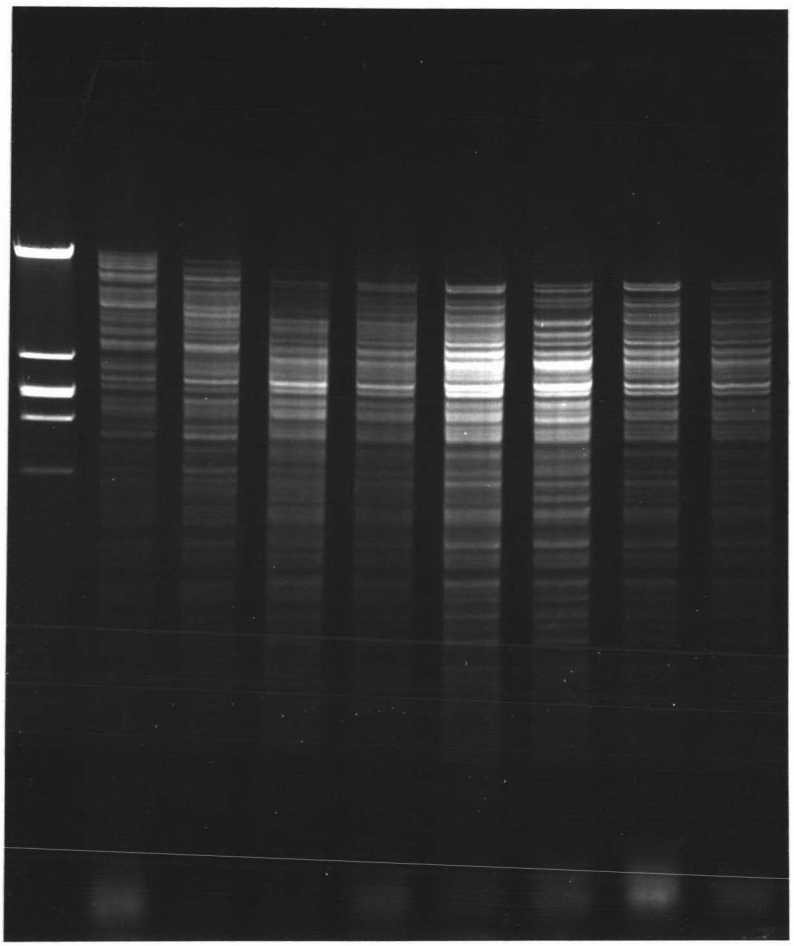

Figure 16 : REA of 8 sub-clones of M. ovipneumoniae strain 5 taken following 20 passages in vitro: Lane 1: Lambda; Lanes 2 to 9: strain 5 (P20), sub-clones 1-8. Patterns fell into 6 sub-groups:

Sub-group a: Lanes 6, 8 and 9
Sub-group a": Lane 7
Sub-group a"": Lane 5
Sub-group a"":Lane 4
Sub-group b: Lane 2
Sub-group c: Lane 3

Note: Differences between sub-groups 'a', 'b' and 'c' are slightly greater than differences within sub-groups 'a', i.e. a', a",a"',a"",but are, nevertheless, considered minor compared with differences exhibited by isolates from different sources.

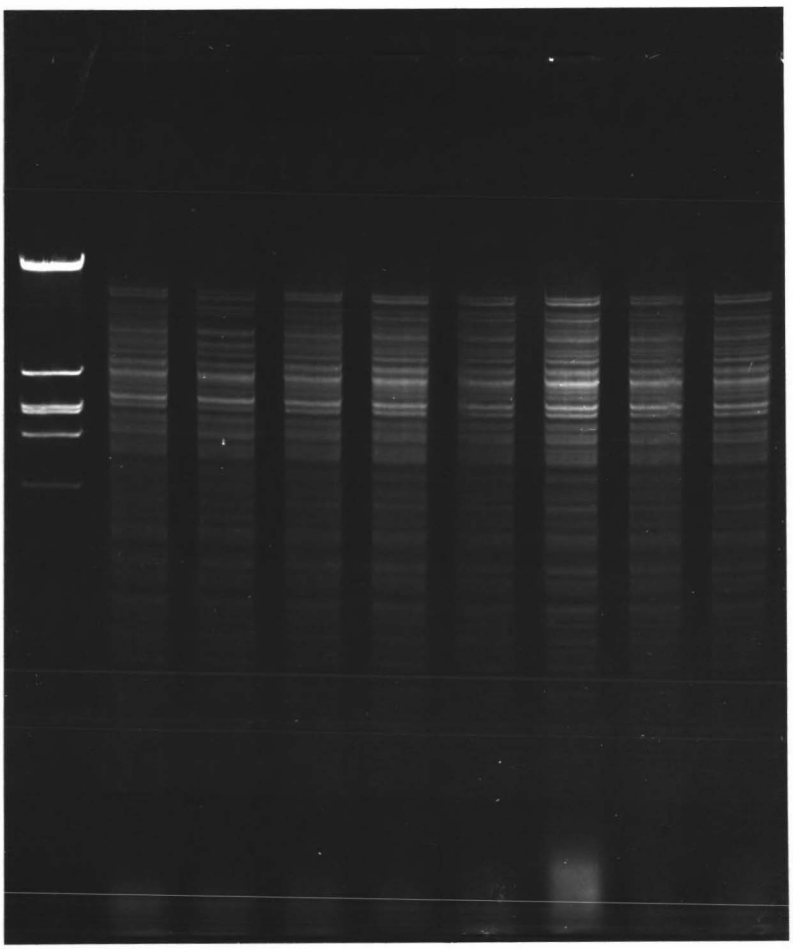

Figure 17 : REA of 8 sub-clones of M. ovipneumoniae strain 5 taken following 3 passages in vitro in the presence of sub-inhibitory concentrations of antiserum.
Lane 1: Lambda; Lanes 2 to 9: strain 5, (P3+Ab) sub-clones 1-8. Patterns fell into 2 sub-groups:
Sub-group a: Lanes 2, 4, 5, 6, 7, 8 and 9
Sub-group a": Lane 3
Note: Differences exhibited between sub-groups a, a' and a" are considered minor compared with differences exhibited by isolates from different sources.

5.1.4 Discussion

Several observations can be made:

1. All 24 patterns (8 before passage; 8 after 20 passages *in vitro* and 8 after 3 passages in the presence of antiserum) show clear areas of identity and hence are closely related. Nevertheless:

2. the 8 sub-clones taken before passage *in vitro* (Fig. 15) are not totally homogeneous, but patterns fall into one basic group (pattern a) which includes patterns with only a few (up to 3 bands) differences, e.g. pattern a' (lane 3) appears to have an extra band at approximately 13-7 megadaltons but lacks a band at approximately 2.1 megadaltons when compared to pattern a (lane 4).

3. After 20 passages *in vitro*, 8 sub-clones (Fig. 16) showed 3 patterns that revealed more than 3 differences. These are represented respectively by lane 2; lane 3; and lanes 4 to 9. However, the patterns in lanes 4 to 9 show some minor differences, i.e. changes in not more than 3 bands.

4. After triple passage *in vitro* in the presence of sub-inhibitory concentrations of antibody (Fig. 17), 8 sub-clones appear to be slightly more homogeneous than before passage. That is, all patterns fell into one group (pattern a), with only one sub-clone showing a minor difference (lane 3, pattern a").

It is concluded that the heterogeneity of the original cloned culture has increased after 20 passages *in vitro* but decreased following 3 passages in the presence of antiserum. The significance of this is considered in the General Discussion.

CHAPTER 6: General Discussion

Resolution of bands in restriction patterns. The resolution of fragments of DNA (produced on digestion with a restriction endonuclease) in an agarose gel, depends on 3 major factors:

1. The agarose gel electrophoresis technique (which is standard in this study and which should resolve up to 30-100 bands).
2. The complexity of the DNA.
3. The average size of DNA fragments, which will depend on the number and distribution restriction endonuclease recognition sequences.

Mycoplasma DNA has a molecular weight of approximately 5×10^8 daltons (Bergey's Manual of Determinative Bacteriology, 8th Ed.) and M. ovipneumoniae has a G+C content of 28% (Major, 1977). Calculations based on this data, (and assuming random distribution of bases), show that, the number of fragments expected on digestion of M. ovipneumoniae DNA with EcoRI would be approximately 230. Experimentally, only 30 to 40 bands of varying resolution and intensity were produced. There are a number of explanations for this difference between the observed and expected number of bands (not mutually exclusive). It is most likely that some bands represent more than one fragment, however, bases may be markedly non-random: thus, there may be fewer recognition sequences than expected; or, some bases may be methylated. The last two explanations, if true, would result in fewer fragments, but the average molecular weight of these fragments would be proportionally higher. When patterns were compared to the pattern produced by lambda DNA (as a molecular weight marker) there was no obvious bias towards the higher molecular weight bands. Moreover, differences in intensities of bands of similar molecular weight, supports the conclusion that some bands represent multiple fragments. That is, the intensity of fluorescence in a band is proportional to the amount of DNA present, and in bands containing equal amounts of fragments, the intensity of fluorescence is proportional to the molecular weight (thus, a decrease in intensity of bands is observed in the gel

towards the lower molecular weight bands). Therefore, differences in intensities of bands of similar molecular weight, would indicate that different amounts of DNA are present; thus, if the lighter band represents a single fragment, the more intense band must represent multiple fragments. This also means that changes in 1 or 2 fragments may not be detectable in the overall pattern.

Application of REA to the identification of strains of M. ovipneumoniae

For REA to be useful for the identification of strains, 3 requirements must be fulfilled. They are:

1. REA must produce a recognisable and reproducible pattern.
2. The pattern must be stable after at least limited passage in vitro.
3. Variation in restriction pattern must exist between strains.

Despite the likelihood that many bands, in the restriction pattern of a strain of M. ovipneumoniae, may represent multiple fragments, recognisable patterns were observed, and were found to be reproducible (Fig. 5). These patterns were shown to be stable after a limited number of passages in vitro (Fig. 9), and different strains gave different restriction patterns (Fig. 6). Therefore, the three requirements were fulfilled and it was concluded that REA would be useful for identifying strains of M. ovipneumoniae.

REA of M. ovipneumoniae compared to that of other micro-organisms

Robberson et al.,(1980) examined the DNA of one strain of Mycoplasma hominis type I using several restriction endonucleases. EcoRI gave approximately 23 fragments, but there appeared to be no Bam HI cleavage site in this mycoplasmal DNA. Darai et al.,(1981) examined the DNA of several strains of Mycoplasma hyorhinis using the restriction endonuclease, Bst EII, which revealed only 4 distinct fragments. Both workers concluded that REA was

useful in the species identification of these mycoplasmas. Mielenz et al.,(1979) concluded that 3 species of Rhizobium could be identified on the basis of their restriction patterns, and Marshall et al.,(1981) found that REA of Leptospira interrogans could distinguish serovars. These workers found that strains within a species of Rhizobium or a serovar of L. interrogans were largely homogeneous with respect to restriction pattern, although some minor differences did occur.

These results are in marked contrast to those of the present study, in which the restriction patterns exhibited by strains of M. ovipneumoniae allows REA to be useful for strain identification, but not in species identification. However, in the present work, only one restriction endonuclease was used, and possibly other restriction endonucleases may be useful for identifying the species, M. ovipneumoniae.

Use of REA in an epidemiological study.

The present study shows that many strains of M. ovipneumoniae exist within a flock of lambs, but not all isolates are different. REA can therefore be used to examine the epidemiology of M. ovipneumoniae within a flock of lambs.

Examination of isolates from one flock of lambs led to the following conclusions:

1. Several strains of M. ovipneumoniae can be recovered from the nasal cavities of one flock of lambs. However, at any one sampling time, one strain may predominate.

2. The colonisation of the nasal cavity of a lamb is often transitory, but can persist for at least 6 weeks.

3. Strains of M. ovipneumoniae recovered from the lungs of the flock of lambs at slaughter can be relatively homogeneous, and can correspond to those strains present in the nasal cavity, 20 days earlier.

These conclusions were derived from one flock of lambs, in one year, and in one part of New Zealand. A full epidemiological study of M. ovipneumoniae in New Zealand sheep, would require an examination of different flocks, in different geographical locations, and should take place over a number of years. Any conclusion reached by extrapolation of the present results must, therefore, be tentative. Nevertheless, it is interesting that isolates of M. ovipneumoniae obtained from the lungs of the lambs at slaughter, were more homogeneous than those obtained from the nasal cavities. There could be two explanations for this:

1. A limited number of strains of M. ovipneumoniae are capable of invading the lungs of lambs.

2. The strain of M. ovipneumoniae predominant in the nasal cavity at the time of maximum stress factors, invades the lungs, i.e. the invasion of the lower respiratory tract by M. ovipneumoniae may be stress-dependent rather than strain-dependent.

The extensive heterogeneity observed among lung isolates of different flocks (Fig. 6) favours the second explanation. However, both hypotheses are testable, by extending the study to more flocks, and by comparing nasal and lung isolates within a flock over the summer/autumn period.

Use of REA in determining the role of M. ovipneumoniae in the pathogenesis of CNP.

Further investigation is required in order to establish the exact role that M. ovipneumoniae plays in the pathogenesis of CNP. For this purpose, REA could be useful in the following:

1. An epidemiological study of lung isolates of M. ovipneumoniae from different flocks, in different geographical locations, in different years, together with a study of pneumonic lesions in those lambs - to investigate whether any correlation exists between the severity of lung lesions and the

strain of M. ovipneumoniae.

2. Further transmission experiments using different strains of M. ovipneumoniae, particularly with any strain that is consistently associated with extensive pneumonic lesions.

3. In transmission experiments using non-Specific Pathogen Free sheep, the isolates obtained following slaughter could be examined by REA to show unequivocally that the inoculated strain, rather than a resident one, had colonised the lungs.

Origin of heterogeneity of strains.

The unexpectedly large number of different restriction patterns exhibited by isolates of M. ovipneumoniae raised questions as to the origin of this heterogeneity. Several possibilities exist:

1. Multiple stable strains may be independently maintained;

2. Frequent genetic interchange between strains give rise to a multiplicity of REA patterns;
or,

3. The DNA of M. ovipneumoniae may have a high potential for change, e.g. by mutation.

An in vitro experiment (using a multiply-cloned strain of M. ovipneumoniae) was performed to investigate the origin of heterogeneity, revealing that:

1. Sub-clones, taken immediately following cloning, were not totally homogeneous, but revealed minor differences in restriction pattern.

2. Sub-clones, taken following multiple passage in vitro, showed an increase in the heterogeneity when compared to (1) above.

3. Sub-clones, taken following limited passage in the presence of antiserum, were less heterogeneous than the sub-clones from (1) above.

It should be noted here, that all but 3-5 bands (out of 30-40 discernable bands) of a pattern from one sub-clone, were in the same position and of equal intensity when compared with patterns produced by the other sub-clones. Therefore, the differences observed between sub-clones were considered minor when compared to the differences seen between the patterns of random isolates (Fig. 6). Hence, these findings do not invalidate the use of REA in epidemiological studies.

The increase in heterogeneity of restriction patterns of the sub-clones after multiple passage in vitro does, however, indicate that a strain of M. ovipneumoniae has the potential for change. The origin of variation in the DNA of microorganisms can be:

1. Mutation
2. Interchange of genetic material with other micro-organisms, i.e. via transduction, conjugation, transformation, plasmids.
   Note: this may be important in the field but is not applicable to the minor changes which occurred in the pure cultures used in this in vitro experiment.
3. Rearrangement of genetic material within the cell i.e. transposons.

Since mutations occur in the DNA of all living organisms, and neither (2) nor (3) have been shown to occur in M. ovipneumoniae, perhaps the most conservative hypothesis is that, changes in restriction pattern observed after multiple passage in vitro, represent random mutations. Limited growth would allow these random mutations to occur between the time of colony formation and subsequent growth in culture, giving rise to limited variation, as was observed. Moreover, many sub-cultures would provide the scope for greater variation as was also observed.

The relative homogeneity after passage in the presence of antibody might be explained if certain mutations were selected against by the antiserum used. However, this would

require that changes in the DNA restriction pattern would correlate with changes in surface proteins.

While mutation is the most conservative explanation of the origin of heterogeneity among strains of *M. ovipneumoniae*, the presence or absence of genetic interactions, and transposons, is amenable to investigation, and further work will be required to decide this. It has been reported, that variation in restriction patterns occurs following passage of vaccinia virus *in vitro*. Panicali *et al*. (1981) reported two major variants of the WR strain of vaccinia virus following serial propagation of plaque-purified virus stocks, *in vitro*. These variations were discovered to be due to a) a 6.3 megadalton deletion, and b) addition of unique viral sequences.

Conclusion: From this study, we have arrived at several conclusions:

1. REA gives reproducible results when applied to *M. ovipneumoniae*.
2. *M. ovipneumoniae* DNA is stable after limited passage *in vitro*.
3. Multiple serial passage *in vitro* shows some differences between multiple sub-clones. Therefore, *M. ovipneumoniae* appears to have the potential for variation.
4. To date, every isolate from independent sources (i.e. different flock of sheep) has exhibited a unique restriction pattern.
5. REA is useful for "fingerprinting" strains of *M. ovipneumoniae* in epidemiological studies within a flock.

Areas where further work should be undertaken fall into 2 categories:

1. A more detailed examination of strains of *M. ovipneumoniae* in the upper and lower respiratory tracts of a number of flocks of lambs. A study is

in progress by George Ionas, using PAGE in an attempt to correlate differences in proteins with differences in bands of restriction patterns. Serological work would also be useful in the study of these strains.

2. The origin of heterogeneity, e.g. a search for genetic interaction between strains; transposons; plasmids; a study of mutation rates, etc.

Perhaps this may be regarded as more than the normal number of "loose ends", since the number of questions asked at the end of this thesis, outnumbers those answered by the work. In the circumstances, comfort may be taken from one of my more illustrious predecessors, who noted:

> "It is characteristic of Science and Progress that they continually open new fields to our vision."
>
> Louis Pasteur

APPENDIX

FM4 Broth

This medium is the fourth of several formulations of Frey, Hanson and Anderson (1968). The formula is:

NaCl	5.0g
KCl	0.4g
$MgSO_4.7H_2O$	0.2g
$Na_2HPO_4.12H_2O$	4.03g
KH_2PO_4	0.1g
Glucose	10.0g
Peptone CS (Bacto)	10.0g
Yeast Autolysate (Albimi)	5.0g
NAD	0.1g
L-cysteine HCl	0.1g
Eagles Vitamin Solution (X100)	25ml
0.4% phenol red	2.5ml
Penicillin	10^6 units
Thallous Acetate	0.5ml
Deionised water to	1000.0ml

Note:

1. The thallous acetate was dissolved in 10ml of deionised water and added to the medium dropwise to prevent precipitation.
2. The basal medium was supplemented with 150ml of pig serum.
3. The pH of the medium was adjusted to pH 7.8 with 1.0 N NaOH.
4. The complete medium was clarified by filtration through non-sterile 5.0, 0.45 and 0.2um pore-size filters; and sterilised by filtration through a sterile 0.2um filter.
5. The formula for Eagle's Vitamin Solution (X100) is:

D-biotin	20mg
Calcium pentothenate	20mg
Choline chloride	20mg
Folic acid	20mg
Riboflavin	2.0mg

Myo-Inositol	40mg
Niacinamide	20mg
Pyridoxine	20mg
Thiamine-HCl	20mg
Distilled water to	100ml

The solution was stored in 25ml aliquots at -20°C until required.

6. Growth of M. ovipneumoniae (a glycolytic mycoplasma) in FM4 broth was indicated by a colour change in the pH indicator from red to yellow. The medium was used immediately or stored at 4°C for not more than 2 weeks.

FM4 Agar

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

The BHI-agar was autoclaved at 121°C for 15 minutes. It was then cooled to approximately 50°C and added to 80ml of FM4 at 46°C. The medium was mixed thoroughly, and 4ml volumes were pipetted into 50mm plastic petri-dishes with tight-fitting lids. Once the medium had solidified, the plates were dried at 37°C for 15 minutes. The plates were then used immediately, or stored at 4°C for not more than 2 weeks.

Phosphate Buffered Saline (PBS)

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
Distilled water to	1000.0ml

The solution was autoclaved at 121°C for 15 minutes. Final pH = 7.2 to 7.4.

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