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**Colonisation of the ovine respiratory tract by  
*Pasteurella (Mannheimia) haemolytica***

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A thesis presented in partial fulfilment of the  
requirements for the degree of **Master of Veterinary  
Studies (MVS)** at Massey University

**2003**

## Abstract

*Pasteurella (Mannheimia) haemolytica* is a member of the normal bacterial flora of the nasopharyngeal, tonsillar and oral mucous membranes of sheep. The history, characteristics and pathogenicity of this organism are reviewed and the associated diseases of the ovine respiratory tract are discussed. In New Zealand, *P. haemolytica* is associated with two major disease entities; acute pneumonic pasteurellosis and chronic non-progressive pneumonia (CNP). Clinical or acute pneumonic pasteurellosis occurs as a sporadic disease with low prevalence on certain farms whereas CNP is very widespread and economically important as it causes poor growth rates and downgrading of carcasses during slaughter.

The epidemiological relationship between the nasal carriage of *P. haemolytica* in healthy ewes and their lambs was investigated and it was found that although lambs occasionally became infected from their dams they were more commonly infected from other sources. A very significant difference between the rate of nasal carriage on four farms in the Manawatu district was observed and a peak prevalence of *P. haemolytica* was seen in the February-March period. A close relationship between nasal carriage and pneumonia was found on one farm (Farm 4), which initially had a pure and vigorous growth of *P. haemolytica* from the nasal swabs obtained from young lambs. When 6 lambs were kept in close contact for a period on one farm, all developed a high rate of nasal carriage of *P. haemolytica* within 5 days.

DNA fingerprinting of the isolates from ewes and their offspring showed a variety of restriction endonuclease patterns using pulsed field gel electrophoresis (PFGE). The pulsed field profiles of isolates from the nasal cavity of ewes and their new-born lambs showed that lambs are more likely to obtain the first strain of *P. haemolytica* from in-contact ewes, lambs or the environment rather than from their mothers. The pattern of isolation of *P. haemolytica* in lambs on three farms without pneumonia showed that most strains of the organism were present on only one occasion and within two months the nasal cavity was occupied by other strains. On one farm (Farm 4), some strains of *P. haemolytica* were present throughout the whole life of the lambs and one these strains was later isolated from pneumonic lesions at slaughter.

# Acknowledgements

I am particularly grateful to my chief supervisor, Associate Professor Maurice Rewi Alley for his patience, encouragement, invaluable contribution of ideas, advice and particularly for guiding and preparing me from farm to laboratory throughout the course of the study and in completion of my thesis.

I am also grateful to my co-supervisor Dr. Stan Fenwick for his great help, advice and scientific contribution to the microbiological studies and particularly pulsed field gel electrophoresis.

I greatly appreciate the considerable support from the four farm owners – Dr. Robert Bruce and family, Glen Oroua; Roger Voss and family, Mirawai Farms Ltd, Kopane; Assoc. Prof. M. R. Alley and Mrs. Dorothy Alley, Tikoke farm, Pahiatua Track and Tim Harvey, Alister MacDonald and Rodney Grubb, Tuapaka, Forest Hill Rd.

I would like to thank to the staff and veterinarians at Richmond Meat works, Oringi; Lambpackers, Fielding and AFFCO, Wanganui.

I gratefully acknowledge technical assistance from a number of technical staff and postgraduate students of IVABS particularly – Magda and Kylie Walker (Bacteriology lab.), Jan Schrama (Media lab.), Megan Leyland (PFGE lab.), Pam Slack, Pat Davey, Faris Sharpe (sample collection) and postgraduate students F. Ahmed and Naomi Boxal.

I want to thank to Duncan Hedderley, Statistics Dept. Massey University for his statistics advice.

I would like to specially thank my father and mother, my wife (Rukhshana) and my two lovely daughters (Nabila and Nazia) for their encouragement, patience and help during the course of the study.

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# Chapter 1

## Introduction

Pasteurellosis is one of the most common respiratory infections of sheep throughout the world. Although this disease may initially involve the interaction of physiological stress with more than one infectious agent, *Pasteurella (Mannheimia) haemolytica* and less commonly *Pasteurella multocida* are considered to be the most pathogenic organisms present in ovine respiratory tract. This disease is a major economic problem to the sheep industry in New Zealand costing at least \$23 million each year (Anon, 1999).

In New Zealand, two forms of ovine respiratory disease have been recognised, namely acute pneumonic pasteurellosis and chronic non-progressive pneumonia. Acute pneumonic pasteurellosis occurs in a sporadic form with low prevalence. It causes deaths in sheep of all ages, although in the UK lambs during the first few months of life and ewes at lambing are particularly susceptible (Rodger, 1989). In Australia and New Zealand sporadic outbreaks also occur in older lambs aged 5-8 months and up to 10% of a flock may be affected (Sorenson, 1976; Alley, 1991). The outbreaks are usually associated with some form of management stress or close contact between animals prior to the disease occurring.

Chronic non-progressive pneumonia (CNP) is a disease of lambs, which is seen in New Zealand during the late summer and autumn months. The aetiology of CNP is less well understood and has been reviewed recently by Alley *et al* (1999). Most workers believe that in CNP, *M. ovipneumoniae* acts as a primary pathogen which can cause mild lung injury and ciliostasis thus allowing *P. haemolytica* to colonize and proliferate in the respiratory tract which leads to the development of lesions of clinical

significance. This pneumonia is widespread in the North Island of New Zealand and up to 70% of lambs on some farms are affected. This disease may predispose to acute pneumonia but it can also become severe enough to cause poor growth rates of lambs, lung abscessation and pleurisy in lambs and hoggets. Alley (1987) estimated that pneumonic lambs had a mean liveweight gain of 1.74 kg per month less than normal controls.

*Pasteurella haemolytica* is therefore closely associated with most forms of pneumonic disease in sheep. It may even colonize lungs with jaagsiekte and maedi. Although these bacteria can act very effectively as primary pathogens, severe lung infection is more likely to follow predisposing factors such as stressful mustering, shearing, drenching or dipping (Harris, 1974; Alley, 1991; Bruere and West, 1993). Field and experimental evidence has confirmed that infections with other microorganisms are important predisposing factors and act by impairing respiratory defense mechanisms. Some viruses such as parainfluenza type 3 (PI3) and adenoviruses have been shown to have a temporal relationship with *P. haemolytica* in ovine pneumonia while reovirus and respiratory syncytial virus have a questionable significance.

*Pasteurella haemolytica* organisms are part of the normal bacterial flora of nasopharyngeal, tonsillar and oral mucous membranes of sheep. The colonisation mechanism in the upper respiratory tract is still not clearly understood. The role of induced virulence factors in the pathogenesis of pasteurellosis could be critical in mediating upper respiratory tract adhesion and colonisation (Brogden *et al.*, 1998). Such factors may promote an increase in the number of *Pasteurella* organisms deposited through infective droplets into the lungs, beyond that efficiently cleared by normal lung defenses. The seeding of these organisms into the lungs may provide

numerous foci of infection that eventually progress into the characteristic pneumonic lesions seen in the disease.

Very little work regarding the source of infection in lambs both at the epidemiological and molecular levels has been undertaken to date. Early work in the UK suggested that lambs probably acquire infection soon after birth due to intimate contact with their dams. This has not been confirmed under New Zealand pastoral conditions. There is therefore a need to investigate the isolates carried in the respiratory tract by healthy lambs and the process of establishment of such isolates in the nasal cavity in relation to the age of lambs and their contact with other potential carriers.

## General Review of Literature

### 1.1 Classification, Nomenclature and Early History of *Pasteurella (Mannheimia) haemolytica*:

The Pasteurellaceae are a family of Gram-negative, facultatively anaerobic coccobacilli, which are non-motile, non-sporeforming and exhibit pleomorphism. The family is divided into 3 genera: *Pasteurella*, *Haemophilus* and *Actinobacillus*. Recently Angen *et al.*, (1999) proposed a new genus *Mannheimia* under this family for the trehalose-negative *Pasteurella haemolytica* complex. This genus would consist of five named species: *M. haemolytica*, *M. glucosida*, *M. granulomatis*, *M. ruminalis* and *M. varigena*.

*Pasteurella (Mannheimia) haemolytica* is associated with pneumonia in ruminants particularly sheep. It is a Gram-negative, small rod to coccobacilli, non-motile encapsulated organism. It exhibits slight pleomorphism and occasional bipolar staining. Structurally, it resembles other Gram-negative organisms by having capsular polysaccharides, outer and inner membrane proteins and peptidoglycan (Adlam, 1989).

Rivolta in 1877 and Revolet in 1879 first described the disease pasteurellosis after investigating a mortality outbreak in poultry later designated as fowl cholera. An Italian, Count Trevisan later suggested the genus be named *Pasteurella* in recognition of Louis Pasteur who was probably the first to grow the organism in pure culture (Mutters *et al.*, 1989). Early investigators used a zoological classification to classify Pasteurellae, therefore organisms of widely divergent characteristics were included in this group. In the early 20<sup>th</sup> century many researchers such as Baumgarten 1911, Mohler and Eichhorn 1913, Hutty 1925, Migge 1933 and Manninger 1934

(Rosenbusch and Merchant, 1939) questioned the value of this classification because of the experimental and field cross pathogenicity and cross immunity of this group of organisms. Jones (1921) divided the Pasteurellae group into an atypical group (group 1) and typical group (group 2) based on cultural, biochemical and serological characteristics such as haemolysis of rabbit blood agar, indole production and pathogenicity to rabbits, a method which remains in use until the present day.

Early researchers failed to acknowledge *Pasteurella sp.* as the causative agent of sheep pneumonia because they depended entirely upon the nature of the lesions, bipolar staining and pathogenicity to characterize the organism. Newsom and Cross (1932) studied the two groups of Pasteurellae identified by Jones (1921) in detail and suggested that the atypical strains should be classified as a separate species and were first to propose the name *P. haemolytica* be applied to this group. But confusion regarding the name of the genera continued for another decade as some researchers such as Lovell and Hughes, 1935; Bosworth and Lovell, 1944 (Smith, 1961) used the name 'haemolytic coccobacilli' for haemolytic strains of the organism.

Smith (1959, 1961) divided *P. haemolytica* strains into two biotypes named as A and T based on phenotypic characteristics, epidemiological and pathological differences. The arabinose fermenting biotype became known as *Pasteurella arabinose* and the trehalose fermenting biotype was called *Pasteurella trehalose*. Frederiksen (1973) proposed a third group of the strains of *P. haemolytica*, which did not fit into these two groups.

Biberstein *et al.* (1960) and Biberstein and Gills (1962) found a consistent association between biotypes and serotypes using an indirect haemagglutination test (IHA). On the basis of its capsular polysaccharide, they divided *P. haemolytica* strains into 15



serotypes. Serotypes 1, 2, 5, 6, 7, 8, 11, 12, 13 & 14 are within A biotype. The remaining serotypes viz. 3, 4, 10 & 15 are T biotype.

Bisgaard, 1984 cited many conflicting results from different laboratories in obtaining L-arabinose fermentation by some biotype A strains. Investigators found difficulties in the identification of *P. haemolytica* strains, which could affect studies on its epidemiology and pathogenesis. Mutters *et al.*, (1989) classified *P. haemolytica* isolated from ruminants into 12 biogroups based on phenotypic investigations. The genomic study partly resolved the problem. *Pasteurella haemolytica* was excluded from the genus *Pasteurella sensu stricto* in 1985 on the basis of DNA-DNA hybridisation (Mutters *et al.*, 1985: 1986) and its separation from the three recognised genera within the Pasteurellaceae was later supported by DNA-rRNA hybridisation (De Ley *et al.*, 1990) and 16S rRNA sequencing (Dewhirst *et al.*, 1992).

Angen *et al.*, (1997) investigated the phenotypic and genotypic diversity of the trehalose-negative *P. haemolytica* complex using quantitative evaluation of phenotypic data, ribotyping and multilocus enzyme electrophoresis (MEE) and proposed that the trehalose-negative group might represent a new genus within the family Pasteurellaceae. In 1999, Angen *et al.*, concluded their previous investigation of the taxonomy of the trehalose-negative *P. haemolytica* complex. The results obtained using 16S rRNA sequencing and DNA–DNA hybridization indicated that this group contains distinct genetic and phenotypic groups. They proposed a new genus named Mannheimia for the trehalose-negative *P. haemolytica* complex based on polyphasic investigations. They transferred two previously named species *P. haemolytica* and *P. granulomatis* to *M. haemolytica* and *M. granulomatis* respectively and proposed 3 new species – *M. glucosida*, *M. ruminalis* and *M. varigena* within the new genus.

## 1.2 Biochemical characteristics, Biotyping and Serotyping of *Pasteurella (Mannheimia) haemolytica*:

### 1.2.1 Biochemical characteristics:

*Pasteurella (Mannheimia) haemolytica* produces a narrow zone of haemolysis on 5-7% ovine or bovine blood agar plates. After 24 hours incubation at 37°C, the characteristic colonies are 0.2 x 1-2 µm, have a smooth surface, an entire edge, are low convex in shape and are translucent with a zone of beta haemolysis directly underneath the colony (Quinn *et al.*, 2002).

*Mannheimia haemolytica* has the ability to grow on MacConkey's agar producing pinpoint, pink or red colonies. The organisms can be distinguished from other *Pasteurella* species by the absence of urease activity and their inability to produce indole. They are catalase positive and can produce yellow slant and yellow butt without H<sub>2</sub>S on TSI agar slopes (Quinn *et al.*, 2002).

The strains of *Mannheimia haemolytica* can produce acid from lactose, sucrose, maltose and D-xylose but no strains ferment L-arabinose or D-trehalose. Whereas *Pasteurella trehalose* are catalase negative, can produce acid from sucrose, maltose, D-trehalose but not from lactose, D-xylose or L-arabinose (Angen *et al.*, 1999; Quinn *et al.*, 2002).

### 1.2.2 Biotyping:

Because of its colonial characteristics *Pasteurella haemolytica* was considered to be a homogenous species. Studies of variational phenomena have been confined to the search for serological subdivisions. Montgomerie *et al.*, (1938) first described two subgroups and Florent and Godbille in 1950 later described three distinct subgroups based on agglutination reactions although their results could not be confirmed (Biberstein *et al.*, 1958). Subsequently 'biotyping' and 'serotyping' were adopted as the two most widely accepted typing systems.

Biberstein *et al.*, (1958) were first to recognize the heterogeneity of *P. haemolytica* and detected two types of organisms. They referred to these two types of *P. haemolytica* as 'S' and 'R', similar to rough or smooth brucella but serological tests confirmed that the R variant was not a rough mutant like rough brucella or salmonella. They distinguished the two types by colonial characteristics, crystal violet uptake and serological properties. Although they failed to differentiate the two types by biochemical behaviour and agglutination tests, the only detectable difference was the presence of an abundant surface substance in the S type and a relatively deficient substance in the R type.

Smith (1959) studied different isolates of *P. hemolytica* from cases of ovine pneumonia and septicemia and found A types were associated with enzootic pneumonia and T types with lamb septicaemia. Smith (1961) confirmed this typing system, termed 'biotyping' using colonial morphology, fermentation reactions, growth curves and sensitivity to antibiotics correlated with pathogenic and epidemiologic differences between these organisms. He showed that although both biotypes produced similar degrees of haemolysis, A-biotype had small, even, grey colonies,

clearly demarcated with central thickening whereas T-biotype colonies tended to be slightly larger and possessed large, dark brownish centers. Both strains fermented fructose, maltose, dextrin and sorbitol within 2 days without the production of gas and none fermented inulin, erythritol, adnitol or dulcitol in 14 days. He observed all T strains gave a positive reaction with trehalose within 2 days whilst their reactions with arabinose were negative until 10 days. Type A strains gave a positive reaction with arabinose by the 7<sup>th</sup> day but remained negative with trehalose up to the 10<sup>th</sup> day. He also found that type A strains lost their viability more rapidly in ageing broth cultures and were more sensitive to penicillin and tetracycline than T strains. Smith also observed epidemiological differences between these two strains. Type A strains were associated with enzootic pneumonia in lambs and sheep as well as septicaemia in lambs within the first week of their lives whereas T strains were associated with septicaemic disease of older lambs.

### 1.2.3 Serotyping:

Several workers found difficulty with Smith's biotyping of the organism as well as the pathogenic and epidemiological classification. The main problem was that of colonial dissociation within strains as well as inconsistency and variability of arabinose and trehalose fermentation (Biberstein, 1978).

A second typing system, called serotyping, was initiated by Biberstein *et al.*, (1960) who confirmed 10 serotypes using indirect haemagglutination (IHA) test and this method quickly became the most widespread method of *P. haemolytica* typing. Biberstein and Gills (1962) subsequently identified two more strains and found a consistent relationship between biotypes and serotypes.

Later, five more serotypes were identified by several workers using an IHA technique (Pegram *et al.*, 1979, Fraser *et al.*, 1982, Younan and Fodar, 1995). Fraser *et al.*, (1983) modified the IHA technique by using microtitre trays and glutaraldehyde fixed cells which made the test easier and faster. Frank and Wessman (1978) found similar results to IHA by using a slide agglutination test. Fraser *et al.*, (1982) reported that 17 serotypes could be identified but 6-11% of identical *P. haemolytica* isolates were untypeable and could not be assigned using this system.

Biberstein, (1978) demonstrated that most untypeable isolates were closely similar to 'A' biotypes based on the criteria of sugar fermentation and were associated with the naso-pharynx of healthy animals. Donachie *et al.*, (1984) introduced counter current immuno-electrophoresis and successfully differentiated nine serogroups of 'untypeable' strains. Fodor *et al.*, (1996) recently achieved 94% success by introducing a simple, fast and reliable serotyping technique for the detection of type-specific antigens in lung lesions even in the absence of viable *P. haemolytica*.

## **1.2 Virulence factors for *P. haemolytica*:**

*Pasteurella haemolytica* has a number of virulence factors such as fimbriae, capsules, protease, neuraminidase, endotoxin and leukotoxin although there is at present no evidence that these are restricted to strains recovered from clinical cases of pasteurellosis.

### **1.3.1 Extracellular *P. haemolytica* products:**

#### **1.3.1.1 Fimbriae and Glycocalyx:**

Fimbriae of gram-negative bacteria are important for their specific adhesion. Morck *et al.*, (1987) examined the presence of glycocalyx and fimbriae on the surface of *P. haemolytica* using an electron microscope. They were successfully able to detect both fimbriae and glycocalyx but only in the logarithmic phase of growth. Although their role as a virulence factor is unknown, they may be important in the colonisation of bacteria in the upper respiratory tract and subsequent pathogenesis of pasteurellosis.

#### **1.3.1.2 Neuraminidase:**

Scharmann *et al.*, (1970) first detected neuraminidase enzyme from untypeable strains of *P. haemolytica*. Frank and Tabatabai (1981) also detected neuraminidase enzyme from almost all A biotypes except A11 and not from any T biotype strains. These workers found this enzyme was cell associated with little present in culture supernatant. Otulakowski *et al.*, (1983) was able to isolate the enzyme in crude cytotoxin prepared from culture supernatant. Later Straus *et al.*, (1993) confirmed the findings of Otulakowski and demonstrated that all A biotypes produce neuraminidase

of 150-200 KDA with the exception of serotype 11. The actual significance of neuraminidase in the pathogenesis of pneumonic pasteurellosis has not been confirmed but it probably reduces the protective function of epithelial mucous in entrapment and removal of invading microorganisms.

### **1.3.1.3 Sialoglycoprotease:**

Otulakowski *et al.*, (1983) first demonstrated a protease enzyme cytotoxic for bovine pulmonary macrophages in the culture supernatants of *P. haemolytica*. Abdullah *et al.*, (1992) identified this as a glycoprotease (O-sialoglycoproteine endopeptidase) secreted by serotypes 1, 2, 5, 6, 7, 8, 9 & 12 which degrades glycophorin –A, whereas serotypes 3, 4, 10 & 11 showed no glycoprotease activity. Recently Lee and Shewen (1996) detected IgG1 protease activity in partially purified *P. haemolytica* culture supernatant's that can cleave immunoglobulins such as IgA and IgG.

#### 1.3.1.4 Cytotoxin (Leukotoxin A):

Earlier workers found both *P. haemolytica* culture supernatants and washed *P. haemolytica* cells were toxic to bovine alveolar macrophages (Markham and Wilkie 1980). Several groups of workers then attempted to purify and characterize this cytotoxin and confirmed its cytotoxicity to ruminant leukocytes and platelets (Baluyut *et al.*, 1981; Kaehler *et al.*, 1980; Shewen and Wilkie, 1982; Sutherland, 1985). Maheswaran *et al.*, (1992) experimented with neutrophils and mast cells exposed to leukotoxin (LKT) and found they released oxygen free radicals, proteolytic enzymes, eicosanoids and histamine.

Shewen and Wilkie (1983) studied the presence of this cytotoxin using immunodiffusion tests and their work suggested that all strains of *P. haemolytica* contain the same toxin. However Chang *et al.*, (1987) failed to isolate this cytotoxin from four untypeable strains and concluded that heterologous serotypes might not produce this cytotoxin. Moore *et al.*, (1985) found that when the cytotoxin is produced *in-vivo* it evokes the production of neutralizing antibodies. Further purification studies have shown that the cytotoxin is a calcium dependent pore-forming protein thought to play a central role in the disease process (Lainson *et al.*, 1996). Whiteley *et al.*, (1991) showed that purified LKT induces consolidation and oedema of interlobular septa with haemorrhages in the lungs of ruminants.

Recently vaccination of lambs with an anti-leukotoxin vaccine has been successful in protecting animals against experimentally challenged *Pasteurella haemolytica* serotype A2 (Alexander *et al.*, 1995).



### 1.3.2 Cell associated *P. haemolytica* products:

#### 1.3.2.1 Capsular Polysaccharide:

Corstvet *et al.*, (1982) examined *P. haemolytica* capsules using a light microscope and suggested that young log phase cultures showed better encapsulation than older cultures. Gilmour *et al.*, (1985) demonstrated morphological differences in the capsular structures between A and T biotypes using the electron microscope. After scanning electron microscopy they found biotype A strains have numerous protrusions on the cell walls whereas T strains had only a few. Furthermore, the capsule of *P. haemolytica* was found to vary in composition between serotypes. It was shown that, the capsule inhibits complement-mediated serum killing as well as phagocytosis and intracellular killing of *P. haemolytica*. The capsule also enhances neutrophil directed migration and adhesion of *P. haemolytica* to alveolar epithelium.

Adlam (1989) characterized five different capsular polysaccharides of A1, A2, A7, T4 and T15 that are responsible for serological specificity. He further compared the structure of the five polysaccharides and found A1 capsular polysaccharide was structurally similar to 'enterobacterial common antigen', A2 was identical with capsular polysaccharide of *Neisseria meningitidis* B and *E. coli* K1. Serotype T15 polysaccharide was found to be similar to pathogenic *E. coli* K62 and A7 was structurally identical to *N. meningitis* and *Haemophilus influenzae* type F.

Adlam (1989) noted that the production of colaminic acid and a dextran polymer by serotype A2 may allow *P. haemolytica* to mimic host membranes rich in sialic acid residues and thereby effectively go undetected by host defenses.

Brogden *et al.*, (1998) postulated that *P. haemolytica* A1 capsular polysaccharide has a lectin type interaction with pulmonary surfactant, which may facilitate bacterial

attachment to the lining of the lung alveoli. In this way A1 capsular polysaccharide could impair phagocytosis and killing.

### **1.3.2.2 Lipopolysaccharide:**

The dried cell weight of *P. haemolytica* consists of approximately 12 to 25% lipopolysaccharide (LPS). Adlam (1989) studied the structure of LPS and found it varies between serotypes. He suggested that LPS makes a major contribution to the pathogenesis of *P. haemolytica* infection in the lungs of sheep and is probably responsible for high mortality observed in acute pasteurellosis. He also reported that A biotype strains have rough LPS whereas T biotypes have smooth LPS.

LPS is an endotoxin, which induces local and systemic pathophysiological changes in the cardiovascular system, modifies pulmonary function, damages endothelium, stimulates polymorphonuclear (PMN) adherence and modifies leukocyte function.

Brogden and coworkers (1986) detected more marked effect of smooth LPS than rough LPS. The effects in sheep included adverse reactions in the lung such as inflammation, oedema, hyperemia and haemorrhage in the lungs as well as adverse effects in the circulatory system often leading to metabolic failure and lethal shock.

Cutlip *et al.*, (1998) studied the changes in the lungs of lambs after intratracheal injection of lipopolysaccharides from *P. haemolytica* A1 and found much resemblance to field cases of pneumonic pasteurellosis. They observed rapid sequestration of neutrophils in the capillaries of lungs and aggregation of surfactant in the alveoli, followed by swelling of the alveolar epithelium and capillary endothelium, oedema, haemorrhage, and emigration of neutrophils into the interstitium and small air spaces of the lungs. They detected necrosis of neutrophils as a constant feature. Alveolar, interstitial and intravascular macrophages and lymphoid cells increased

slowly to become the predominant inflammatory cells at 72 hours after LPS injection whereas multinucleated cells appeared at 2-6 days after inoculation of LPS into the lungs.

### **1.3.2.3 Membrane proteins and enzymes:**

Rowe *et al.*, (1997) detected a metalloenzyme, superoxide dismutase, in serotype A1 and A2 that catalyzes the conversion of highly toxic superoxide radicals to two kinds of molecules such as hydrogen peroxide and oxygen in different states of oxidation. Although the role of this enzyme is yet to be clarified, Rowe and coworkers (1997) and Brogden *et al.*, (1998) thought that it might protect *P. haemolytica* from oxygen radicals generated at the tissue and mucous surface during colonisation as well as during the respiratory burst of phagocytosis.

## **1.4 Diseases associated with *Pasteurella haemolytica* in sheep:**

In New Zealand, *P. haemolytica* is mainly associated with two major ovine diseases. These are pneumonic pasteurellosis and chronic non-progressive pneumonia. Early literature showed some confusion regarding the terminology of these diseases partly due to the use of different names for similar pathological changes. Montgomerie *et al.*, (1938) used the term 'enzootic pneumonia' to describe an outbreak of acute pneumonia in adult sheep in Wales. Salisbury (1957) used the name 'Southland pneumonia' or 'enzootic pneumonia' in New Zealand to describe pneumonic pasteurellosis or acute fibrinous pneumonia because of the outbreaks reported in adult sheep being fed turnips in Southland. Subsequently this pneumonia was found throughout New Zealand. Hore (1970) attempted an anatomical or aetiological classification of pneumonias but this is also controversial because one agent may cause more than one anatomical form due to time and host response and many pneumonias are complex in aetiology e.g., initial viral infection exacerbated by bacteria.

### **1.4.1 Pneumonic pasteurellosis:**

This is usually caused by biotype A strains of *P. haemolytica* and also known as acute fibrinous pneumonia, enzootic pneumonia, and acute necrotizing or exudative pneumonia. The disease is more common in the late winter and early spring in the United Kingdom and continental Europe, while in Australia and New Zealand the peak prevalence is in the late summer and autumn. The clinical signs include dullness, coughing, anorexia, and pyrexia above 40.6°C with nasal and ocular discharges. Animals usually die within 36 hours of the onset of clinical signs.

#### **1.4.1.1 Epidemiology:**

*Pasteurella spp.* are normal inhabitants of the upper respiratory tract of sheep. The epidemiological picture of pasteurellosis in sheep suggests that it is caused by the spread of a particular serotype of *P. haemolytica* through the flock (Blood and Radostits, 1989). In some flocks the development of the disease appears to occur in carrier animals which are already infected. Pasteurellosis is the main cause of sheep mortality in Great Britain and is a problem in most countries of the world where sheep are raised intensively including New Zealand. Biotype A strains can cause pneumonia in all ages of sheep at any time of year and septicemia in young lambs, biotype T strains can cause acute and usually fatal infection in lambs of 6 to 12 months of age which generally occurs from August to November (Gilmour and Gilmour, 1989).

Biberstein and Thompson (1966) compared the *P. haemolytica* strains isolated from the United States with those of the British Isles and found them to be similar except in relative frequency. In their epidemiological studies they found the normal flocks tend to have a relatively low incidence of *P. haemolytica* carriers compared to flocks with

outbreaks of pneumonia. Later Shreeve *et al.*, (1972) confirmed that the number of different serotypes and incidence of untypeable strains were higher in normal flocks than in outbreak flocks. Biberstein and Thompson (1966) also inoculated high dose rates of *P. haemolytica* into the nasal cavity of lambs and observed that most inoculated strains survived for only a few days while one strain persisted 10 weeks. This work highlighted the instability of nasal isolates.

Shreeve and Thompson (1970) studied the carriage of *P. haemolytica* in lambs and detected the first typeable colonies 48 hours after birth. They were able to isolate a range of serotypes, both A and T, and they found that the number of serotypes carried by individual animals increased with age. They recovered similar strains from almost all lambs and their dams or other in-contact ewes in the same batch. Accordingly, they suggested that intimate and prolonged contact with adult sheep might facilitate the transfer of strains from the dam, although they also found serotype '3' from seven lambs but not from their dams or other ewes in that batch. They offered no explanation for this finding. Al-Sultan and Aitken (1985) found that serotype A and untypeable strains first colonize the tonsils of lambs of under 3 days of age but subsequently T strains succeeded in colonizing the tonsils at around 3 weeks of age.

Gilmour *et al.*, (1974) surveyed 100 adult sheep heads obtained from an abattoir in Scotland and isolated *P. haemolytica* of 95% from tonsils and from 64% at nasopharynges. Alley (1975) reported that more than 60% of pneumonic lungs collected in a slaughter house survey contained *P. haemolytica* whereas only 6% of normal lungs had *P. haemolytica*. He also demonstrated that normal and pneumonic sheep both contained a high proportion (73% and 78% respectively) of *P. haemolytica* in their nasal cavities.

Biberstein *et al.*, (1970) surveyed the nasal carriage of sheep flocks in the Edinburgh area over a 12-month period. They found a bimodal curve of carrier rates where the number of carriers peaked in late spring and early summer and again in late autumn, which coincided with the seasonal pattern of enzootic pneumonia in that region. Further work in Scotland by Gilmour and Gilmour (1989) found May-June were the months with the highest rates of nasal carriage. In most parts of the world serotype A2 is most common (Biberstein and Thompson, 1966; Pegram *et al.*, 1979; Fraser *et al.*, 1982; Gilmour and Gilmour, 1989) and the same is true in New Zealand. Prince *et al.*, (1985) serotyped 40 isolates of *P. haemolytica* recovered from 60 lungs with lesions of chronic non-progressive pneumonia in which 55% were serotype A2, 25% serotype A1 and 7.5% were serotype A8 and A9. Gilmour and Gilmour (1989) serotyped 6500 isolates collected from 1982-86 at Moredun Research Institute. They found 65% of serotypes were biotype A of which A2 was the commonest, 28% were T biotypes and 7% untypeable strains. Recently Al-Tarazi and Dagnall (1997) found A7 and A9 predominated in Jordan.

In Scotland, Gilmour and Gilmour (1989) summarized reports of pneumonia from Moredun Research Institute and described it as a sporadic disease in all ages of sheep although outbreaks did occur throughout the year and those in late spring and early-summer involved high morbidity and mortality. They reported 450 outbreaks from 1983-1985 due to *P. haemolytica* Types A. Sixty percent of outbreaks were associated with lambs and 30% in ewes. The mean mortality they observed was 2.49% and the mortality rate was higher in hill / upland flocks than low land flocks.

Very little detailed information is available on pneumonic pasteurellosis in New Zealand. It is now widely accepted that all ages of sheep can be affected by acute pneumonic pasteurellosis although early workers found it only in adult sheep (Alley,

1991). Hopkirk (1936) reported a lobar type of pneumonia in an outbreak in Southland during the summer months where a great number of adult sheep died and was the first to recover the haemolytic organism responsible.

In New Zealand, septicaemic pasteurellosis does not appear to be as important as overseas. However Hartley and Boyes (1955) reported some cases of septicaemic pasteurellosis with 7% of neonatal lamb mortality but did not identify the biotype involved. Salisbury (1957) reported another pneumonia outbreak in Southland in 1949 where 236 ewes died on 10 properties carrying 6570 sheep. This was also in the summer months coinciding with high pasture growth or when the pasture was in seed. He isolated a haemolytic *Pasteurella*-like organism from outbreaks of the pneumonia. Davis (1974) reported 9% of deaths due to pneumonia in a survey of sheep mortality in Hawke's Bay and a slightly higher prevalence was reported in a smaller King Country survey by Pyke (1974). Although these workers recovered *P. haemolytica* from all pneumonic lungs, lack of a full laboratory examination made interpretation of their findings difficult (Alley, 1987). A subsequent report from Sorenson (1976) indicated that this type of pneumonia could also affect lambs/hoggets. He reported 600 deaths (21%) of 2850 hoggets in the March-April period and 353 (16%) of animals were downgraded or rejected for pleurisy/pneumonia at slaughter from the surviving 2238 animals.

Davies *et al.*, (1980) reported that the prevalence of pneumonia among slaughtered lambs was low in November-December but rose to 20-30% in March and June after a virus infection passed through the flocks in the Hawke's Bay region. Pfeffer *et al.*, (1983) surveyed the prevalence and microbiology of pneumonia in a flock of lambs between August 1978 and June 1979. Serological examination indicated that most



lambs were infected by parainfluenza type 3 (PI3) virus between January and February whereas adenovirus was present between March and June.

Midwinter (1987) surveyed the CNP lesions from 60 sheep of four widely separate districts of New Zealand i.e., Auckland, Gisborne, Christchurch and Invercargill during March – April period. The isolation rates were much higher in the South Island (77.5%) than in the North (38.4%). She isolated nine A biotypes and found A2 was the predominant serotype.

Alley (1987) described in detail an outbreak of acute pneumonia in lambs aged 5-8 months in the Manawatu region during March-April period. He reported that 60% of total losses (36 lambs) were due to pneumonic pasteurellosis and this occurred in the first week of the outbreak. A further 25% loss occurred over the next fortnight with fibrinous pleurisy and pericarditis lesions. He recovered *P. haemolytica* alone or with mixed bacteria from most of the pneumonic lungs.

Black (1997) re-evaluated the role of *Pasteurella* spp. in ovine pneumonia. He reported isolations from 509 lung samples with pneumonia of which 359 yielded *P. haemolytica*, 73 *P. multocida* and 26 samples contained both. He found the distribution of *P. haemolytica* was widespread and identified 11 serotypes including small numbers of T serotypes.

Davis (1989) investigated the principal causes of mortalities during transportation of live sheep from New Zealand and found pneumonia alone (18.87%) and pneumonia with other conditions (9.85%) as one of the major causes of mortalities. Black and Duganzich (1995) reported an outbreak of pneumonia causing high mortalities during a sea voyage and noted that this raised serious welfare and economic issues. He pointed out the lack of any scientific investigation into the possible causes of the pneumonic lesions. The pneumonia outbreak was subsequently found to be caused by *Pasteurella multocida* (Alley, 2002).

#### 1.4.1.2 Source of *P. haemolytica* infection:

Very little work has been done on how lambs acquire nasal infection. Shreeve and Thompson (1970) suggested that lambs might acquire infection soon after birth due to close contact with their dams. Whether these same strains of *P. haemolytica* are later able to cause pneumonia in their hosts is questionable. The source of *P. haemolytica* isolates that are able to undergo explosive proliferation in the respiratory tract has been debated but there is scant evidence suggesting that sheep/calves become infected by exposure to infected nasal secretions or sputum from other infected animals (Frank *et al.*, 1996).

Frank *et al.*, (1989) were successfully able to inoculate the middle nasal meatus of healthy non-stressed calves with *P. haemolytica* at a steady rate. They found that the duration, frequency and concentration of *P. haemolytica* shed from the inoculated nostrils was significantly greater than from the nostrils of other healthy calves that had been exposed by instilling the culture into the ventral nasal meatus of both nostrils. A group of workers (Frank and Briggs, 1992; Frank *et al.*, 1994) then analyzed field and laboratory reports and concluded that *P. haemolytica* isolates can survive and be carried in healthy calves in sites other than the main nasal passage and that the palatine tonsils might be the most likely alternative site.

Recently Burriel (1997) was able to isolate this organism from grass, water, straw-beddings used by sheep which proved this organism has the ability to survive in the environment and suggested a high probability that this is involved in transmission between animal to animal. Scott and Jones (1998) investigated the possible presence of *P. haemolytica* in the mouth of lambs and skin of the teats. They were able to isolate the organism from the mouth of ewes and lambs soon after lambing as well as

on the teat-skin of ewes thus demonstrating the possibility of transmission from ewes to lambs and vice versa.

#### **1.4.1.3 Gross pathology:**

In peracute cases of pasteurellosis, pulmonary consolidation is absent, the lungs are swollen and heavy and the surfaces are red to dark purple with very dark patches resembling haemorrhagic infarcts. The cut surface of such lungs exudes pinkish froth and the interlobular and sometimes lobar septa are thickened and opalescent (Gilmour and Gilmour, 1989).

In acute cases, the typical gross lesion consists of red consolidation of the apical, cardiac and ventral diaphragmatic lobes of both lungs. Involvement of the diaphragmatic lobes is irregular and often patchy in distribution. The remaining lung is usually congested and oedematous and blood stained frothy exudate is present in the alveoli. The consolidated areas are swollen and protrude above the surface of the surrounding tissue. They vary from dark red to blue in colour and are moderately firm in consistency. Their surfaces are often covered with thick strands of fibrin, which are mildly adherent to the overlying thoracic pleura. In some cases, grey necrotic areas are present within ventral parts of the consolidated tissue. The thoracic cavity contains a variable amount of clear exudate in which occasional strands of fibrin are present (Alley, 1975).

The subacute and chronic pneumonic lesions in surviving animals are usually characterized by abscessation of the lungs and, occasionally, the regional lymphnodes. There may be extensive pulmonary scarring and fibrous adhesions between the parietal and visceral pleura and the pericardium (Dungworth, 1985; Alley, 1987).

#### 1.4.1.4 Histopathology:

Peracute lesions: The most striking feature is intense hyperaemia with marked congestion of all alveolar and interstitial blood vessels. Haemorrhages are present ranging from scattered, interstitial capillary extravasations to prominent foci of intra-alveolar bleeding. Additionally, pale eosinophilic fluid is often seen in alveoli mingled with large numbers of Gram-negative coccobacilli. Sometimes a mild peribronchiolar and interstitial lymphocytic infiltrate can be found in the more intensely hyperaemic areas (Gilmour, 1978).

Acute lesions: The histological features include hyperaemia and oedema, serofibrinous exudate admixed with variable numbers of macrophages and neutrophils in alveoli, bronchioles, bronchi and the interstitium of the lungs. Haemorrhages, thrombosis of blood and lymphatic vessels and multiple foci of coagulative necrosis are also seen (Al-Darraj *et al.*, 1982). The alveolar spaces may be distended with randomly distributed necrotic cellular debris but more often they are filled with fibrinous exudate, neutrophils and dead macrophages which become flattened into the shape of an oat grain. These are the so called 'oat cells' which are very characteristic of the disease (Herceg *et al.*, 1982).

The interstitial tissues are oedematous and the interlobular and pleural spaces are usually distended with fibrinous exudate sparsely infiltrated by neutrophils and large mononuclear cells (Alley, 1975). Areas of necrosis appear to arise primarily from bronchioles although in some cases they extend widely across whole lobules. Necrotic cellular exudate or haemorrhages often marginate such areas. Bacterial colonies are usually found within these necrotic areas and at the periphery of necrotic lobules.

The sub-acute or chronic cases are characterized by organization of the affected areas of the lung, fibroplasia, abscessation, fibrous pleuritis and pericarditis.

#### 1.4.1.5 Experimental transmission:

Experimental transmission is essential to confirm the interaction between pathogen and its host by producing the disease. Early workers consistently failed to transmit pasteurellosis from diseased to healthy animals using sheep of various ages, inoculated with various materials by various routes (Dungal, 1931; Montgomerie *et al.*, 1938; Downey, 1957; Salisbury, 1957). These early workers expressed doubts regarding the primary aetiological ability of this organism and thought that a virus might also be involved in the production of the disease although there was no conclusive evidence. Smith (1964) was first able to transmit the disease successfully using peritoneal fluid of mice infected with a pure culture of *P. haemolytica* A alone, but the doses required were extremely high ( $8.8 \times 10^9$ ).

Confusion regarding the role of *P. haemolytica* as primary pathogen continues until the present time. Although Biberstein *et al.*, (1967) confirmed Smith's findings and reported that *P. haemolytica* alone was capable of producing characteristic lung lesions, the doses required were still so high that they would produce non-specific pathological changes.

A major problem in studying the pathogenicity of *P. haemolytica* is the colonisation and the carriage of *P. haemolytica* by apparently healthy sheep. As *P. haemolytica* is recoverable from young lambs hours after birth and a large proportion of healthy sheep carry the organism in their naso-pharynx or tonsils, these animals may be compromised when used to study most aspects of pasteurellosis. Because of previous exposure to pasteurella organisms conventionally reared sheep have a natural resistance to experimental infection. These problems were overcome by the use of hysterectomy-derived, colostrum-deprived, specific pathogen free (SPF) lambs.

Gilmour *et al.*, (1975) was first to use specific pathogen free (SPF) lambs and successfully produce pneumonia using low doses of *P. haemolytica* alone ( $1 \times 10^{4.8}$ ). However, Sharp *et al.*, (1978a) subsequently claimed that *P. haemolytica* alone produced only a transient, mild illness without overt respiratory signs.

The importance of parainfluenza virus Type III (PI 3) and other viruses as initiating agents of pasteurellosis is still a topic of debate. Hore (1966) first isolated PI3 virus from outbreaks of respiratory disease in sheep and subsequently Hore and Stevenson (1969) succeeded in reproducing lung lesions in young lambs. They found the lesions were more severe than parallel experimental infections produced by Biberstein *et al.*, (1971) using *Pasteurella* alone. However, Shreeve *et al.*, (1972) found no evidence of PI3 virus involvement when they studied the role of PI3 virus in four outbreaks of pneumonia from 6 counties in Scotland. Sharp *et al.*, (1978a) later successfully produced severe respiratory disease similar to that observed in naturally occurring pneumonic pasteurellosis by using combined intratracheal and intranasal inoculation of PI 3 virus and subsequent *P. haemolytica* inhalation.

In New Zealand, Davies *et al.*, (1977) failed to produce clinical illness and consistent lung lesions by combined PI 3 virus and *P. haemolytica* inoculation, probably due to the use of conventionally reared lambs which were believed to have natural resistance to experimental infections. A longitudinal serological survey of respiratory virus infections in lambs in New Zealand, Davies *et al.*, (1980) showed a temporal relationship between outbreaks of pneumonia and the presence of PI 3 virus, and adenovirus in the flock. Although they failed to establish a direct casual relationship they suggested that respiratory viruses might have an indirect role in the development of ovine pneumonia. Further work by Davies *et al.*, (1981) successfully produced an acute necrotizing bronchopneumonia in SPF lambs by combined intratracheal and

intranasal inoculation of PI 3 virus followed after 6 days by *P. haemolytica* inhalation. Davies *et al.*, (1986) suggested that PI 3-virus infection acts by reducing the bactericidal activity of neutrophils in the 4-6 day period following infection, leaving the lower respiratory tract susceptible to *P.haemolytica* aerosols. They suggested respiratory syncytial virus and adeno-virus may also play a similar role to PI 3.

Gilmour *et al.*, (1979) found *P. haemolytica* serotypes 1, 2, 6 and 9 were pathogenic using comparable challenge doses in SPF lambs although Donachie *et al.*, (1984) suggested that there might be differences in pathogenicity between these serotypes. Donachie *et al.*, (1984) found no-pathogenic effects in specific pathogen free lambs challenged with IHA negative strains of biotype A.

### **1.4.1.6 Pathogenesis of pasteurellosis:**

#### **1.4.1.6.1 Muco-ciliary clearance:**

The respiratory passages are covered with a mucoid blanket. This together with the ciliated epithelial cells transport microorganisms from the upper respiratory tract via the pharynx into the stomach where the organisms are killed by the gastric acids.

Stress and respiratory viruses may disrupt this clearance mechanism and allow the explosive proliferation of *P. haemolytica*. Diesel *et al.*, (1991) showed that tracheal mucous velocity may be slowed by cold temperature e.g., calves exposed to cold temperature and then exposed intranasally with *P. haemolytica* developed higher concentrations of this bacterium in nasal passages than calves that were not exposed to cold.

Gilmour and Gilmour (1989) claimed that climatic changes especially warm weather might be particularly important irrespective of any other stressful conditions such as movement or transportation. However, Harris (1974) observed that death of sheep from pneumonia in the United Kingdom was linked to movement, dipping or worming and Alley (1991) mentioned that the outbreaks in Australia and New Zealand were often associated with heat stress and transportation or mustering sheep in hot dry conditions. Bruere and West (1993) summarized the risk factors such as mouth breathing which bypass the respiratory defenses, dry or dusty conditions, cold stress and rainfall, poor parasite control and / or malnutrition, facial eczema, dehydration, reduced heterosis and steep terrain on the farm which may predispose to the disease.

Respiratory viruses such as PI3, adeno-virus type 6 and respiratory syncytial virus and to a lesser extent bovine adeno-virus type 2, ovine adeno-virus types 1 & 5 and reovirus type 1 may injure or destroy ciliated epithelial cells thereby making the



respiratory tract susceptible to secondary bacterial infection. These respiratory viruses may impair the pulmonary clearance mechanisms including phagocytosis by neutrophils and macrophages and cause local immunosuppression, which facilitates subsequent bacterial infection and results in outbreaks of pasteurellosis. Al-Kaissi and Alley (1983) and Brogden *et al.*, (1998) also demonstrated that *Mycoplasma ovipneumoniae* and *Bordetella parapertussis* organisms may affect ciliated cell function in vitro and increase the susceptibility of sheep to secondary *P. haemolytica* infection.

#### **1.4.1.6.2 Mucosal immunoglobulins:**

Immunoglobulins such as IgA and IgG are both found in the pulmonary mucosal secretions and serve as an immune barrier to exclude foreign particles particularly microorganisms. Walker (1979) studied the presence of immunoglobulins and found specific IgA in bovine lungs exposed to *P. haemolytica* may enhance the clearance of *P. haemolytica* and prevent colonisation of the mucosal epithelium. Abdullah *et al.*, (1992) detected *P. haemolytica* extracellular enzymes such as O-sialoglycoprotein endopeptidase in partially purified *P. haemolytica* supernatant. Brogden *et al.*, (1998) described how this enzyme could cleave immunoglobulins and allow *P. haemolytica* to colonize and proliferate in-vitro.

#### 1.4.1.6.3 Pulmonary surfactant:

Surfactant is a complex mixture of phospholipids, fatty acids and small amounts of protein synthesized by type 11 alveolar epithelial cells. Pulmonary surfactant decreases surface tension at the air-alveolar space interface, controlling exudation of fluid into the alveoli, reducing the effort of breathing and protecting the lungs against collapse.

Brogden *et al.*, (1996) studied the lipo-polysaccharides of *P. haemolytica*. The organism releases endotoxin, which forms complexes with pulmonary surfactant altering its surface tension properties and physiologic functions. They found that *P. haemolytica* LPS may produce pulmonary oedema, haemorrhage and atelectasis resulting in a decrease in total lung capacity, static compliance, diffusing capacity and arterial PO<sub>2</sub>.

#### 1.4.1.6.4 Alveolar macrophages:

Under normal conditions, alveolar macrophages will effectively clear *Pasteurellae* from alveoli by phagocytic mechanisms. Pulmonary macrophages release a superoxide anion when exposed to *P. haemolytica* and the response is dependent on the presence of opsonizing antibody and the quantity of organisms presented to the phagocyte (Blood and Radostits, 1989).

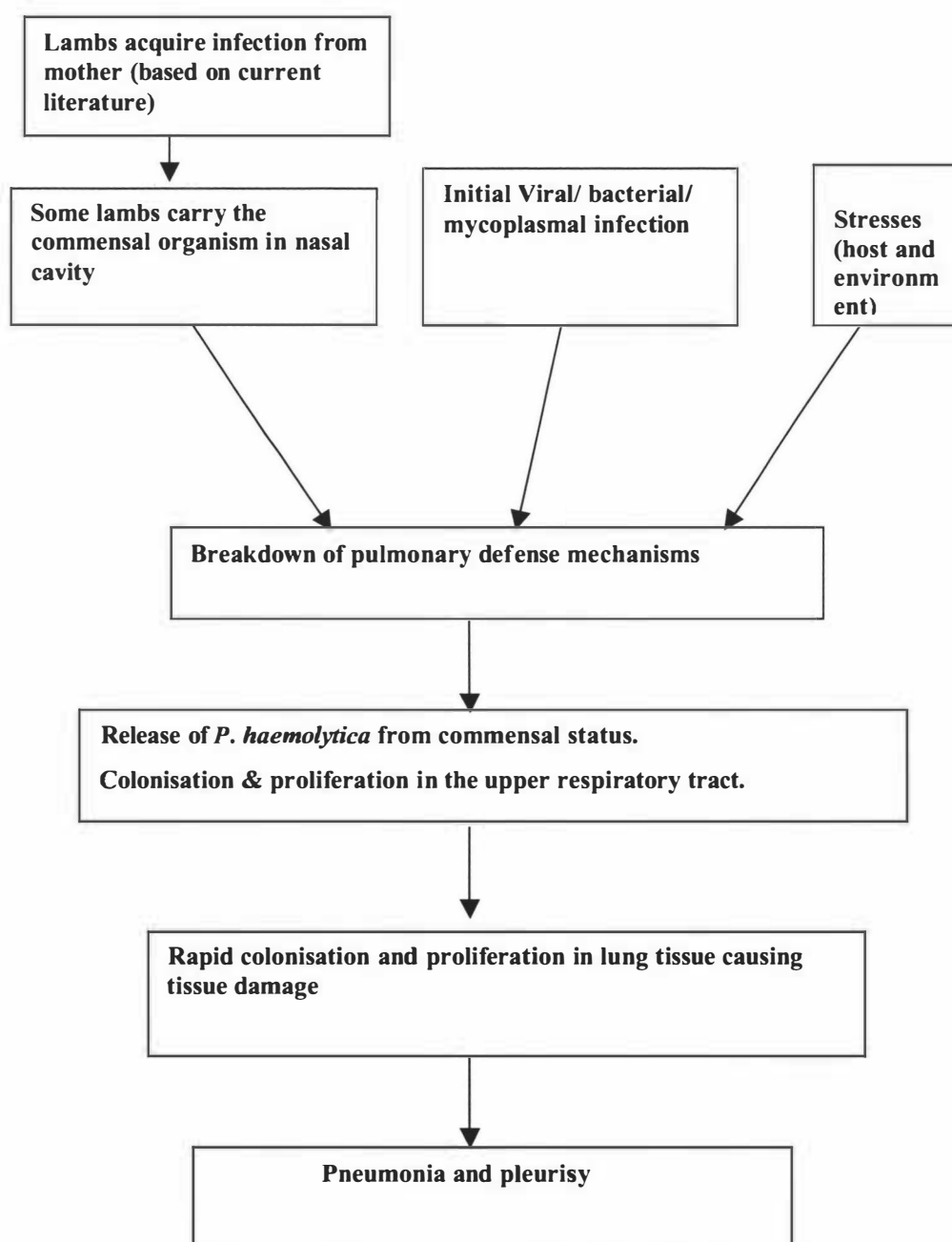
Brogden *et al.*, (1998) pointed out that many respiratory viruses depress macrophage function and many products of *P. haemolytica* (leukotoxin, endotoxin, capsular polysaccharide etc.) can kill alveolar macrophages directly or as well as depress macrophage function. In this way the natural defense mechanisms may be impaired and *P. haemolytica* organisms proliferate in the respiratory tract.

#### **1.4.1.6.5 Antimicrobial peptides:**

Resident mononuclear cells, inflammatory cells, epithelial cells and serous and mucous secretions in the respiratory tract contain a number of antimicrobial peptides such as alpha and beta defensins, which act against bacteria. When functioning properly, these peptides are thought to be capable of maintaining sterility in the tracheo-bronchiolar airways. It is possible that initial infections with viral or primary bacterial agents breakdown the anti-microbial barrier directly by injuring and destroying epithelial cells or indirectly by creating a micro-environment filled with necrotic cellular debris capable of overwhelming antimicrobial activity (Brogden *et al.*, 1998).

#### 1.4.1.6.6 Summary of pathogenesis:

Pasteurellosis is a multifactorial disease involving the interaction between host defenses, the inherent (virulent) properties of the pathogens and environmental factors. The relative roles of the aetiologic agents and stress factors in producing the disease have not been clearly established and may well vary in different situations. The upper respiratory tract is constantly exposed to large number of microorganisms and pulmonary defense mechanisms usually keep the respiratory tract free from these potential pathogens.



### **1.4.2 Chronic non-progressive pneumonia (CNP):**

This is the most common form of pneumonia in New Zealand and elsewhere in the world. It affects mainly young sheep from 2 to 12 months of age. The disease was first morphologically defined by Stamp and Nisbet (1963) in Scotland who introduced the term 'atypical pneumonia'. Subsequently many synonyms have been used, some of which are confusing.

In Scotland, some workers have described the natural form of atypical pneumonia as 'chronic pneumonia' (Gilmour *et al.*, 1982) and experimental form as 'proliferative exudative (PE) pneumonia' (Jones *et al.*, 1982a).

In Australia, some workers used the term 'summer pneumonia' while others have used 'proliferative interstitial pneumonia' (Carmicheal *et al.*, 1972).

In New Zealand, earlier investigators used the term 'enzootic pneumonia' to describe pneumonic pasteurellosis (Salisbury, 1957; Sorenson, 1976) but others used this term to describe the chronic form of pneumonia (Kirton *et al.*, 1976; McGowan *et al.*, 1978). Davies *et al.*, (1980) used the term 'enzootic pneumonia' to describe both forms of the disease. Manktelow (1984) first used the term 'chronic non-progressive pneumonia' to distinguish it from 'progressive pneumonia of viral aetiology' such as Maedi, and this term is now accepted by most New Zealand workers (Alley and Clarke, 1979; Alley, 1987; Alley *et al.*, 1999; Bruere and West, 1993).

### 1.4.2.1 Epidemiology:

Davies *et al.*, (1980) reported that the morbidity and severity of CNP in New Zealand varies considerably between farms with a range of 5-70%. They recorded about 30% of slaughtered lambs affected in the Hawke's Bay district during March-April period. Kirton *et al.*, (1976) conducted a survey over a five-year period and first showed an association between moderate to severe levels of CNP and reduced carcass weights. They found 6.5% of 3243 lambs had reduced carcass weights by a mean of 0.45kg per animal. Alley (1987) carried out a trial where he found the pneumonic lambs had a mean reduction in liveweight gain of 1.74 kg over 30 days and 2.19kg loss over 60 days. Alley (1975) found 70% to 80% of some flocks affected with CNP. Thus, although CNP is rarely fatal it is economically important due to pleural adhesions (Alley, 1975) and diminishing weight gain (Jones *et al.*, 1982b). In New Zealand the prevalence peaks in February to April (Alley, 1991).

The aetiology of CNP is thought to be multifactorial. Most research workers now believe that *P. haemolytica* and *Mycoplasma ovipneumoniae* are the main aetiological agents (Jones *et al.*, 1978; Alley and Clarke, 1979; Gilmour and Gilmour, 1989; Brogden *et al.*, 1998). Alley *et al.*, (1999) recently reviewed the role of *M. ovipneumoniae* in CNP and concluded that mixed strains of *M. ovipneumoniae* are more effective in producing lesions compared to *M. ovipneumoniae* alone. They suggested that *M. ovipneumoniae* initiates colonisation of the lung by *P. haemolytica* by causing ciliostasis. Thus mild lung lesions can be caused by *M. ovipneumoniae* alone but usually the lesions are exacerbated by *P. haemolytica*.

However, Davies *et al.*, (1981) were uncertain about the role of *M. ovipneumoniae* as a primary or secondary lung pathogen as they failed to produce significant pneumonia

by the inoculation of a combination of *M. ovipneumoniae* and *P. haemolytica* into SPF lambs.

However, the role of PI-3 virus, *M. arginini*, *Chlamydia psittci* in this type of pneumonia is questionable as these organisms are only occasionally recovered from this type of disease. Gilmour and Gilmour (1989) suggested that these organisms might be associated with concurrent infections without any primary role in the disease. Cutlip *et al.*, (1993) studied the chronic effects of coinfection in lambs with parainfluenza-3 virus and *P. haemolytica* and concluded that without other factors the coinfection with PI-3 virus and *P. haemolytica* as a cause of chronic enzootic pneumonia was unimportant.

#### 1.4.2.2 Experimental transmission:

Sullivan *et al.*, (1973a) investigated recurrent poor growth and excess mortality problems in prime lambs in Australia. They identified the initial disease as a 'proliferative interstitial pneumonia' associated with a mycoplasma organism later named *M. ovipneumoniae*. The condition was supervened at 5-10 weeks age by secondary *Pasteurella* and *Actinobacillus* infection resulting in overt clinical pneumonia. Sullivan *et al.*, (1973b) were able to reproduce experimental 'proliferative interstitial pneumonia' with intravenous or aerosol inoculation of *M. ovipneumoniae* isolated from the lungs of a pneumonic sheep, but they failed to recover mycoplasma from the lungs of experimentally exposed lambs. Jones *et al.*, (1978) experimentally reproduced 'proliferative exudative (PE) pneumonia' in 10 sheep with endobronchial inoculation of lung homogenate suspension containing *P. haemolytica*, *M. ovipneumoniae* and *E. coli*. The lesions produced were indistinguishable from atypical pneumonia, suggesting that both *P. haemolytica* and *M. ovipneumoniae* have a role in the aetiology of this disease.

In Scotland, Jones *et al.*, (1979) studied a commercial housed flock with an annual occurrence of pneumonia. They examined 34 lambs, 26 of which had pneumonic lesions and recovered *M. ovipneumoniae* and *P. haemolytica* only, from all the pneumonic lungs.

There have been mixed results following the use of conventionally-reared sheep and Sharp *et al.*, (1978b) consistently failed to develop pneumonic lesions in this type of animal. In New Zealand, Alley and Clarke (1979) experimented with three groups of conventionally-reared lambs using intranasal inoculation of pneumonic lung homogenate containing *P. haemolytica*, *M. ovipneumoniae*, *Neisseria*, *Streptococci* and *E. coli* and a cloned isolate of *M. ovipneumoniae*. They found 85% of sheep



inoculated with whole pneumonic lung homogenate developed lesions indistinguishable from field cases of CNP whereas only 20% of sheep inoculated with *M. ovipneumoniae* alone developed mild pneumonia. They suggested that *M. ovipneumoniae* alone could colonize ovine lung and produce mild pneumonic lesions but the production of severe lesions required a further agent which was most probably *P. haemolytica*.

Jones *et al.*, (1982c) were successfully able to reproduce proliferative exudative pneumonia in SPF lambs with cloned cultures of *M. ovipneumoniae* and *P. haemolytica*. They compared the ability of mixed strains of *M. ovipneumoniae* and a single strain of *M. ovipneumoniae* with *P. haemolytica* and observed the mixed strain combination of *M. ovipneumoniae* and *P. haemolytica* produced more severe lesions, which they thought was due to synergistic effects. They confirmed that *M. ovipneumoniae* alone may produce mild PE pneumonia but the inclusion of *P. haemolytica* in the challenge inoculum increased the morbidity and severity of the disease. Gilmour *et al.*, (1982) confirmed these findings using 15 conventionally - reared lambs injected intratracheally with a lung suspension containing *P. haemolytica* and *M. ovipneumoniae*. All lambs developed pneumonic symptoms after 3 weeks of age. The sheep were killed 3 to 28 weeks post inoculation and they recovered *M. ovipneumoniae* from the lungs of every animal up to 28 weeks post inoculation and *P. haemolytica* up to 11 weeks.

In a review of 10 experiments on the reproduction of chronic pneumonia in sheep by the use of pneumonic lung homogenate suspensions, Jones *et al.*, (1986) reported consistent recovery of *M. ovipneumoniae* and *P. haemolytica* but only occasional recovery of PI-3. They strongly argued that *M. ovipneumoniae* and *P. haemolytica* are

the aetiological agents of chronic pneumonia whereas PI-3 virus is an opportunistic invader.

#### **1.4.2.3 Gross pathology:**

The lesions consist of varying degrees of dull-red consolidation, red-grey consolidation, grey-red consolidation and grey consolidation in the antero-ventral parts of both lungs (Alley, 1975). The consolidated tissue varies from finely granular to unevenly granular sometimes with numerous grey-white foci. It is generally very firm and the affected lobes are thicker than normal. The cut surface has a finely mottled texture or is homogenous and pale-grey in appearance. Occasionally, fibrous adhesions are present between the pleural surface of the affected lobes and the parietal pleura or between the caudal surface of the cardiac lobes and the antero-ventral surface of the diaphragmatic lobes of both lungs (Alley, 1975; McGowan *et al.*, 1978).

In addition to these types of consolidation, the ventral margins of apical and cardiac lobes of some cases shows dull-red consolidation which merges dorsally with a zone of emphysematous lung in an irregular and patchy manner (Alley, 1975). Sometimes thin or thick linear bands of collapse are found in the apical lobes. Pleural and pulmonary abscesses are occasionally encountered in sheep of all ages. In young lambs, these are usually multiple lesions, less than 1 cm in diameter disseminated throughout the ventral areas of the lung and surrounded by variable areas of red consolidation. In older lambs the abscesses are invariably associated with lesions of pre-existing chronic pneumonia. Sullivan *et al.*, (1973a) reported the macroscopic changes as grey, less collapsible lungs with lobular atelectasis.

#### 1.4.2.4 Histopathology:

Alveolar collapse and severe infiltration of neutrophils and macrophages into the alveolar spaces characterize the histological lesions. Type-2 cell (pneumocyte) hyperplasia, interstitial infiltration with lymphocytes and plasma cells and peribronchial and perivascular lymphoid hyperplasia are the outstanding histological features. There is also marked hyperplasia of bronchiolar and alveolar epithelium (Alley, 1975). The predominant microscopic changes of proliferative interstitial pneumonia described by Sullivan *et al.*, (1973a) were proliferation of 'alveolar septal cells' and proliferative changes in the epithelium of the bronchioles together with perivascular and peribronchial lymphoid hyperplasia.

In chronic cases marked epithelial hyperplasia as well as peribronchiolar fibrosis and alveolar interstitial thickening are prominent. Proliferation of fibrous tissue in the walls of the bronchioles results in the formation of nodular scars, which partially obstruct the bronchiolar lumen (Alley, 1975; Stamp and Nisbet, 1963).

### 1.4.3 Systemic Pasteurellosis:

Systemic pasteurellosis is usually caused by “T” strains and sometimes by “A” strains of *Pasteurella haemolytica*. It is not a common disease in New Zealand although the organism has occasionally been isolated (Black, 1997). Lambs under 3 months of age are mainly affected by “A” types and the infection is systemic rather than pneumonic although severe pleurisy and lesions in the pericardium are also present (Gilmour and Gilmour, 1989). Systemic infection with “T” types usually occurs in lambs aged 5 to 12 months during autumn. The disease is characterized by sudden death. Predisposing factors include feeding on rape or turnips, a change to better pastures or changes in weather. The literature on this disease has been reviewed in detail by Gilmour and Gilmour (1989).

On post-mortem examination subcutaneous haemorrhages are seen over the neck and thorax. Marked serosal haemorrhages are found in the pleura, epi- and endocardium, heart valves, mesenteric lymphnode and spleen. The trachea and bronchi are congested and contain blood stained froth. The lungs are congested, heavy with bluish discolouration. Blood-tinged exudates are present in the pleural, peritoneal and pericardial cavities and these contain fibrin strands. Death is probably due to toxemia caused by circulating endotoxins (Gilmour and Gilmour, 1989).

## Chapter 2

### Prevalence of *Pasteurella (Mannheimia) haemolytica* in ewes and their lambs

#### 2.1 Introduction

The importance of *Pasteurella haemolytica* as a pathogen of sheep is well established and has been reviewed in Chapter 1. Although the majority of studies of the frequency and recovery rate of *P. haemolytica* have been made overseas, many valuable studies have also been undertaken in New Zealand (Salisbury, 1957; Alley, 1975; Alley and Clarke, 1979; Davies *et al.*, 1980; Alley, 1991; Prince, 1985; Midwinter, 1987). However, no detailed studies have yet been completed on the natural transmission of *P. haemolytica* from ewes to their offspring in New Zealand.

This study was performed to gather information on the following:-

- the transmission of *P. haemolytica* from ewes to their offspring under New Zealand farming conditions
- the difference in the nasal carriage of *P. haemolytica* between sheep flocks on different New Zealand farms
- the seasonal differences in nasal carriage of *P. haemolytica*
- the relationship between nasal carriage of *P. haemolytica* and the subsequent occurrence of pneumonia.

## **2.2 Materials and methods**

### **2.2.1 Selection of farms and animals:**

Four sheep farms using differing management practices, located in different areas of the Manawatu district surrounding Palmerston North were selected for this study. Two of these were intensive flat-land units where lambs were raised for a 3 – 4 month period prior to slaughter and the other two were hill country farms where lambs were raised for at least a 5 – 8 month period before sale or slaughter. Approximately twenty ewes from each farm were selected arbitrarily (total = 87) and their offspring (total = 96) were ear-tagged or numbered for identification and further sampling (see Table 1).

### **2.2.2 Collection of specimens on the farm:**

The external nares of each ewe and their offspring were carefully cleaned with cotton wool soaked in 70% alcohol before inserting the nasal swabs. Samples of nasal mucus were then obtained by inserting cotton tipped, microbiological swabs (sterile culture swab in Amies medium) intranasally to a maximum depth of about 15 cm depending on the size of animal. After insertion into the right ventral nasal cavity, the swabs were first rotated clockwise then anticlockwise and then withdrawn about 10 cm and inserted into the right dorsal nasal cavity where they were rotated in a similar manner. The swabs containing samples of nasal mucus were then placed immediately into transport medium before transportation to the laboratory.

**Table 1: Four Manawatu farms from which *P. haemolytica* samples were collected from the naso-pharynx of ewes and lambs:**

Name of farm	Location	Size of farm	No. of ewes	Breed	Stocking rate	Lambing %	Soils	Pasture	Management practice
Mirawai Farms Ltd.	Kopane	178 ha	850	Romney ewes x Poll Dorset sire	11 Su / ha	115%	Opiki peat, Te Arakura silt loam, Kairanga loam	Tetraploid perennial ryegrass and clover.	Sheep and cattle, early lamb production plus crops. 45% crops and 55% livestock
Robert A. Bruce	Glen Oroua, RD 3, PN	26.5 ha	150	Poll Dorset x Romney	18 Su / ha	135%	Silt, sord, clay	Ryegrass/ Clover	25% sheep & 75% cattle
Tikoke	Pahiatua	200ha	600	Perendale	10 Su/ha	95%	Yellow-brown earths	Browntop	Breeding unit, set stocking
Tuapaka	Forest Hill	365 ha	2000	Romney x Suffolk	11 Su/ ha	110%	Makara steepland, yellow-brown earths	Browntop, ryegrass, prairie grass and white clover	Sheep and bull beef, breeding unit and set stocking

2.2.2.1 Times of sample collection:

The selected lambs from Farm 1 (Mirawai Farms Ltd) and Farm 2 (Robert A. Bruce) were swabbed 3 times at monthly intervals from August 1998 until slaughter in November 1998. Lambs from Farm 3 (Tikoke) were sampled 7 times from August 1998 to June 1999 and Farm 4 (Tuapaka) four times from October 1998 to February 1999. At the time of the initial sampling the mothers of each of the selected lambs were identified by observing their suckling behaviour and these ewes were also swabbed.

Table 2: Times of sample collection from four different farms

Month	Farm 1	Farm 2	Farm 3	Farm 4
August	1 <sup>st</sup> sampling		1 <sup>st</sup> sampling	
September	2 <sup>nd</sup> sampling	1 <sup>st</sup> sampling		
October	3 <sup>rd</sup> sampling and killing	2 <sup>nd</sup> sampling	2 <sup>nd</sup> sampling	1 <sup>st</sup> sampling
November		3 <sup>rd</sup> sampling and killing	3 <sup>rd</sup> sampling	2 <sup>nd</sup> sampling
December		Killing		3 <sup>rd</sup> sampling
January			4 <sup>th</sup> sampling	
February		Killing		Killing
March			5 <sup>th</sup> sampling	
April			6 <sup>th</sup> sampling	
May				
June			Killing	



## 2.2.2.2 Microbiological analysis of the nasal samples:

### 2.2.2.2.1 Plating of nasal swabs:

After transportation to the laboratory each swab was smeared over the surface of a blood and Mac-Conkey agar plate (see Appendix I) and aerobically incubated at 37°C for about 24 hours. The growth pattern of *P. haemolytica* on blood agar was recorded. Typical *P. haemolytica* colonies of 0.1 to 0.2 cm diameter with a smooth surface, entire edge, low convex shape, translucent appearance and surrounded by a distinct narrow zone of beta haemolysis (Biberstein, 1958) were selected for subculture. A selected typical (single) colony was subcultured on blood agar and incubated at 37°C for 24 hours to isolate the organism in pure culture.

### 2.2.2.2.2 Isolation and identification:

One colony from the purified plate was inoculated into heart infusion broth (HIB) at 37°C for 24 hours. Three to four drops of the inoculum from the heart infusion broth (HIB – Appendix I) were then inoculated onto Triple sugar iron (TSI – Appendix I) agar slants, urease agar, arabinose, trehalose and galactose sugar broth and half of a blood agar plate. A heavy inoculum (15 – 20 drops) was also inoculated onto the half of a Mac-Conkey agar plate. This was incubated aerobically at 37°C for 24 hours. Isolates, which showed the following characteristics, were considered to be *P. haemolytica* and stored at – 70°C (Appendix II). The isolates: -

- Showed typical colony morphology (as above)
- Were Gram-negative cocco-bacilli on microscopy
- Had the ability to grow on Mac-Conkey agar and produce small pink or reddish colonies.
- Were oxidase positive (see Appendix I)
- Were urease negative (see Appendix I)
- Produced acid throughout TSI agar but no gas or hydrogen sulphide (see Appendix I).

### **2.2.3 Collection of specimens at slaughter:**

The lungs from the lambs in each of the study groups together with their corresponding heads were collected from the local meat works (Lambpackers, Fielding, AFFCO, Wanganui and Richmond Meat works, Oringi) immediately after slaughter and transported to the laboratory for examination. Samples of nasal mucus were obtained by cutting the head sagittally, slightly to one side of the midline with an electric band saw and removing the nasal septum with bone forceps. A sterile swab was then drawn over the exposed turbinate and inserted into both the dorsal and ventral nasal meatus. Incising the trachea transversely midway along its length and inserting the sterile swab distally as far as bifurcation of the main bronchi allowed the collection of bronchial swabs. A three to four centimetre area of the right apical lung lobe was removed from each animal with clean forceps and scissors for culture.

The nasal and bronchial swabs were plated out initially onto 5% sheep blood agar and Mac-Conkey agar. The surface of the lung sample was heat seared before plating out in a similar manner. A similar protocol for the identification and storage of *P. haemolytica* was followed to that described earlier.

### **2.2.4 Statistical methods:**

The Minitab statistical software programme was used to determine the significance of the results.  $P < 0.05$  was considered significant and  $P < 0.01$  highly significant.

2.3 Results

2.3.1 Prevalence of *Pasteurella haemolytica* in ewes and their lambs:

2.3.1.1 Prevalence of *P. haemolytica* in ewes:

The prevalence of *P. haemolytica* in the nasal swabs of ewes on the four different farms at the time of lambing was recorded. The results are summarized in Table 3. The highest rate of carriage was seen on Farm 1 (59%) and the lowest rate was seen on Farm 3 (33%).

**Table 3: Prevalence of *P. haemolytica* in the nasal swabs of ewes from four Manawatu farms**

Farm ID	Month	No. of ewes tested	No. of positive ewes	% of positive ewes
No. 1 (Mirawai Farms Ltd.)	August	22	13	59%
No 2 (Robert A. Bruce)	September	22	12	55%
No. 3 (Tikoke)	August	21	7	33%
No. 4 (Tuapaka)	October	22	9	41%
Total		87	41	47%

2.3.1.2 Prevalence of *P. haemolytica* in lambs:

The monthly prevalence of *P.haemolytica* in lambs on four farms was recorded and has been summarized in Table 4. The swabs were obtained at approximately monthly intervals starting at the age of 5 – 10 days and extending until the time of slaughter which varied on each farm. Farm 4 showed a high rate of nasal carriage (55%) from an early age. The high rate was slightly lower during December but reached a peak again in February. Farm 1 also showed moderately high nasal carriage of *P. haemolytica* at an early age, but this reduced considerably as the lambs aged.

Farm 2 showed a reasonably low rate of nasal carriage throughout the 4 to 6 month period of the animal’s lifetime. In contrast, Farm 3 showed a very low rate of nasal carriage initially but this increased gradually throughout the summer to a peak in March, then fell away again in autumn and early winter (April to June).

**Table 4: Monthly prevalence of *P. haemolytica* in the nasal swabs of lambs from four Manawatu farms:**

Month	Farm 1	Farm 2	Farm 3	Farm 4
August - 98	9 / 22 (41%)	na	1 / 24 (4%)	na
September - 98	4 / 22 (18%)	7 / 26 (27%)	na	na
October - 98	9 / 19 (47%)	10 / 26 (38%)	5 / 24 (21%)	12 / 22 (55%)
November – 98	na	10 / 26 (38%)	6 / 24 (25%)	12 / 21 (57%)
December – 98	na	na	na	8 / 21 (38%)
January – 99	na	na	6 / 22 (27%)	na
February – 99	na	na	na	13 / 21 (62%)
March – 99	na	na	14 / 19 (74%)	na
April - 99	na	na	10 / 19 (53%)	na
June - 99	na	na	4 / 19 (21%)	na

Na = not available

**2.3.1.3 Prevalence of *P. haemolytica* in lambs after slaughter:**

The prevalence of *P. haemolytica* in the respiratory tract of the lambs at the time of slaughter was recorded. The swabs were obtained from the nasal cavity, bronchus and lungs as well as from any pneumonic lesions that were recorded and the results are summarized in Table 5. Farm 4 showed a very high rate of the carriage of *P. haemolytica* in nasal swabs (62%), bronchial swabs (62%) and lungs (62%). On postmortem examination a very large number of lambs (57%) showed severe pneumonic lesions. The other farms showed a lower rate of nasal carriage (21 to 39%) which was almost half that of Farm 4. Carriage rate of *P. haemolytica* in the bronchi and lungs varied from 0% to 9% in all other farms except Farm 4.

**Table 5: Prevalence of *P.haemolytica* in the respiratory tract after slaughter of lambs on four Manawatu farms:**

Farm ID	Nasal Swab	Bronchus	Lungs	Pneumonic lesions
No. 1	3 / 11 (27%)	0 / 11 (0%)	1 / 11 (9%)	0 / 11 (0%)
No. 2	9 / 23 (39%)	0 / 23 (0%)	0 / 23 (0%)	0 / 23 (0%)
No. 3	4 / 19 (21%)	1 / 19 (5%)	0 / 19 (0%)	0 / 19 (0%)
No. 4	13 / 21 (62%)	13 / 21 (62%)	12 / 21 (57%)	12 / 21 (57%)

2.3.2 Seasonal distribution of *P. haemolytica* carriage:

The seasonal pattern of *P. haemolytica* carriage in the nasal cavity was recorded on two farms that kept lambs for a 5 - 8 months period and this is summarized in Table 6 and 7.

Table 6: Seasonal distribution of *P. haemolytica* in lambs on Farm 3:

Sampling no.	Month	No. of lambs	No. of positive lambs
Ist sampling	Aug-98	24	1 (4%)
2nd sampling	Oct-98	24	5 (21%)
3rd sampling	Nov-98	24	6 (25%)
4th sampling	Jan-99	22	6 (27%)
5th sampling	Mar-99	19	14 (74%)
6th sampling	Apr-99	19	10 (53%)
After killing	Jun-99	19	4 (21%)

Table 7: Seasonal distribution of *P. haemolytica* in lambs on Farm 4.

Sampling no.	Month	No. of lambs	No. of positive lambs
1 <sup>st</sup> sampling	Oct-98	22	12 (55%)
2nd sampling	Nov-98	21	12 (57%)
3rd sampling	Dec-98	21	8 (38%)
After killing	Feb-99	21	13 (62%)

Farm 4 showed a high rate of nasal carriage (55%) from an early age. This high rate was slightly lower during December but peaked again in January and at slaughter in February. In contrast, Farm 3 showed a very low rate of nasal carriage initially which increased gradually throughout the summer to a peak in March but again fell in autumn and early winter (April to June).

Statistical analysis using the basic logistic regression test indicated a significant difference ( $p < 0.0001$ ) in carriage rates between months on Farm 3. The infection level was found to be significantly lower in August, and then increased significantly in the October – January – March period. Within the March - April period, differences between individual months were not significant

2.3.3 The transmission of *P. haemolytica* from mother to offspring:

The results obtained after swabbing the nasal cavity of ewes shortly after lambing and their lambs are summarized in Table 8.

Table 8: Presence of *P. haemolytica* in the nasal cavity of mother and offspring at 1 to 2 weeks after birth

Relationship	Farm 1 (22 lambs)	Farm 2 (26 lambs)	Farm 3 (24 lambs)	Farm 4 (22 lambs)
Mother and lamb both +	5 (23%)	4 (15%)	0 (0%)	6 (27%)
Mother -ve & lamb +ve	4 (18%)	3 (12%)	1 (4%)	6 (27%)
Mother +ve & lamb -ve	7 (32%)	8 (31%)	7 (29%)	3 (14%)

Only a small number of ewes and lambs (15/80) were found to be both positive for the presence of *P. haemolytica* when their nasal cavities were swabbed less than 2 weeks after birth. A similar number of lambs (14/80) were found to be positive without evidence of *P. haemolytica* in the nose of their mother. On the other hand 25/87 ewes were found to be positive to *P. haemolytica* without evidence of infection of their offspring.

Statistical analysis using the binary logistic regression test showed no significant difference in the infection rate between farms and it also indicated that whether the mother was infected or not did not affect whether the lamb was infected ( $G = 16.967$ ,  $DF = 7$  and  $p= 0.018$ ).



## 2.4 Discussion

### 2.4.1 Prevalence of *P. haemolytica* in ewes and their lambs:

This is the first study of the transmission of nasal organisms between ewes and their offspring in New Zealand and the first study to compare nasal carriage rates in lambs on different farms in the same district. It has shown that the rate of nasal carriage of *P. haemolytica* in ewes and their lambs on the four different farms varied considerably. In ewes, it varied from 33% to 59% in the month of August (Table 3). In lambs, the variation was more remarkable. The lowest recovery rate observed at 1 to 2 weeks after birth was 4% on Farm 3 and the highest was 55%, recorded on Farm 4 (Table 4). There have been a number of studies in New Zealand and overseas where *P. haemolytica* has been isolated from the nasal cavity of adult sheep in abattoirs or on farms. Samples taken in the abattoir revealed a high carriage of *P. haemolytica* between 60 - 70% of sheep. Alley (1975) examined the distribution of bacteria in the normal and pneumonic ovine respiratory tract from abattoirs during 1971 and 1972 and recovered slightly higher levels (73% and 78% respectively) of *P. haemolytica* from the nasal cavities than those seen in the present study. Gilmour *et al.*, (1974) surveyed 100 adult sheep heads from an abattoir in Scotland where they recovered *P. haemolytica* from 64% of nasopharynges. Hamdy *et al.*, (1959) studied the microbial agents in slaughtered lambs and their mothers in abattoir and isolated *P. haemolytica* from 68.3% of lamb throat swabs and 63.8% from the throats of their mothers.

Pfeffer *et al.*, (1983) investigated the prevalence and microbiological aspects of pneumonia in a flock of lambs at the abattoir where they isolated 61% of *P. haemolytica* from the nasal cavity of a surveillance group of lambs and 55% from a random group lambs.

Our lower recovery rate of *P. haemolytica* may be attributed partly to the fact that this was an on farm study. Carriage rates are likely to increase during travel or within the abattoir yards where animals are kept in very close contact and these carriage rates may therefore not represent the true on-farm situation. Never-the-less, abattoir studies may have the advantage of sampling a wider area of nasal cavity depending on the technique used but it was felt that the collection technique alone was not sufficiently important to account for the increases in rates of carriage seen at slaughter.

Our results were more in keeping with the findings of workers who have collected samples from live animals held on farms in New Zealand (Thurley *et al.*, 1977 and Prince *et al.*, 1985). Thurley *et al.*, (1977) studied the prevalence of pneumonia in lambs from November 1973 to March 1974 and recovered *P. haemolytica* from 9 of 27 (33%) animals. Prince *et al.*, (1985) cultured 110 isolates of *P. haemolytica* from nasal swabs of 200 sheep (55%) from four different farms and 40 isolates from 60 lungs (66%).

The overseas on-farm studies have shown a much lower frequency of *P. haemolytica* isolation than in New Zealand. These differences could be due to environmental, husbandry or breed differences. The nasal carriage of *P. haemolytica* in normal and pneumonic flocks was investigated by Biberstein and Thompson (1966) in Scotland who found a relatively low frequency (10.7% to 31%) in normal flocks and a much higher level in clinically normal sheep in pneumonic flocks (12% to 69%). More recently, Al –Tarazi and Dagnall (1997) studied the nasal carriage of *P. haemolytica*

in sheep and goats in Jordan and they recovered *P. haemolytica* from 11 – 27% of sheep during a 1-year survey.

### **2.4.2 Seasonal distribution of *P. haemolytica* carriage:**

The current study has suggested there may be a seasonal variation in the pattern of *P. haemolytica* carriage within the nasal cavity of lambs. Although the nasal carriage rate on Farm 1 and Farm 2 was almost consistent over the 3 - 4 month period of sampling (August – November), Farm 3 and Farm 4 showed a very different scenario. Farm 3 showed a very low rate of nasal carriage initially which increased gradually throughout the summer to a peak in March but fell away again in autumn and early winter (June). On Farm 4 the initial high rate (55%) continued throughout the whole period (October to February) although it was slightly lower during December (38%). Season variations have been studied previously by Pfeffer *et al.*, (1983) who carried out a study on lambs from August 1978 to June 1979 on a property in Hawke 's Bay. They found a large increase in carrier rates from January to June (84 – 93%) with the exception of March (57%). Thurley *et al.*, (1977) earlier investigated the incidence of enzootic pneumonia at Wallaceville over the two seasons 1973-75. They found that the isolation of *P. haemolytica* was lower during November to January, after which the rate increased abruptly (February – June). They also monitored 15 lambs at monthly intervals from November 1974 to June 1975. The initial recovery rate was 27% in November and this increased gradually, until from February to June all the 15 lambs (100%) had *P. haemolytica* in their nasal cavity.

In Scotland, Biberstein *et al.*, (1970) discovered a bimodal curve in carrier rates of *P. haemolytica* over a 12 month period in several flocks. They found the first peak occurred in late autumn and the second in late spring to early summer and the peaks coincided with the seasonal pattern of enzootic pneumonia in that region. Samples

submitted for a monthly count of *P. haemolytica* at the Moredun Research Institute during 1982 – 1986 found May and June were the months which consistently had the highest isolation rates and this coincided with outbreaks of pneumonia (Gilmour and Gilmour, 1989).

The results of the present study confirm the work of these previous investigators. A possible reason for the increase in nasal carriage rates during the summer months is the hot-dry weather during the February – March period accompanied by nutritional stress and dehydration which could lower the innate resistance to *P. haemolytica* and allow it to proliferate in the respiratory tract and spread to other sheep. After drought when the pasture growth has improved, the sheep may regain their resistance and clear the *P. haemolytica* from the respiratory tract (as in Farm 3). Late summer and autumn are also peak periods for the occurrence of other debilitating diseases such as facial eczema and high parasite burdens in lambs.

A second important factor likely to affect the seasonal increase in the carriage rates is an increase in management procedures. At this time of the year lambs are often mustered regularly and confined to yards or sometimes housed overnight for shearing or crutching, fly-strike control, drenching and weighing. These procedures would provide excellent opportunities for the spread of nasal micro-organisms. If a particular strain of *P. haemolytica* is virulent, the stocking intensity is high, and the animals are under physiological stress, the pulmonary defense mechanisms may fail to clear the *P. haemolytica* from the respiratory tract and allow it to rapidly colonize and proliferate in lung tissue causing pneumonia and pleurisy.

This present study has also indicated that a seasonal increase in carriers on some farms may not necessarily be the cause of overt pneumonic disease; but might only reflect an increased weight of infection. After killing the lambs from all 4 farms, no

overt pneumonic lesions were found in lambs from 3 of these. Farm 4 was an exception and 57% of lambs exhibited pneumonic lesions (Table 5). This suggests that the presence of certain strains of *P. haemolytica* may be important in the pathogenesis of pneumonia and highlights the need for further work in identifying and studying the different strains of the organism. However, the between farm differences may also reflect differences in farm environment and management.

### **2.4.3 The transmission of *P. haemolytica* from mother to offspring:**

Earlier work in Scotland claimed that a transfer of nasal organisms from ewe to lamb occurred within the first few days of the lamb's life due to the close contact between lambs and their mother (Shreeve and Thompson, 1970). Thereafter the carriage rate of the organism in the flock increased with age. Hamdy *et al.*, (1959) investigated *P. haemolytica* isolates from throat swabs of lambs and their mothers and noted that all *Pasteurella* organisms isolated from lambs were morphologically and biochemically similar to those from their dams.

In the present study, the nasal carriage of *P. haemolytica* in 87 ewes and their 96 lambs was monitored at monthly intervals on four different farms. We found only 15 ewes and their lambs which both carried *P. haemolytica* concurrently. The rest of the ewes and lambs were either positive to *P. haemolytica* individually or both negative. Statistical analysis indicated that infection of the mother did not affect the chance of infection of the lamb.

This work was carried out on four farms in the Manawatu region only and therefore needs to be repeated on a wider scale and for a longer time period before weaning to confirm these findings. Never-the-less, it has shown that infection of lambs from their mothers is not consistent and suggests that lambs may also get infected from other in-contact sheep at an early age. Recent work by Frank *et al.*, (1996) has suggested that lambs could become infected from nasal contact with other ewes or from the sputum of infected animals and Buriel (1997) recovered *P. haemolytica* from the grass, water or straw-bedding used by infected sheep.

## Chapter 3

### A Study of *Pasteurella haemolytica* isolates using pulsed-field gel electrophoresis.

#### 3.1 Introduction

Schwartz and Cantor (1984) first developed pulsed field gel electrophoresis (PFGE) to separate large DNA molecules. This method revolutionized both microbial genetics and the study of larger genomes of microorganisms and is now widely used in the epidemiological and ecological investigation of microbial disease.

Olive and Bean (1999) compared PFGE with other molecular typing methods and found it to be a unique technique, which they suggested should become the gold standard for discriminating bacteria at a strain level. Pulsed field gel electrophoresis is a modified technique for restriction endonuclease analysis of the chromosome (REAC) that has a greater resolving power for strain analysis. DNA fragments larger than 40 kb are not efficiently resolved or filtered through the largest agar pores during conventional agarose gel electrophoresis. In addition, with conventional technique, there are also difficulties in obtaining suitable fragments of chromosomal DNA because of the shearing of large DNA molecules in solutions. Therefore the physical characterization of bacterial DNA has been limited at the genomic level.

Pulsed field gel electrophoresis has been able to overcome these constraints by preparing unit length chromosomal DNA using the in situ lysis of bacteria embedded in agarose and subsequent digestion of the DNA directly in the agarose with rare-cutting restriction endonucleases. This results in the generation of a small number of large DNA fragments that are resolved into a pattern of discrete bands in an agarose

gel by pulsed field gel electrophoresis. Thus, total genomic DNA can be resolved into a limited number of restriction fragments with distinct electrophoretic mobilities. The patterns are then finally examined by UV transillumination of ethidium bromide stained gels (McClelland *et al.*, 1987).

The main principle of PFGE is to separate the DNA fragments by alternating the electric field between pairs of electrodes. In this way the fragments are forced to re-orient and move at different speeds by changing the field direction. The large fragments are more slowly re-oriented than smaller fragments, and thus different patterns of DNA fragments are generated.

There is no gold standard of pattern for interpretation of pulsed field types. Tenover *et al.*, 1995 proposed a standardized criteria to interpret PFGE patterns from bacterial strains recovered during epidemics. They termed the bacterial isolates 'closely related' if the isolates differed by one to three bands, and 'possibly related' if the isolates had four to six band differences. Isolates were considered 'unrelated' if there were six or more band differences in the pattern produced.

Recently, overseas workers have found the pulsed field gel electrophoresis technique is highly suitable to discriminate strains of *P. haemolytica* (Kodjo *et al.*, 1999). The aim of the present study was to apply this technique to strains of *P. haemolytica* isolated from ewes and their lambs from four different farms around the Manawatu region in order to gain further information on the colonisation of the ovine respiratory tract and its relationship to the occurrence of pneumonia.



## 3.2 Materials and Methods

This PFGE method used follows that described by Fenwick (1997) with some minor exceptions.

### 3.2.1 Selection of isolates:

Seventy-three isolates were selected for PFGE study from a total 244 frozen isolates of *P. haemolytica* stored at  $-70^{\circ}\text{C}$  that were obtained from the four study farms (Chapter 2). These consisted of 28 isolates of *P. haemolytica* from 14 ewes and their lambs (obtained just after birth) in which both the ewe and her lamb were positive on microbiological investigation. Thirty-three isolates of *P. haemolytica* from lambs obtained over the spring and summer months to examine similarity in the pattern of isolates. Twelve isolates of *P. haemolytica* were selected from the two nostrils of two lambs from Farm 4 to examine the consistency of recovery of *P. haemolytica* during three consecutive days.

### 3.2.2 Preparation of DNA plugs from *P. haemolytica* strains:

The frozen pure culture of each *P. haemolytica* strain was inoculated onto a blood agar plate and incubated at  $37^{\circ}\text{C}$  for 24 hours. The isolates were checked for cell morphology and their Gram stain reaction. Three to four *P. haemolytica* colonies were selected and inoculated into brain heart infusion broth (Difco) and incubated at  $37^{\circ}\text{C}$  overnight.

Between 500 to 700  $\mu\text{l}$  of the overnight culture (optical density of 1.4 at 610nm) was centrifuged at 13,000 rpm for 5 min. The pellet was resuspended in 150- $\mu\text{l}$  of ice-cold PETT IV buffers (1 M NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) and

centrifuged again at 13,000 rpm for 5 min. The pellet was finally resuspended in 50  $\mu$ l of cold PETT IV buffer.

The cell suspension (50  $\mu$ l in PETT IV buffer) was mixed with 75  $\mu$ l of 1% molten (60 – 80°C) low melting point PFC agarose (Pulsed-field certified, Bio-Rad). Seventy-five microlitre aliquots of the agarose-cell suspension were added to plug moulds and left to solidify for 15 min at 4°C.

The agarose plugs were added to the 1 ml of urea-ESP buffer (6 M urea, 50 mM Tris pH 8.0, 50 mM EDTA pH 8.0; 1% sodium lauroyl sarcosine, 0.2% sodium deoxycholate, 0.5 mg ml<sup>-1</sup> proteinase k) and incubated in a water-bath at 56°C overnight.

The plugs were washed eight times in 10 ml of TE buffer pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) for one hour on a rocking machine. The plugs were transferred into an eppendorf containing 1 ml of TE buffer and stored at 4°C until required.

### **3.2.3 Restriction endonuclease digestion of DNA embedded in agarose plugs:**

A number of restriction enzymes such as Sma 1, Not 1, Sal 1 and Apa 1 were assessed to find out the appropriate enzyme for digesting *P. haemolytica* isolates. After assessing the cutting ability and the number and size of bands produced, Sal 1 was found to be the most appropriate enzyme.

A 2 mm strip of DNA plug was excised using a sterile scalpel blade and placed in an eppendorf containing 100  $\mu\text{l}$  of 1 x restriction buffer (12  $\mu\text{l}$  of Sal 1 buffer, 1  $\mu\text{l}$  100 mg ml<sup>-1</sup> Bovine Serum Albumin (BSA), 87  $\mu\text{l}$  of sterile distilled water) and equilibrated for one hour on ice.

The restriction buffer was then removed and 80  $\mu\text{l}$  cutting buffer (8  $\mu\text{l}$  10 Sal 1 buffer, 0.8  $\mu\text{l}$  100 mg ml<sup>-1</sup> BSA, 15 units (2  $\mu\text{l}$ ) restriction endonuclease, 61  $\mu\text{l}$  distilled water) was added.

The plugs and buffer were equilibrated for 45 min on ice and incubated overnight in a water-bath at 37°C.

### **3.2.4 Pulsed-field gel electrophoresis of digested DNA:**

Standard electrophoresis protocols were used for all isolates using a Contour Clamped Homogeneous electric field (CHEF) electrophoresis system (CHEF Mapper, Bio-Rad Laboratories, Richmond, California, USA).

The DNA-plug slices were loaded into the wells of a 1% agarose gel (800 mg in 80 ml of 0.5 x TBE buffer 1 M TRIS base, 1 M H<sub>2</sub>BO<sub>3</sub>, 2 mM di-Na<sup>+</sup> EDTA pH 8.0). The gel was equilibrated in the electrophoresis chamber with the buffer (0.5 X TBE) circulating for 2 h. Lambda marker (#340, New England Biolabs Inc., USA) was also run in the first and last run of each gel to provide DNA size standards. The wells were filled with 1% PFC agarose and the gel was left at room temperature for 10 min to allow the agarose to harden.

Two litres of 0.5 X TBE buffer were poured into the electrophoresis chamber. The water chiller and pump were turned on to equilibrate the buffer to 14°C and the gel was placed in the chamber. Electrophoresis was conducted under the following conditions: 6.0 V/cm; switch times - initial 3 secs, final 25 secs; ramp 79%; duration of run – 22 hours.

### **3.2.5 Staining, photographing and interpretation of pulsotype patterns:**

After electrophoresis, the gels were stained in fresh aqueous ethidium bromide (80  $\mu$ l of 10 mg ml<sup>-1</sup> stock solution in 800 ml MQ water) for 20 min. The gels were de-stained in water for 30 min, examined under UV light and photographed.

The photographic image of each gel was scanned and the graphic image enlarged for ease of visual interpretation. The analysis of the degree of dissimilarity of patterns of bands between isolates was performed using the criteria set forth by Tenover *et al.*, (1995) and Fenwick (1997):

Identical: all bands are in similar position

Closely related: differed by one to three bands

Possibly related: four to six band differences

Unrelated: six or more band differences

### 3.3 Results:

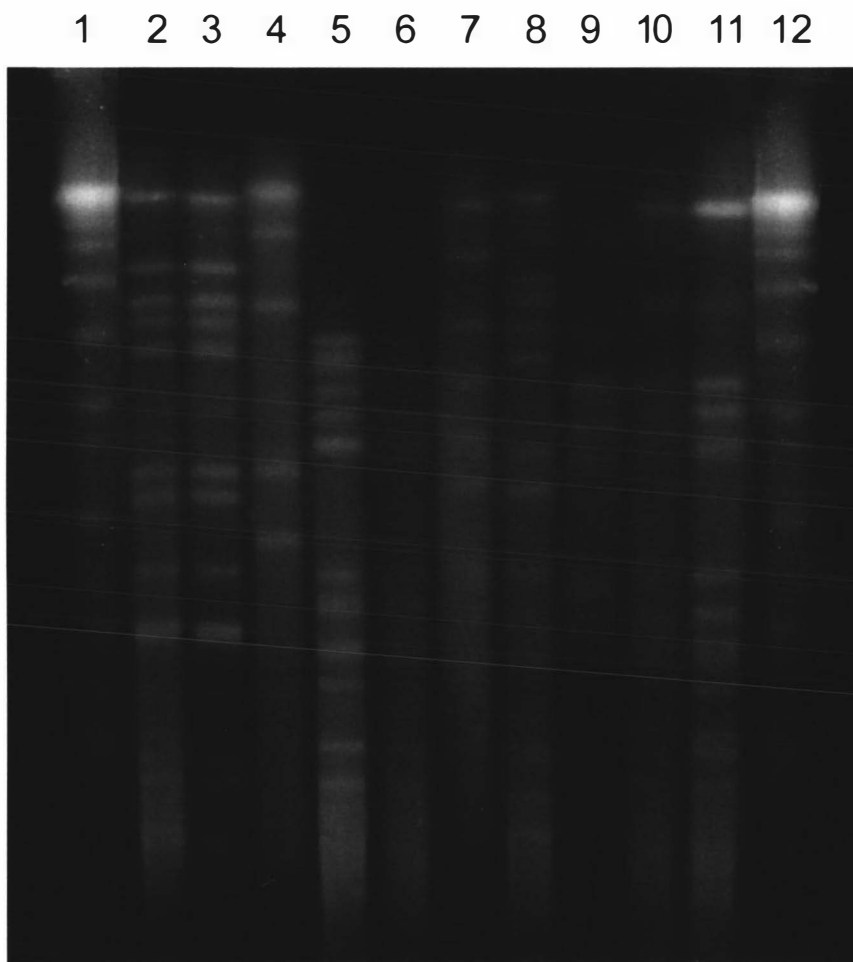
#### 3.3.1 Pulsed field profiles of isolates from ewes and their lambs:

Farm 1: the pulsotypes from five ewes and their lambs from Farm 1 is shown in Figure 1. Following the standardized criteria for interpreting the PFGE patterns described by Tenover *et al.*, 1995, isolates No. 2 and 14 (E 2 and L 2) were closely related pulsotypes. However, the strains of *P. haemolytica* isolates from the other four ewes and their lambs were found to be completely unrelated.

Farm 2: The banding pattern of five ewes and their lambs from Farm 2 is shown in Figure 2. The pulse field pattern of isolates from ewes and their lambs on this farm were totally unrelated. The strain of *P. haemolytica* obtained from one lamb (isolate No. 43) was found to be similar to that from another ewe (isolate No. 36) in the flock which was not her mother. Interestingly, the strain of *P. haemolytica* in the respiratory tract of one ewe was found to have a close similarity to the strain obtained from another ewe in the flock (isolates no. 34 and 35).

Farm 4: The pulsed field profiles of isolates from four ewes and their lambs on Farm 4 is shown in Figure 3. The profile of *P. haemolytica* from isolate No. 79 (TE 4) was found to very closely resemble that obtained from her lamb (TL 4). However, the banding pattern of isolates from other ewes and their lambs were totally unrelated.

Figure 1: Photograph of a PFGE gel containing isolates from ewes and their lambs from Farm #1

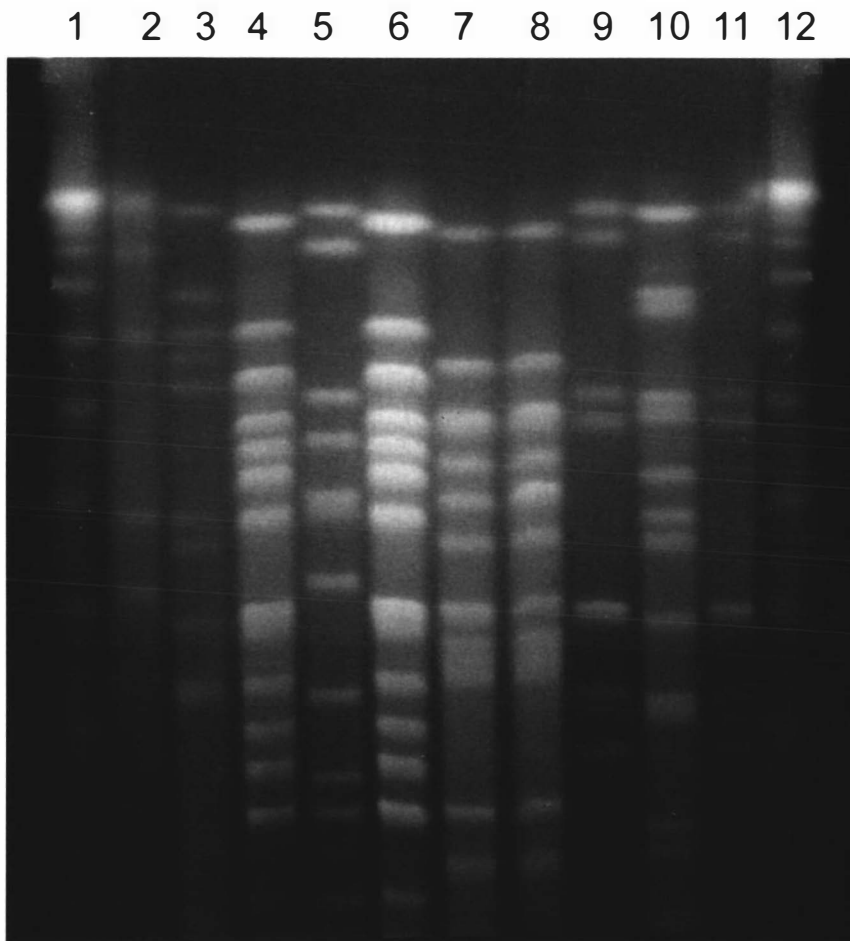


**Index: Figure 1: Isolates from ewes and their lambs from Farm #1**

Lane # (from right)	Isolate no.	Animal ID	Sample date
1	Lambda Marker		
2	2	E 2	12 – 8 - 98
3	14	L 2	12 – 8 -98
4	9	E 13	12 – 8 - 98
5	18	L 13	12 – 8 - 98
6	13	E 20	12 – 8 - 98
7	21	L 20	12 – 8 - 98
8	10	E 14	12 – 8 - 98
9	9	L 14	12 – 8 - 98
10	8	E 12	12 – 8 - 98
11	17	L 12	12 – 8 - 98
12	Lambda Marker		



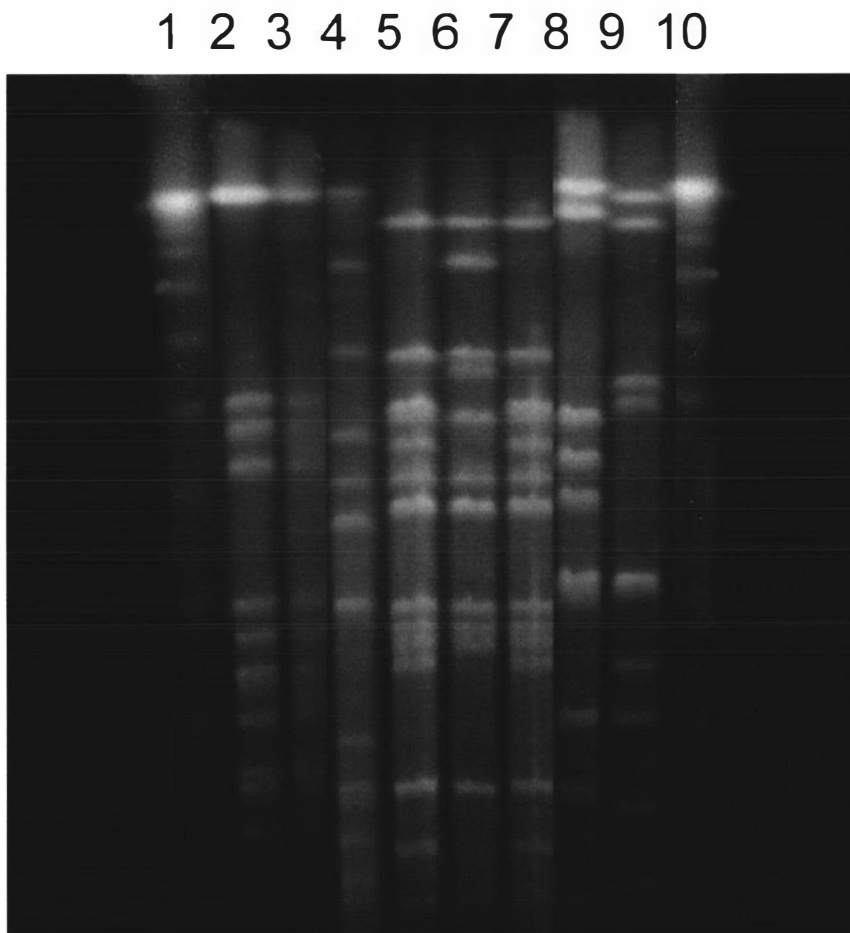
Figure 2: Photograph of a PFGE gel containing isolates from ewes and their lambs from Farm #2



**Index: Figure 2: Isolates from ewes and their lambs from Farm #2**

Lane # (from right)	Isolate no.	Animal ID	Sample date
1	Lambda Marker		
2	32	E 12	7 – 9 - 98
3	38	L 12	7 – 9 - 98
4	34	E 15	7 – 9 - 98
5	39	L 15	7 – 9 - 98
6	35	E 20	7 – 9 - 98
7	43	L 20	7 – 9 - 98
8	36	E 21	7 – 9 - 98
9	42	L 21	7 – 9 - 98
10	37	E 22	7 – 9 - 98
11	44	L 22	7 – 9 - 98
12	Lambda Marker		

Figure 3: Photograph of a PFGE gel containing isolates from ewes and their lambs from Farm #4



**Index: Figure 3: Isolates from ewes and their lambs from Farm #4**

Lane # (from right)	Isolate no.	Animal ID	Sample date
1	Lambda Marker		
2	79	E 4	12 – 10 - 98
3	67	L 4	12 – 10 - 98
4	80	E 5	12 – 10 - 98
5	68	L 5	12 – 10 - 98
6	85	E 20	12 – 10 - 98
7	87	L 20	12 – 10 - 98
8	83	E 21	12 – 10 - 98
9	74	L 21	12 – 10 - 98
10	Lambda marker		

### **3.3.2 The similarity of *Pasteurella haemolytica* strains recovered from lambs from birth until slaughter:**

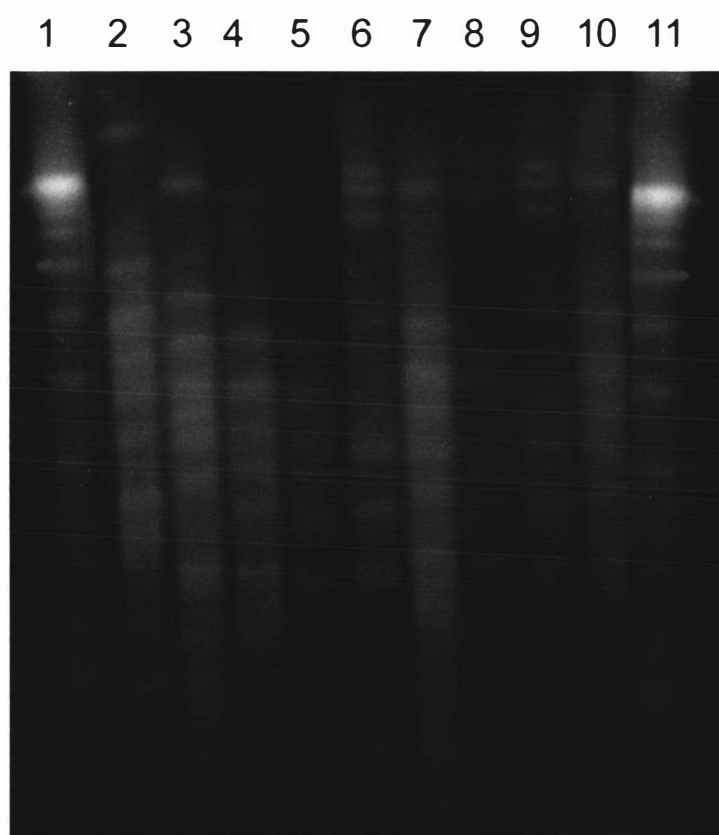
The similarity of *P. haemolytica* isolates from the nasal cavity of lambs from 3 farms was investigated from birth until the animals were slaughtered. The results showed that the persistence of *P. haemolytica* strains within the nasal cavity of individuals varied between farms. Some pulsotypes were consistently recovered from individuals over a 2-3 months period and could therefore be considered persistent members of the respiratory tract microbial flora (e.g., isolate no. 68, 119, 138, 189, 190). However, the majority of the strains could only be detected in the nasal cavity on one occasion during the period of lambs life.

The similarity of *P. haemolytica* strains from the respiratory tract of lambs from Farm 2 is shown in Figure 4. The results showed that isolate No. 16, 53, 46, 64, 42, 65 and 115 were only once present in the respiratory tract during the 3 months period. Only one *P. haemolytica* strain (isolate No. 15) was consistently present for two consecutive months.

The consistency of recovery of *P. haemolytica* isolates from Farm 3 is shown in Figure 5. Most of the *P. haemolytica* isolates from this farm were only detected once during a 5- 6 months period. One strain of *P. haemolytica* (isolate No. 131) from the respiratory tract of a lamb persisted consistently for two consecutive months.

The consistency of recovery of *P. haemolytica* isolates from the lambs from Farm 4 is shown in Figure 6. A distinctly different scenario was observed on this farm. Some strains of *P. haemolytica* were consistently isolated from the nasal cavity of these lambs. They dominated the respiratory tract microflora and persisted throughout the lifetime of the individuals.

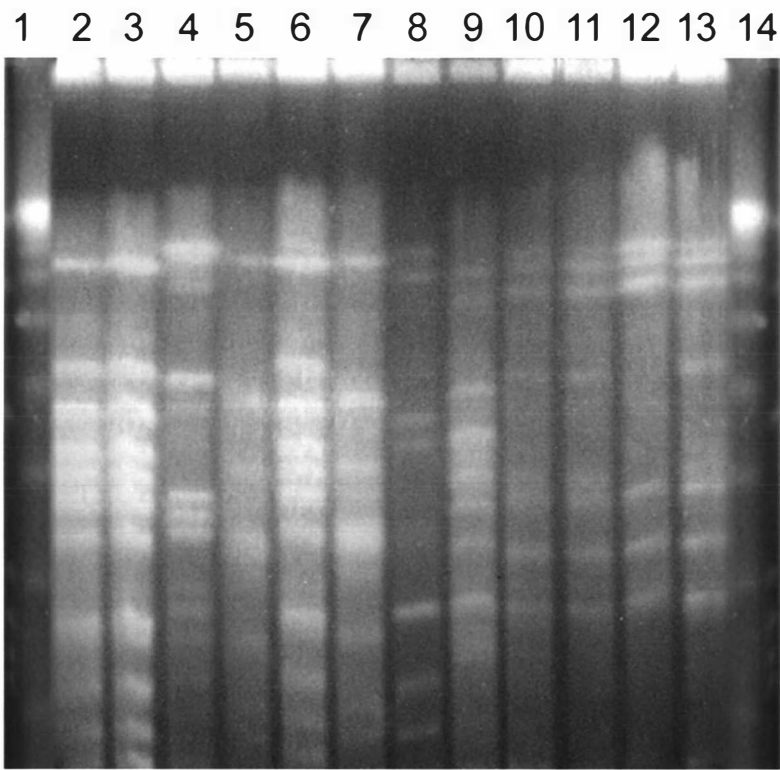
Figure 4: Photograph of a PFGE gel containing isolates recovered from lambs  
from Farm #2



**Index: Figure 4: Isolates recovered from lambs from Farm # 2**

Lane # (from right)	Isolate no.	Animal ID	Swabbing time
1	Lambda marker		
2	16	L 9	1st
3	53	L9	2nd
4	39	L 15	1st
5	112	L 15	3rd
6	46	L 18	1st
7	64	L 18	2nd
8	42	L 21	1st
9	65	L 21	2nd
10	115	L 21	3rd
11	Lambda marker		

Figure 5: Photograph of a PFGE gel containing isolates recovered from lambs from Farm #3

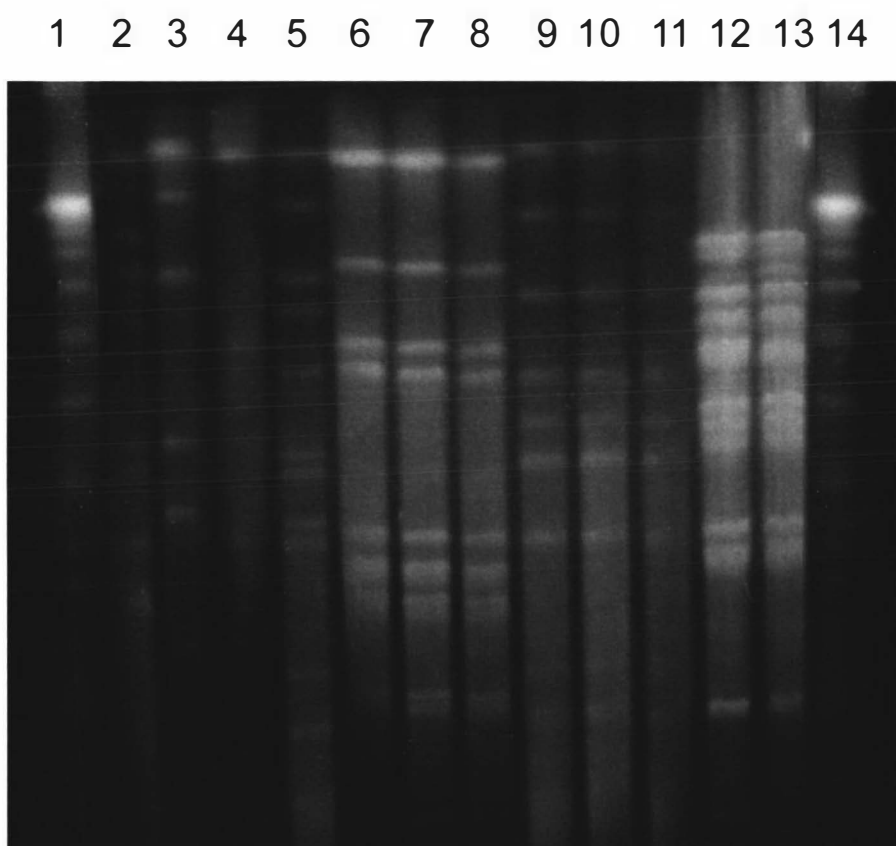




**Index: Figure 5: Isolates recovered from lambs from Farm # 3**

Lane # (from right)	Isolate no.	Animal ID	Swabbing time
1	Lambda marker		
2	131	L 17	3 <sup>rd</sup>
3	151	L 17	4 <sup>th</sup>
4	256	L 17	5 <sup>th</sup>
5	268	L 17	6 <sup>th</sup>
6	148	L 3	4 <sup>th</sup>
7	246	L 3	5 <sup>th</sup>
8	262	L 3	6 <sup>th</sup>
9	132	L 18	3 <sup>rd</sup>
10	248	L 18	5 <sup>th</sup>
11	269	L 18	6 <sup>th</sup>
12	251	L 10	5 <sup>th</sup>
13	265	L 10	6 <sup>th</sup>
14	Lambda marker		

Figure 6: Photograph of a PFGE gel containing isolates recovered from lambs  
from Farm #4



**Index: Figure 6: Isolates recovered from lambs from Farm # 4**

Lane # (from right)	Isolate no.	Animal ID	Swabbing time
1	Lambda marker		
2	67	L 4	1 <sup>st</sup>
3	137	L 4	3 <sup>rd</sup>
4	211	L 4	After killing (NS)
5	213	L 4	After killing (L)
6	68	L 5	1 <sup>st</sup>
7	119	L 5	2 <sup>nd</sup>
8	138	L 5	3 <sup>rd</sup>
9	219	L 5	After killing (B)
10	220	L 5	After killing (L)
11	70	L 12	1 <sup>st</sup>
12	230	L 12	After killing (B)
13	231	L 12	After killing (L)
14	Lambda marker		

After killing (NS) – *P. haemolytica* isolates from nasal septum after killing the lamb

After killing (B) - *P. haemolytica* isolates from bronchus after killing the lamb

After killing (L) - *P. haemolytica* isolates from lungs after killing the lamb

### **3.3.3 The consistency of recovery of *P. haemolytica* and the effect of close contact on the lambs:**

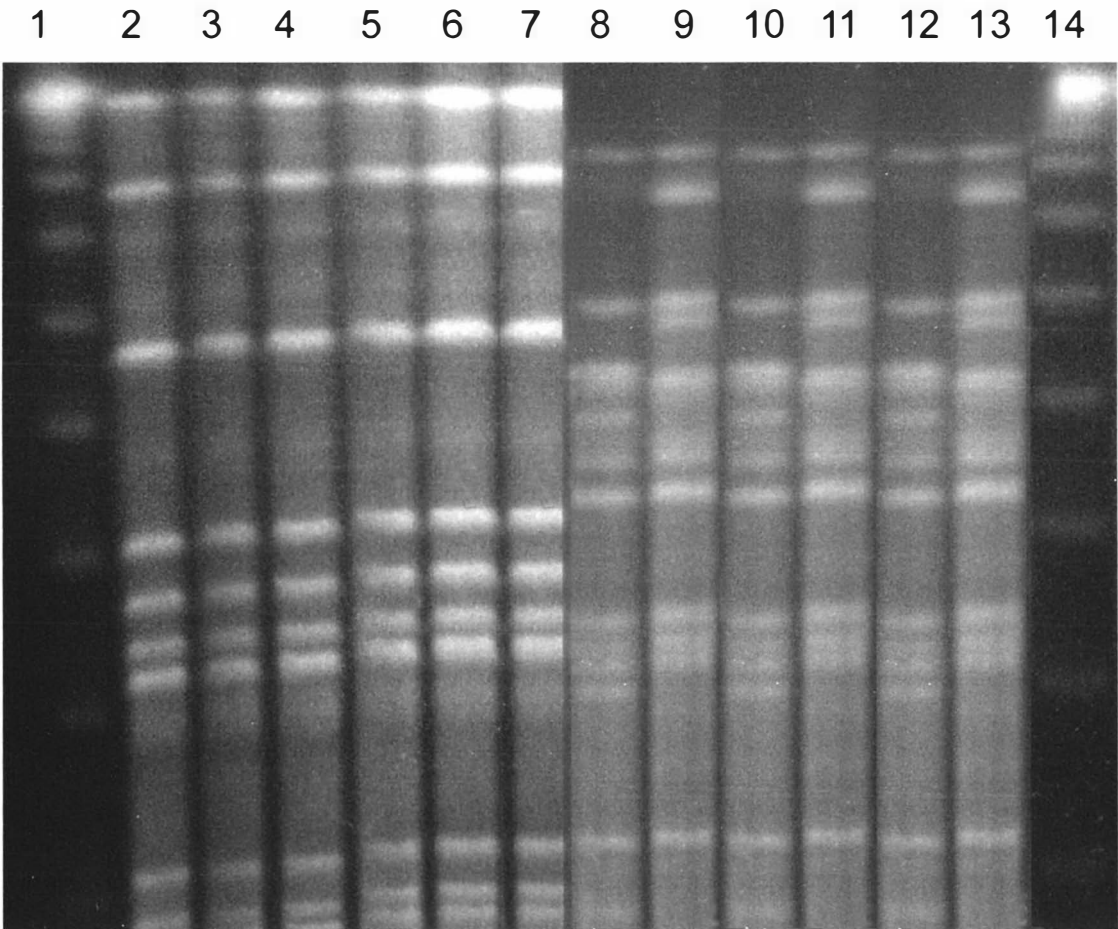
The accuracy of the swabbing technique and its ability to consistently recover the same isolate from nasal cavities on consecutive days was studied on Farm 4. Since the animals were yarded every day and kept in close contact a by-product of this study was to observe the effect of this degree of contact on the nasal flora. The results are shown in Table 9. All of the lambs except Lamb No 21 yielded *P. haemolytica* isolates with a similar PFGE pattern from both nostrils. The growth pattern of organisms on blood agar was also found to be very similar.

The effect of daily close contact was remarkable. Lambs that were negative at the initial swabbing became positive to *P. haemolytica* on the 4<sup>th</sup> day of the swabbing experiment.

The pulsed field profiles of isolates obtained from both nostrils of two of the lambs were studied in detail. The results illustrated in Figure 7 show that the same strain of *P. haemolytica* was present in both nostrils of both individuals for 3 consecutive days.



Figure 7: Photograph of a PFGE gel containing isolates from both nostrils of two lambs from Farm 4



**Index: Figure 7: Isolates from both nostrils of two lambs from Farm # 4**

Lane # (from right)	Isolate no.	Animal ID	Swabbing time
1	Lambda marker		
2	162	L 5	1 <sup>st</sup> day; left nostril
3	163	L 5	1 <sup>st</sup> day; right nostril
4	171	L 5	2nd day; left nostril
5	172	L 5	2 <sup>nd</sup> day; right nostril
6	183	L 5	3rd day; left nostril
7	184	L 5	3 <sup>rd</sup> day; right nostril
8	164	L 8	1 <sup>st</sup> day; left nostril
9	165	L 8	1 <sup>st</sup> day; right nostril
10	173	L 8	2nd day; left nostril
11	174	L 8	2 <sup>nd</sup> day; right nostril
12	185	L 8	3rd day; left nostril
13	186	L 8	3 <sup>rd</sup> day; right nostril
14	Lambda marker		

### 3.4 Discussion

It is now well established that pulsed field gel electrophoresis is a highly effective molecular method for discriminating bacterial strains. Over the last 10 years it has been used increasingly to identify the inter-strain relatedness of many bacterial genera (Lai and Birren, 1995; Shimizu *et al.*, 1997).

The technique has proven to be the most efficient subtyping and epidemiological study method currently available for several pathogenic bacterial strains (Fenwick, 1997; Kodjo *et al.*, 1999). Maslow *et al.*, (1993) also described PFGE as a highly effective molecular epidemiological technique because of its highly discriminatory physical mapping ability for bacterial chromosomes using known genes as probes. It was superior to both phenotypic and southern blotting techniques in discriminating isolates of *Escherichia coli*, *Staphylococcus aureus* and other species.

To date however, very little work has been performed on the subtyping and epidemiology of *P. haemolytica* using molecular methods. Kodjo *et al.*, (1999) analysed 130 strains of *P. haemolytica* and compared the discriminatory capability of different methods such as ribotyping, random amplified polymorphic DNA (RAPD) analysis and pulsed field gel electrophoresis. They recorded 15 ribotype patterns after digestion with Hind III for the *P. haemolytica* biotype A strains. They also found a total of 44 and 15 unique RAPD patterns for biotypes A and T respectively. They observed the same ribotype pattern for strains belonging to several distinct A serotypes and found a combined ribotype-RAPD pattern for biotype A strains of different serotypes from different animals and geographic regions. They identified 69 and 26 PFGE patterns for biotypes A and T respectively that showed their residual heterogeneity and the strain or group of strains appeared to be distinct. They did not detect any common clone using this technique from two different hosts or from



different locations. They found that PFGE was more efficient than ribotyping and RAPD analysis in discrimination of *P. haemolytica* strains.

The ribotyping and RAPD assays have proven less discriminatory in earlier work in cattle. Chaslus-Dancla *et al.*, (1996) studied the validation of RAPD assays and compared the method with ribotyping for epidemiological surveys of *Pasteurella* strains in cattle from a variety of sites and from different and independent feedlots. They only observed 3 ribotype and RAPD patterns after digestion of total DNA by Hind III and Pvu II out of 40 *P. haemolytica* strains.

In this study, several restriction enzymes including Sma I, Not I, Apa I, Xba I and Sal I were used for digesting the agarose embedded chromosomal DNA of *P. haemolytica*. The results showed that Sal I restriction endonuclease was the most effective for discriminating between *P. haemolytica* strains. This restriction enzyme produced 8-14 bands sufficient for discrimination between strains. This is in agreement with the recent overseas study of Kodjo *et al.*, 1999 who also identified this enzyme as most effective and appropriate for use with *P. haemolytica* isolates.

The detection of *P. haemolytica* strains in the nasal cavity within the first 1-2 weeks of the lambs life is in broad agreement with earlier studies of lambs in both Scotland and Jordan (Shreeve and Thompson, 1970; Al-Sultan and Aitken, 1985). Shreeve and Thompson (1970) detected the first typeable *P. haemolytica* colonies in the nasal passage 48 hours after birth. Al-Sultan and Aitken (1985) recovered both typeable and untypeable colonies from the tonsils 3 days after birth.

The method of acquisition of *P. haemolytica* by the respiratory tract of lambs is still debated. Several studies have proposed that newborn lambs receive the first *P. haemolytica* strains from their dams (Hamdy *et al.*, 1959; Shreeve and Thompson, 1970). This view was not supported by the results obtained in our study. Our results

showed that at the time of first swabbing of ewes and their lambs, most of the strains of *P. haemolytica* obtained from lambs were completely unrelated to those obtained from their mothers. However, in a few cases the ewe and lamb strains were found to be very closely related to each other with similar but not identical PFGE profiles. On one occasion, the strain of *P. haemolytica* recovered from the respiratory tract of the lamb was very similar to the strain recovered from another ewe in the flock, which was not its mother.

These results suggest several potential routes of infection may be involved and that new born lambs are more likely to become infected by *P. haemolytica* from in-contact ewes, other lambs or perhaps their environment rather than from their mother.

The establishment of *P. haemolytica* in the nasal cavity in relation to the age of lambs is still not clear. Earlier work found instability of the isolates of *P. haemolytica* in the nasal cavities of lambs. Biberstein and Thompson (1966) inoculated high doses of *P. haemolytica* into the nasal cavity of lambs and observed that most inoculated strains survived for only a few days, although one strain persisted for 10 weeks. This ability of certain strains to persist was partly confirmed in the current study. Our results demonstrated that most of the *P. haemolytica* strains on three of the farms were unstable and were present on only one occasion. Later sampling of the same lambs found the nasal cavity was occupied by a different strain.

On Farm 4 however, a different picture of colonisation emerged. Most of the strains of *P. haemolytica* obtained from the nasal cavities of lambs on this farm were present from birth to slaughter. This finding is particularly interesting because this was the only one of the four farms in which lambs were subsequently shown to have pneumonia when they were slaughtered at 5 months of age. The strains of *P. haemolytica* obtained from the pneumonic lungs and bronchus of two lambs were

however, not the same as the strains that had persisted for 3 months in the nasal cavity. These results show that after acquisition, some strains of *P. haemolytica* can persist in the nasal cavities of lambs for more than a 3- month period

The present study was performed by swabbing of only the right nostril of both lambs and ewes. There is little information on the distribution of the microbial flora within the nasal cavity and it was important to know whether swabbing of the right nostril was representative of the flora in the entire nasal cavity. The findings obtained by swabbing both the nostrils of 7 sheep over 3 consecutive days showed a consistent growth and isolation pattern from both nostrils.

The pulsed field profiles of two subjects studied in detail were also similar and the same PFGE strain was found in both nostrils over 3 consecutive days of nasal swabbing. These findings supported the view that the organisms are widely enough distributed within the nasal cavity to be adequately sampled using the technique employed in this study. These findings also demonstrated the effects of close contact at daily yarding and mustering on the transmission and carriage of the organism. The effect of introducing a virulent strain of *P. haemolytica* to a naïve population of sheep has been studied previously by Foreyt (1989). He raised 6 Rocky Mountain bighorn sheep in captivity over a year then placed them with 6 clinically normal domestic sheep. On initial swabbing of the nasal cavity of all sheep, he isolated *P. haemolytica* only from the domestic sheep. He observed fatal pasteurella pneumonia within 4 days of this contact and all bighorn sheep died within 4-71 days. *Pasteurella haemolytica* was isolated from the respiratory tract of all bighorn sheep. In our study, the isolation of a single pulsotype from 2 lambs over 3 consecutive days suggests that this strain of the organism had a superior ability to colonise and proliferate in the ovine upper respiratory tract of these individuals.

Further work in this area is necessary as it has important practical implications in preventing cross colonisation of potential pathogens in sheep husbandry procedures.

## Chapter 4

### General Discussion

This investigation has succeeded in combining conventional field microbiological techniques with new molecular techniques for the DNA fingerprinting of *Pasteurella haemolytica* to provide useful information on the nasal carriage of the organism in sheep and the epidemiology of ovine pneumonia. Although the study was limited in scope and concentrated on four farms in the Manawatu region, the results obtained provide evidence that ewe to lamb transmission of *P. haemolytica* is less common than previously thought and that considerable differences in rates of nasal carriage exist between farms. These findings suggest that further large-scale investigations of ovine nasal carriage are warranted.

A major criticism, which could be made of the field microbiological technique used, was a possible lack of accuracy of the method of swabbing employed for the recovery of *P. haemolytica*. The method used was a modified version of that previously used in New Zealand (Alley, 1975; Alley, 1991; Prince *et al.*, 1985; Pfeffer *et al.*, 1983) and workers overseas (Biberstein *et al.*, 1970; Biberstein, 1978; Gilmour and Gilmour, 1989). Other workers have used intranasal washing to recover microorganisms from the respiratory tract (Brockmeier *et al.*, 2002). Rohn *et al.*, (1998) studied the comparative evaluation of bacteriological findings from nasal swabs, tracheal swabs and tracheal washes from 107 calves with chronic respiratory diseases. They observed the rate of isolation of total bacteria was improved by 11.2% by using tracheal washings, except in the case of *P. haemolytica* where the organism was isolated most frequently from nasal swabs (39 nasal swabs, 35 tracheal swabs and 34 tracheal washes were positive).

In view of this criticism a small trial was set up to find how consistent the nasal swabbing technique was in recovering organisms (Chapter 3). Because the present study was performed by swabbing only the right nostril of both lambs and ewes the validity of this method of sampling required confirmation. There is little information on the distribution of the microbial flora within the nasal cavity and it was important to know whether swabbing of the right nostril was representative of the flora in the entire nasal cavity. The findings obtained by swabbing both the nostrils of 7 sheep over 3 consecutive days showed a consistent type of growth and isolation pattern from both nostrils.

The pulsed field profiles of two subjects studied in detail were also similar and the same PFGE strain was found in both nostrils over 3 consecutive days of nasal swabbing. These findings supported the view that the organisms are widely enough distributed within the nasal cavity to be adequately sampled using the technique employed in this study.

In the epidemiological investigation, the overall prevalence of *P. haemolytica* in ewes (33-59%) ranks New Zealand among those countries reported to have high isolation rates in on-farm studies (Chapter 2). However, the isolation rates of *P. haemolytica* in lambs were markedly variable (4 – 55%). It is important to recognise that the prevalence reported in this study may not reflect the total New Zealand picture. Only four farms within the Manawatu region were included in this study and one (Farm 3) showed a very low initial isolation rate (4%). The isolation rates on the other 3 farms followed a similar pattern (27 – 55%) to that detected in the earlier studies in New Zealand (Prince *et al.*, 1985; Pfeffer *et al.*, 1983).

The nasal sampling was undertaken at monthly or two-monthly intervals but unfortunately, the data does not provide enough detail to allow firm conclusions about

the seasonal variation of *P. haemolytica*. A more intense investigation would be necessary to accurately map the changing composition of the nasal flora throughout the year. Never-the-less, the current study indicates that the rate of carriage of *P. haemolytica* within the nasal cavity of lambs increases gradually throughout summer to a peak in March but falls away again in autumn and early winter (June). These findings confirm the work of the previous investigators in New Zealand (Pfeffer *et al.*, 1983; Thurley *et al.*, 1977).

A possible reason for the increase in carriage rates during the summer months is the hot- dry weather during the February – March period. This is often accompanied by nutritional stress and dehydration which could lower the innate resistance of lambs to *P. haemolytica*, allow the organism to proliferate throughout the respiratory tract and then spread to other sheep. When the pasture growth improves during late autumn and early winter, the affected lambs may regain their resistance and clear the *P. haemolytica* from the respiratory tract (as in Farm 3). Late summer and autumn are also peak periods for the occurrence of other debilitating diseases such as facial eczema and high parasite burdens in lambs.

A second important factor likely to affect the seasonal increase in carriage rates is an increase in number and variety of management procedures. At this time of the year lambs are often mustered regularly and confined to yards or sometimes-housed overnight for shearing, crutching, fly-strike control, drenching and weighing. These procedures would provide excellent opportunities for the spread of nasal microorganisms. If a particular strain of *P. haemolytica* is virulent, the stocking intensity is high, physiological stress may compromise the pulmonary defense mechanisms which may fail to clear *P. haemolytica* from the respiratory tract.

Evidence that some of these procedures are associated with high levels of pneumonia have been provided by the work of Van der Logt *et al* (1995).

Like the digestive tract, the respiratory tract is presumably sterile until the time of birth, as the foetus is surrounded by a completely sterile environment (Ballongue, 1993). After birth, the respiratory tract is rapidly colonized by bacteria, which increase in number and complexity within the first few days of life. The mixed culture of organisms recovered from the nasal swabs and the variation between individuals suggests that different lambs establish a different nasal microflora at the early age of one to two weeks after birth. This is in broad agreement with the findings of previous studies (Alley, 1991; Ugochukwu and Agwu, 1991).

In this study, pulsed field gel electrophoresis has been applied to identify selected strains of *P. haemolytica* isolated from the respiratory tract of ewes and their lambs. The major weakness of this study was the absence of species identification. The combination of PFGE patterns of *P. haemolytica* strains with species identification using DNA sequencing would have provided detailed information on the strain differentiation capability of PFGE among *P. haemolytica* strains. This would also have helped to accurately record the current *P. haemolytica* strains present in New Zealand. However, the different pulsed field patterns found in ewes and their lambs confirmed that the acquisition of *P. haemolytica* by young lambs was more likely to occur from other ewes and their lambs or perhaps from the environment rather than from their mother. The PFGE study also confirmed that carriage of most of the strains of *P. haemolytica* was unstable apart from those strains present on Farm 4, which persisted in the nasal cavity of lambs for 3-5 months.



The main disadvantages of the PFGE technique is that it requires a long and tedious procedure for isolating and restricting the genomic DNA. It also requires large quantities of expensive enzymes and reagents. It also has the disadvantage that only numerically predominant bacterial strains can be differentiated because isolates are obtained by picking discrete colonies from dilution plates (Tannock, 1999).

In conclusion, this project has highlighted the value of combining molecular techniques, which are able to identify strains of bacteria with conventional methods of field investigation. The results have indicated useful areas of future research, which could improve our understanding of the nasal carriage of *P. haemolytica* in sheep. The knowledge obtained would be valuable in identifying farms, environments and pathogenic strains of the organism and eventually allow future outbreaks of ovine pneumonia to be prevented by vaccination or improved management techniques.

## Appendices:

### Appendix I: Media used for bacteriological examination

#### A. Blood agar plates:

- Blood agar plates were prepared as double layers.
- The base was Difco Blood Agar Base No. 2 with no blood added.
- The upper layer was the same, but supplemented with horse defibrinated blood (Gibco) to 5%.

#### B. MacConkey agar (Difco):

- 50g Bacto MacConkey Agar was suspended in 1 litre distilled water and heat to boiling to dissolve completely.
- The dissolved media was autoclave for 15 min at 121 °C, cooled to 45-50 °C and dispensed in 20 ml amounts in sterile 90-100 mm petri dishes.
- The plates were stored at 4 °C for 2-3 weeks.

#### C. Heart Infusion broth (HIB):

- 25 g dehydrated bacto heart infusion broth (Difco) was suspended in 1 litter distilled water. The medium was boiled to dissolve completely.
- The aliquot's were dispensed into 3 ml bijou bottles and autoclave for 15 min at 121 °C and stored at 4 °C.

#### D. Triple Sugar Iron Agar (TSI – Difco):

- 65 g triple sugar iron agar (TSIA) was suspended in 1 litre of distilled water and dissolved by heating.
- 6 ml of the aliquot's been dispensed into tubes and autoclave for 15 min at 121 °C.
- The tubes were solidified in a slanting position so that a generous butt was formed.

- The isolates were inoculated with a straight wire by stabbing to the base of the butt and streaked the slant.
- The tubes were incubated overnight at 25 °C with caps loosened.

**E. Urea hydrolysis:**

- To rehydrate, 29 g Bacto Urea Agar Base (Difco) was suspended in 100 ml distilled water and mixed thoroughly to dissolve completely.
- The mixture was then filter-sterilised and stored at 4 °C.
- 15 g Bacto Agar was dissolved in 900 ml distilled water by boiling and autoclave for 15 min at 121 °C.
- The dissolved bacto agar then cooled to 50-55 °C and aseptically added 100 ml of the filter-sterilised, concentrated Bacto Urea Agar Base to the cooled Bacto agar.
- The mixture was mixed thoroughly and dispensed 3 ml amounts into sterile bijou bottles and cooled in a slanting position and stored at 4 °C.
- The bacterial culture was inoculated into slants and incubated overnight at 37 °C.

**F. Indol test:**

- One suspected *P. haemolytica* colony was inoculated into heart infusion broth (HIB) and incubated at 37 °C for overnight.
- A few drops of Kovac's reagent was added to the broth and observed the colour change.

**G. Brain heart infusion broth (BHI):**

- 37 g dehydrated bacto brain heart heart infusion (Difco) was suspended in 1 liter distilled water. The medium was boiled to dissolve completely.
- The aliquot's were dispensed into 3 ml bijou bottles and autoclave for 15 min at 121 °C and stored at 4 °C.

**Appendix II: Storage of *Pasteurella haemolytica* isolates:**

- 15% glycerol solution was made in tryptone water and dispensed 3 ml amounts into bijoux bottles. The solution was autoclave for 15 min at 121<sup>0</sup>c, which was then cooled and stored at 4 °C until required.
- A blood agar plate was inoculated with a single colony of *P. haemolytica* and incubated overnight at 37°C.
- The growth off the plate was scraped with a sterile cotton swab and was suspended it in a bijoux bottle containing the glycerol broth.
- 1-1.5 ml of the suspension was deposited into 2 X 2 ml screw-capped tubes and freezed immediately at -70 °C.

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