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THE EPIDEMIOLOGY OF INTERMEDIATE FOOTROT

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DECLARATION

Apart from the assistance stated in the acknowledgements and where reference is made in the text this thesis represents the original work of the author. The investigations presented here have not been presented for any other degree or diploma at any other university.

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March 2000

DEDICATION

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This work is dedicated to

Lesley, Claire, Bonnie and Zac

SUMMARY

This thesis reports the results of a number of studies of the epidemiology of intermediate footrot (IFR). Natural outbreaks of IFR occurred in two flocks, one near Yass, in the southern Tablelands of New South Wales and one near Cooma, in the Monaro district of New South Wales. These natural outbreaks provided the basis for the work recorded here.

Observations in the first flock demonstrated that the form of footrot was of limited virulence and could be classified as a low grade intermediate form. A controlled experiment in the first year showed that it was possible to eradicate the disease using methods based on repeated inspections in a non-transmission period and culling of affected sheep. In the following year, these methods were applied to the whole flock of over 3 000 sheep, except for a control flock of 100 sheep, and footrot was eradicated. Repeated inspections up to five years later confirmed the eradication. Observations made during the course of the eradication program suggested that latent infections occur with IFR, which can persist in an undetectable state for some months. Treatment with antibiotics was contra-indicated when eradicating IFR and it was hypothesised that antibiotics encourage the persistence of latent infections.

Observations in the second flock described the natural epidemiology of IFR in a region which is considered to be less than fully favourable for the expression of footrot. These observations demonstrated that conditions which favour transmission of footrot between sheep are more restricted than the conditions which encourage the extension of footrot lesions within an infected foot. Further, under conditions which limit transmission, particularly late in an outbreak, a higher proportion of infected feet may develop advanced lesions in unfavourable environments than in more favourable conditions. This observation has significance for the diagnosis of IFR based on calculations comparing the number of severe lesions in a flock to the number of infected sheep, or the total number of sheep at risk. Observations in this flock confirmed that the proportion of a flock which will develop score 4 lesions with IFR is limited;

in this case to 7% or fewer.

Infected sheep from this flock were moved to another environment which is considered more favourable for the transmission of footrot and were mixed with uninfected sheep and grazed together for two years. Observations were continued and bodyweights and wool production of the sheep were recorded and analysed with respect to the degree they suffered from footrot. When conditions again favoured transmission, the incidence in both flocks of sheep was similar, suggesting that transmission, rather than the recrudescence of latent infections, was responsible primarily for the rapid rise in footrot prevalence. Again, despite highly favourable environmental conditions and a high flock prevalence, the proportion of the flock which developed severe foot infections was no greater than in the Monaro.

Observations in these two flocks over two years showed that an outbreak of IFR will reduce annual greasy wool production by approximately 2% and reduce the average bodyweight of the flock by 2.5 kg at the peak of the outbreak.

An artificial infection, using a strain of D nodosus isolated from sheep in the Monaro outbreak, was established in a controlled experiment at Bringelly, New South Wales. This experiment included sheep infected with a virulent strain of D nodosus and some sheep infected with both the virulent strain and the strain causing IFR. Some control sheep remained uninfected. This experiment confirmed the expected difference between IFR and VFR. VFR was characterised by a relatively high proportion of score 3 and score 4 foot infections, while IFR was characterised by a relatively high proportion of score 2 lesions, with some score 3 and a few score 4 infections. Measurements of wool production, using dye-banding, suggested that IFR may depress wool growth rates by about 11% on average in an infected flock, for the time that footrot was spreading and at a high prevalence in the flock. VFR, on the other hand, is expected to cause approximately twice as much reduction in wool growth. Predictive equations were developed which can be used to assess the economic impact of footrot of a variety of severity classifications.

Throughout the studies, PCR-RFLP of the *omp* gene was employed as an epidemiological tool to demonstrate continuity of infecting strains between related outbreaks. This technique was evaluated and reviewed. The technique showed promise as a molecular epidemiological marker and demonstrated the highly complex nature of the *D nodosus* bacterial flora in some field outbreaks of footrot. Evidence was also presented of genetic exchange of fimbrial genes between strains of *D nodosus* in natural outbreaks, which has implications for control of footrot with serogroup-specific vaccines.

Finally, the findings of these studies were reviewed and the implications presented. Information has been presented which demonstrates the limited virulence of IFR strains which is expressed in similar ways in a range of environments. To correctly diagnose footrot, the importance of proper assessment of the environment, an understanding of the dynamic relationship between the environment and footrot expression and the use of meaningful mathematical indices of disease severity is essential. Predictions of the economic impact of IFR can now be made and the financial benefit of eradication of low virulent strains can now be compared to the expected cost of doing so.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

D nodosus	*Dichelobacter nodosus*
F necrophorum	*Fusobacterium necrophorum*
HA	Hoof agar
IDS	Interdigital skin
OID	Ovine interdigital dermatitis
PBS	Phospate buffered saline
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RE	Restriction endonuclease
Da	Daltons
ATCC	American Type Culture Collection
MW	Molecular weight
NSW	New South Wales
OE	Oil emulsion
ORF	Open reading frame
omp	Outer membrane protein

BACTERIOLOGICAL TAXONOMY

Throughout this thesis the names used for the bacteria always present in footrot lesions are *Dichelobacter nodosus* and *Fusobacterium necrophorum*. These bacteria have previously been referred to as *Bacteroides nodosus* and *Fusiformis nodosus* for *D nodosus*, and *Fusiformis necrophorus* and *Sphaerophorus necrophorus* for *F necrophorum*.

Where other authors have used names other than those currently used the names have been changed except where the name is used in the title of a published paper.

CHAPTER 1

CONTENTS

LITERATURE REVIEW

1.1.   Introduction

1.1.1.   Footrot as a disease

Footrot is a disease of sheep which results from bacterial infection of the skin between the digits and the epidermal tissues of the hoof laminae. Separation of the horn tissue from the sensitive laminae is a distinguishing characteristic of footrot and is called under-running. The disease causes lameness which, when severe, is debilitating and compromises the welfare and productivity of the sheep.

The disease has been known and described in sheep for more than 200 years but recognition of the complex bacterial nature of the disease has only been clarified in recent decades. *Fusobacterium necrophorum* is always present in footrot lesions and was thought to be the organism primarily responsible for the disease (Mohler and Washburn 1905) but some workers recognised that the organism was ubiquitous in the environment of sheep, and may be a secondary invader (Murnane 1933), or associated with footrot, rather than its cause (Beveridge 1934a). In the 1920s, French workers claimed that a spirochaete was involved with the disease (Gregory 1939) and Beveridge (1935; 1936) described the organism and named it *Spirochaeta penortha*. While believing that the spirochaete had an aetiological role in footrot, Beveridge (1936) was unable to reproduce the disease. 'Organism K', a large, gram negative rod, was identified by Beveridge (1938a) and was then observed by a number of workers in Australia and overseas to be present in smears from footrot lesions (Gregory 1939).

The gram negative anaerobe *Fusiformis nodosus*, now *Dichelobacter nodosus* (Dewhirst *et al* 1990), was named by Beveridge (1941) and identified as the essential transmitting agent of ovine footrot. Beveridge (1941) isolated the organism from lesions and induced footrot in other sheep by applying the organism to the scarified interdigital skin.

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D nodosus cannot establish in the healthy skin of the interdigital space. Beveridge (1934b) and Cross (1978a) recognised that predisposition of the interdigital skin was necessary for footrot to develop, and investigated the possible role of *Strongyloides papillosis* larvae in preparing the interdigital skin for footrot transmission. The necessary association of a condition called ovine interdigital dermatitis (OID) with the development of footrot was first identified by Egerton *et al* (1966) and Parsonson *et al* (1967). They established that OID, marked clinically by erythema and epidermal necrosis of the posterior interdigital skin (IDS), was caused by *F necrophorum* although *Actinomyces pyogenes* was dominant amongst the bacterial flora in the superficial parts of the skin lesions. OID can exist in the absence of *D nodosus* but separation of the hoof does not occur in the absence of *D nodosus*. Mild footrot, with lesions restricted to the IDS, is indistinguishable from OID without laboratory confirmation of the presence of *D nodosus*.

The synergistic association between *D nodosus* and *F necrophorum* in cases of footrot was further elucidated by Egerton *et al* (1969) and Roberts and Egerton (1969). They demonstrated, with histological studies, that these two organisms were the only ones present at the advancing edge of lesions. In new lesions of the IDS, *F necrophorum* invades the epidermis first, followed by *D nodosus* but, at the forefront of lesions under-running the horn, *D nodosus* was often the sole bacterial species present. Subsequent to separation, *F necrophorum* invades, causing severe inflammation and necrosis and adding markedly to the severity of the lesions. *D nodosus* cannot persist in the dermis, possibly due to the presence of a humoral bactericidal factor secreted into that part of the integument (Egerton and Merritt 1970), but *F necrophorum* can persist there. Further adding to the mutuality of the infection, *D nodosus* apparently produces a factor which increases the activity of *F necrophorum* in footrot lesions.

Electron microscopic studies (Hine 1984) support the essential involvement of *F necrophorum* in the advance of footrot lesions but also have demonstrated the presence of a motile filamentous rod-shaped bacterium in close association with *F necrophorum* and the general disposition throughout footrot lesions of a smaller, non-motile organism. The role of these two organisms in the development of footrot is unknown (Stewart 1989).

Separation of the horn of the hoof in footrot lesions occurs within the layers of the epidermis. Deane and Jensen (1955) showed that degeneration, and hence separation, occurred in the *stratum granulosum* and superficial layers of the underlying *stratum spinosum*. Stewart and Parsonson (1976, cited by Stewart 1989) showed that the separation occurred between the *stratum granulosum* and the more superficial *stratum lucidum*. The process causing the separation within the epidermis may be digestion by keratolytic proteases elaborated by *D nodosus* (Thomas 1962b), inflammatory destruction of the epidermal matrix (Egerton *et al* 1969), necrosis caused by metabolites of *F necrophorum* and the un-named motile organism (Hine 1984), or direct bacterial attack on the mid-epidermal tissues, possibly assisted by extracellular proteases (Stewart 1989).

Variations between strains of *D nodosus* in virulence (discussed below) and between individual sheep in resistance to infection lead to differences in the appearance, extent and severity of footrot lesions. The term *expression* is used to describe the variable prevalence and degree of damage done to sheep's feet within a flock by a footrot infection. When footrot is highly expressed within a flock, a high proportion of the flock has extensive under-running of the horn of the feet, with substantial necrotic material in the under-run tissue and severe inflammation of the interdigital skin.

To assist with the objective description of lesions, a lesion scoring system was developed by Egerton and Roberts (1971) and is still widely used, often in a modified form. In this system, a score of 1 indicates limited mild interdigital dermatitis, score 2 indicates more extensive interdigital dermatitis, score 3 indicates severe under-running of the horn of the heel and sole and score 4 indicates that the under-running extends to the outside wall of the hoof. A modification of this scoring system (Stewart *et al* 1982b) divided score 3 lesions into three sub-categories. Score 3a lesions have separation up to 0.5 cm of the plantar horn of the heel, score 3b lesions have more advanced separation of the plantar horn of the heel and score 3c lesions have complete separation of the plantar horn of the heel and extension into the posterior sole region. Cases were considered severe when lesions were 3c or 4. These scores are illustrated in Plate A. A further extension of these two scoring systems is used by NSW Agriculture (Anon 1995). This system includes a score 5 lesion as a severe form of the disease involving the sole, with extensive inflammation and under-running of the hard horn of the hoof.

Plate A

The lesion scoring system used for ovine footrot developed by Egerton and Roberts (1971) and modified by Stewart *et al* (1982b). Scores range from 0 (normal foot) to score 4. Score 1 is a non-specific inflammation of the interdigital skin which may or may not be associated with *D nodosus* infection. Score 2 lesions are restricted to the interdigital space. Score 3 and score 4 lesions are characterised by underrunning of the sole of the foot.

Score 3 has been sub-divided into 3a, 3b and 3c by Stewart *et al* (1982b) and those sub-categories are illustrated here. The most severe lesion illustrated is called a score 5 lesion by some workers (Anon 1995).

Normal foot. There is normal skin between the claws, with no reddening or inflammation and no loss of hair. There is no exudate present.

Score 1: Slight to moderate inflammation with some erosion between the claws. There is no underrunning or erosion of the skin or horn.

Score 3b: Underrunning no more than halfway across the heel or sole.

Score 3c: More extensive underrunning of the heel or sole but not extending to the outside edge of the sole of the claw.

Score 2: The skin between the claws is inflammed and raw. This condition may involve part, or all, of the soft horn of the inside of the claws. There is no underrunning of the horn.

Score 3a: Separation of the skin horn junction, with underrunning extending no more than 5 mm.

Score 4: The underrunning extends to the outside edge of the sole of the claw and involves hard horn.

This is a severe form of the disease involving the sole, with extensive inflammation and underrunning of the hard horn of the hoof.

1.1.2.   Variability in the severity of footrot outbreaks

1.1.2.1.   Terms used to describe footrot

The three descriptors '_benign_', '_intermediate_' and '_virulent_' are applied to both the inherent virulence of specific strains of _D nodosus_ and to the severity of the outbreak of footrot under investigation. Virulence is the ability of a particular strain of _D nodosus_ to cause severe lesions under environmental conditions suitable for the development and persistence of the disease. The specific meaning of each descriptor is discussed below.

1.1.2.2.   Recognition of variations in severity of footrot outbreaks

Thomas (1957) described the differentiation of footscald from footrot. He described the clinical appearance of footscald cases and how, unlike true footrot, footscald lacks advanced separation of the horn and an accumulation of foul-smelling necrotic material. Thomas (1957) found organisms resembling _D nodosus_ in smears but was unable to grow the organisms from footscald cases, although it could be isolated from footrot cases.

Thomas (1962a) described 'footscald' as a condition similar to early footrot, with inflammation of the IDS which becomes ulcerated and covered with a thin film of moist necrotic material. There is limited separation of the horn at the skin-horn junction of the axial aspect of the hooves. He identified the presence of an organism similar to _D nodosus_ isolated from cases of footrot but noted that the organisms of footscald produced less protease than those of footrot, as measured by digestion of azocasein.

Alexander (1962) described three forms of footrot (scald, mild footrot and severe footrot) although he differentiated mild footrot from severe footrot principally on the basis of the more rapid self-cure in mild footrot and the lower prevalence of chronic lesions. He also believed that milder forms of the disease could change into more severe forms without superimposed infection, which suggests that he may have in some cases confounded the influence of environment and that of innate virulence of the organism.

Egerton and Parsonson (1969) proposed that the disease known as 'scald' and associated with less proteolytic strains of *D nodosus* be known as benign footrot and the more serious disease with extensive separation be called virulent footrot. They used the term 'proteolytic index' to describe the difference in proteolytic activity between benign isolates and virulent isolates. This index was the ratio of the width of the cleared area to the width of the area of precipitate surrounding 48 hour cultures of isolates on casein agar culture media. A number of sheep were infected with benign strains and most developed benign footrot. In one sheep with no visible lesion, organisms were visible on histological sections of the IDS, suggesting that benign organisms can survive under hyperkeratotic skin without producing inflammation or necrosis. Benign footrot could be readily converted to virulent footrot by the introduction of virulent isolates onto the benign footrot lesions. Clinically, benign footrot is indistinguishable from the lesions of the IDS which precede and accompany virulent footrot. Observations of benign lesions over two and one half years showed that they do not become virulent, that they regress spontaneously when the environment dries and they recur in the next warm, wet period.

Stewart *et al* (1982a) described a strain or group of strains of *D nodosus* which was intermediate in virulence between those causing benign footrot and those causing virulent footrot. These strains still produce chronic lesions, unlike benign strains, but there is less tendency for chronic lesions than is the case with virulent strains, because fewer sheep develop severe underrunning lesions. These authors proposed the existence of a spectrum of virulence for *D nodosus* which results in a range of clinical expressions in affected flocks, from the mildest (benign) forms to the most severe (virulent) forms. While some reports, based on laboratory submissions, suggest that intermediate footrot is much less common than benign and virulent forms (Links and Morris 1995), a survey of 17 properties in southern New South Wales estimated that 18% of properties had intermediate footrot, compared to 23% with virulent and 41% with benign (Allworth 1994).

1.1.2.3.   Clinical characteristics of benign, intermediate and virulent footrot

It is now well recognised that, within footrot-infected flocks, there is a variation in the extent to which individual sheep are affected by the disease. Thus, in an infected flock, some sheep may have severe lesions (score 3c or score 4) while others may be unaffected or display only

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mild lesions (score 2) or remain unaffected (Egerton *et al* 1983). At least part of the apparent variation in the degree to which individual sheep are affected is due to genetic variation in the susceptibility of sheep within a flock (Raadsma *et al* 1993; 1994a; 1995; Litchfield *et al* 1993).

The variation in the severity of lesions between sheep, independent of any variation in inherent virulence of the infecting strain of *D nodosus*, makes it essential that a large and randomly sampled portion of a flock be examined when attempting to diagnose the form of footrot present in an outbreak (Egerton 1989).

Virulent footrot is characterised by progression of lesions from the IDS to involve extensive underrunning of the horn matrix of the foot in a high proportion of the sheep which are affected by the disease (Egerton and Parsonson 1969; Stewart *et al* 1982a; Stewart *et al* 1986a; 1986b). In some sheep these lesions remain as chronic, deforming infections of the hoof even after environmental conditions are no longer suitable for disease transmission.

Benign footrot is characterised by a high proportion of affected sheep having lesions restricted to the interdigital skin and a low proportion with severe and chronic lesions. Compared to OID, benign footrot is relatively persistent (Morgan *et al* 1972) but, compared to more virulent forms of footrot, it shows a propensity for self-cure when environmental conditions are unfavourable for the disease. Benign footrot is also very responsive to topical treatments (Egerton and Parsonson 1969; Glynn 1993).

Strains of *D nodosus* of different virulence also show differences in cultural characteristics and effects on the productivity of affected flocks, and these are discussed in sections 1.2 and 1.4.

The presence of a continuous spectrum of virulence between benign and virulent strains, including a group with intermediate characteristics, can present difficulties of diagnosis. These difficulties can be significant in state disease control programs, where government regulations require producers to take action to eradicate virulent footrot but not benign footrot (Roycroft 1986; Egerton and Raadsma 1993; Allworth and Egerton 1999).

Intermediate footrot is distinguished from virulent footrot in field outbreaks in the following ways.

- Intermediate footrot causes some score 4 lesions which are clinically indistinguishable from those caused by virulent footrot but, compared to virulent footrot, a smaller proportion of the flock is affected with severe lesions (Stewart *et al* 1984; Roycroft 1986; Dobson 1986; Stewart 1989). Typically fewer than 10% (Egerton 1989) or 20% (Allworth and Egerton 1999) of a flock affected with intermediate footrot develop score 4 lesions.

- Underrun lesions are qualitatively less severe than those of virulent footrot, with less necrosis evident (Egerton 1989)

- Overall, intermediate footrot is a milder disease than virulent footrot on a flock basis and all but the few severely affected sheep recover spontaneously when climatic conditions become dry (Stewart 1989; Dobson 1986)

Benign footrot is distinguished from intermediate footrot in field outbreaks in the following ways.

- Score 4 lesions are rarely present in benign footrot. Typically, no score 4 lesions are found or fewer than 1% of affected sheep are found to have score 4 lesions (Stewart *et al* 1984; Egerton 1989)

- Score 3 lesions occur at a low prevalence in benign footrot, but may occur at a high prevalence in intermediate footrot (Egerton 1989). Fewer than 5% (Allworth 1995) or 10% (Allworth and Egerton 1999) of sheep are expected to have score 3 or score 4 lesions in benign footrot,

- Benign footrot is principally a disease of the IDS, characterised by score 2 lesions (Morgan *et al* 1972; Allworth and Egerton 1999)

Environmental conditions affect the transmission of footrot within a flock and therefore the proportion of sheep which become affected with the disease. Allworth (1995) and Allworth and Egerton (1999) have suggested that a correct differentiation of the disease could still be made under less suitable environmental conditions if the proportion with lesions of any one score is calculated with the number of affected sheep as the denominator, rather than the number of sheep examined. Classification of the form of footrot present using this approach will not, however, be consistent across a range of environmental conditions if environmental conditions also affect the progress of the disease within the infected foot and, therefore, the proportion of affected feet which develop severe lesions.

1.2.   Characterisation of *Dichelobacter nodosus*

1.2.1.   Microbiological characteristics

1.2.1.1.   General cultural characteristics

D nodosus is a Gram negative rod, about 6 μm long and 0.8 μm wide with characteristic terminal enlargements, usually at both ends, most pronounced in organisms taken from footrot lesions rather than cultures and which are visible in light microscopy with Gram stains (Beveridge 1941) or with electron microscopy (Walker *et al* 1973; Stewart 1973).

In culture, *D nodosus* is strictly anaerobic and is usually grown in an atmosphere containing 10% hydrogen and 10% carbon dioxide. The organism is fastidious in growth requirements on media, requiring either 10% horse serum (Beveridge 1941) or ground hoof material (Thomas 1958) in agar, or trypticase, arginine and serine (TAS agar) as additives to agar or broth (Skerman 1975).

Thorley (1976) noted that concentrations of agar above 3% restricted the size and spreading of bacterial colonies other than *D nodosus*. Consequently 4% agar is currently recommended for primary isolation of the organism from lesion material, and 2% agar is used for sub-cultures (Stewart and Claxton 1993).

The organism grows best at 37°C (Beveridge 1941) and, at that temperature, colonies appear on agar plates in four to six days (Stewart and Claxton 1993). Colony morphology has been described by Thorley (1976), Skerman *et al* (1981), Stewart *et al* (1986b) and Stewart and Claxton (1993). On subculture on 2% agar, colony types can vary. Thorley (1976) has described colonies with fimbriate edges, the fimbriae consisting of migrating microcolonies, which Skerman *et al* (1981) describe as beaded (B) type colonies. Colonies of this type are typically of well-piliated organisms and capable of causing virulent footrot. Non-fimbriate (Thorley 1976) or mucoid (M) (Skerman *et al* 1981) colonies usually consist of organisms with relatively low piliation (see section 1.3.2).

1.2.1.2. Cultural characteristics of *D nodosus* isolates of different virulence

A number of laboratory-assessed characteristics of *D nodosus* isolates have been associated with virulence. These include colony morphology as discussed in the previous section, twitching motility (Depiazzi and Richards 1985; Depiazzi *et al* 1990), agar corrosion (Egerton and Parsonson 1966b; Stewart *et al* 1986b) and the presence and nature of extracellular proteases. It is the latter characteristic, first described by Thomas (1962a), which has received the most attention for its perceived ability to predict, *in vitro*, the *in vivo* virulence characteristics of strains of *D nodosus* (Whittington 1994). The tests that have been developed to measure protease characteristics include the proteolytic index (Egerton and Parsonson 1969), the degrading proteinase test (Depiazzi and Richards 1979) and its derivatives, the elastase test (Stewart 1979), the zymogram test (Every 1982; Kortt *et al* 1982) and the protease ELISA (Links *et al* 1995).

The degrading proteinase test (Depiazzi and Richards 1979) was based on the tendency of the proteinases from virulent strains of *D nodosus* to remain stable during incubation in culture at 37°C, while the enzymes produced by benign isolates were less so. The results were extended by other workers (Stewart 1979; Richards *et al* 1980; Stewart *et al* 1982a). Depiazzi and Rood (1984) reported that the differences in stability of the proteases from virulent and benign strains were detectable more quickly at higher temperatures and the stability varied also varied with the concentration of calcium ions. Subsequently, the test became known as the protease thermostability test (Depiazzi and Richards 1985; Green 1985; Depiazzi *et al* 1990).

Palmer (1983) further developed the protease thermostability test by substituting gelatin for hide powder azure as the substrate used to detect protease activity. Gelatin, compared to hide powder azure, was cheaper, soluble and more easily standardised between batches. The gelatin-gel protease thermostability test is now frequently referred to as the 'gelatin-gel' test and the results are reported as stable (S) proteases, inferring virulence, or unstable (U) proteases, inferring benign characteristics.

Stewart (1979) used a solid culture medium containing elastin to compare the elastase activity of strains of _D nodosus_ isolated from cases of virulent or benign footrot. Elastase-positive isolates produced a clearing of elastin particles in six to seven days of culture or slightly longer in some cases. Elastase-negative isolates produced no clearing within 21 or, in some cases, 28 days. Stewart (1979) found very close agreement between the elastase test result and the degrading proteinase test (Depiazzi and Richards 1979) of the same isolates, and a strong agreement (but less than 100%) between the elastase test result and the reported clinical virulence of the outbreak from which each strain was isolated. At the time when these original reports were published, intermediate strains were not recognised (Stewart _et al_ 1982a). Subsequently, Stewart _et al_ (1986b) showed that the elastase test could also distinguish at least some intermediate strains which showed rates of elastin-clearing between those of virulent and benign strains. The addition of calcium chloride to the elastin media enhanced the elastase activity of some strains.

The zymogram test (Every 1982; Kortt _et al_ 1983) distinguishes between strains of _D nodosus_ on the basis of the patterns produced by electrophoresis of their extracellular protease enzymes. Virulent strains produce some bands which benign strains do not produce, and _vice versa_ (Gordon _et al_ 1985). Depiazzi _et al_ (1991) and Palmer (1993) expanded the range of zymogram patterns to three for thermostable proteases (S1, S2 and S3) and six for unstable proteases (U1 to U6) and reported one isolate with a thermo-unstable protease with an S1 zymogram (Palmer 1993).

The protease ELISA (Links _et al_ 1995) uses monoclonal antibodies against a virulent protease and a benign protease in an ELISA system on microtitre plates to demonstrate the presence or absence of the protease characteristic of virulent or benign strains.

A number of reports have compared the results from a range of laboratory tests of virulence (Stewart *et al* 1986b; Depiazzi *et al* 1991; Palmer 1993; Liu and Yong 1993a; Links *et al* 1995; Links and Morris 1995). In general, there is good agreement between the tests, particularly in classification of benign or virulent strains. Agreement is less clear for isolates which are reportedly intermediate in virulence (Liu and Yong 1993a). Whittington (1994) drew attention to several problems associated with reliance on protease-based laboratory tests. These included

- the absence of a gold-standard determination of virulence. Definitions of virulence differ between states and clinical expression of virulence can be modified by environmental conditions

- the isolates tested in a laboratory may not be from the dominant strain causing footrot in the field

- the absence of controlled studies of *in vivo* virulence in sufficiently large groups of sheep in most reported evaluations of protease-based tests

- a small, but significant level of disagreement between the results of the various tests used.

- a small, but significant level of disagreement between test results and reported field virulence

The pen-challenge system offers some hope of relating laboratory-based tests to *in vivo* virulence (Stewart *et al* 1995) but the relationship between virulence in pens and in the field has not yet been demonstrated.

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1.2.2. **Genomic studies of *Dichelobacter nodosus***

1.2.2.1. **The *vap* region and the *vrl***

Dot-blot hybridisations using the recombinant plasmid pJIR318 (Katz *et al* 1991; 1992) as a probe identified a genomic region which occurs at a high frequency in virulent isolates of *D nodosus* but at a low frequency in benign isolates. This genomic region was designated the *vap* region (Katz *et al* 1992) because of its apparent association with virulence. There are three *vap* regions in the chromosome of the virulent prototype strain A198 and, based on sequence information, there appear to be four *vap* genes (*vap*A, *vap*B, *vap*C and *vap*D) in regions 1 and 2. Region 3 (Katz *et al* 1994) contains only *vap*D. Region 1 also contains the open reading frames (ORFs) *vap*E, *vap*F, *vap*G, *vap*H, *vap*I, *vap*Q, *vap*A', *vap*E', *vap*G', *orf118* and *intA* (Cheetham *et al* 1995; Billington *et al* 1996a). In A198, *vap* regions 1 and 3 are almost adjacent on the genome in a sequence of DNA which is approximately 11.9 kb long (Cheetham *et al* 1995) and referred to as *vap* region 1/3. Regions 1 and 3 are separated by a short segment of DNA which is common to at least one benign strain (C305). Region 2 is at a site distant from regions 1 and 3.

Sequence homology of parts of the *vap* region with sequences of several plasmids and bacteriophages suggest that *D nodosus* may have acquired the *vap* regions by integration into the genome of DNA from a bacteriophage or a plasmid containing bacteriophage-related sequences and that the sequences may have arisen by transfer from enteric bacteria (Cheetham *et al* 1995; Bloomfield *et al* 1997). A plasmid, pJIR896, which occurs naturally in *D nodosus* has been identified (Billington *et al* 1996b), cloned and sequenced. The similarity of the plasmid DNA to *vap* region 1/3 supports the hypothesis that this virulence-related sequence in the *D nodosus* genome arose from an integrative event of plasmid DNA (Billington *et al* 1996a) and offers the potential of a genetic transfer system for further studies of the genetic basis of *D nodosus* virulence.

Studies of the molecular genetics of the *vap* regions has led to a model for the evolution of virulent strains of *D nodosus*. Following the acquisition of *vap* sequences by one or more benign strains of the bacterium, other virulence-related genes were acquired. Thus, all virulent

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strains and some benign strains would contain *vap* sequences. Rarely, the *vap* sequences have been lost from virulent strains, indicating that the *vap* sequences are not essential for virulence (Bloomfield *et al* 1997). On the other hand, small changes to the genetic structure of *vap* genes could result in a loss of virulence without losing the ability to hybridise with *vap* specific probes (Katz *et al* 1991). The integration of *vap* sequences into the *D nodosus* genome occurred in the relatively distant past, in evolutionary terms, and there have been substantial re-organisation of *vap* genes since then, including insertions, deletions, duplications, re-organisation of duplicated genes and additional integrations of *vap*-related sequences forming regions 2 and 3 (Cheetham *et al* 1995; Haring *et al* 1995; Billington *et al* 1996a; Billington *et al* 1996b; Bloomfield *et al* 1997).

The function of any of the products of the *vap* genes has not been determined. The *vap*D protein has been produced in recombinant *E coli* and has been shown to be produced by both virulent and benign strains of *D nodosus* (Katz *et al* 1992). As no transformation system for *D nodosus* has yet been developed, it has not been possible to directly test the role of the *vap* region genes in virulence. Restriction map analysis of the plasmid DNA indicated that the gene regions identified were not part of previously studied virulence-associated genes, the fimbrial sub-unit gene or the putative protease gene (Katz *et al* 1992).

The virulence-related locus or *vrl* (Haring *et al* 1995) is a 27 kb DNA sequence which was first identified in A198 using two recombinant plasmids, pJIR314B and pJIR313 (Katz *et al* 1991). It appears, from a large study of 771 *D nodosus* isolates (Rood *et al* 1996), that the presence of the *vap* region and/or the *vrl* is a reasonable predictor of virulence, whether virulence is determined by clinical evidence, elastase activity, protease thermostability, zymogram pattern or colony morphology. Using the three plasmids pJIR318, pJIR314B and pJIR313 as probes, Rood *et al* (1996) classified isolates into three major categories. Category 1 isolates contain both *vap* region and *vrl*, category 2 isolates contain only *vap* region and category 3 isolates contain neither loci. Of category 1, 88% isolates were classified as virulent or high intermediate, 18% of category 2 isolates were classified as virulent but 70% were classified as intermediate. Of category 3 isolates, 83% were classified as benign or low intermediate.

In the study of Rood *et al* (1996), no isolates were detected with the *vrl* region only. The authors suggested that the presence of the *vap* region is essential either for the insertion or the maintenance of the *vrl* locus. These workers developed a PCR version of the previously described gene probe test for the *vap* and *vrl* regions. They synthesised a set of *vap*-specific primers and a set of *vrl*-specific primers. The *vap*-specific primer was from the *vapAC* region. Strain A198 yielded a 733 bp PCR product, as did a number of other strains, from amplification with the *vap* primers. A number of other strains yielded a 1237 bp fragment, indicating strain variation in the *vapAC* region. The presence or absence of these PCR products, and the variation in the product size with the *vap*-specific primers, offer the potential for epidemiological studies with these techniques.

Liu and Yong (1993b) and Liu (1994) screened panels of isolates of *D nodosus* with *D nodosus* genomic clones and identified two clones which, when used together, would differentiate strains of virulent, intermediate and benign *D nodosus* with dot-blot hybridization. Subsequently, the virulent probe was shown to be from the *vap* region or *vrl* region (Rood *et al* 1996). The specificity of the virulent probe was less than 100% because 15% (3/20) of benign strains, as determined by elastase activity, reacted with the virulent probe as well as the benign probe. The benign-specific probe reacted only with strains which had been characterised as benign or intermediate by elastase tests, demonstrating high specificity, but some intermediate strains did not hybridize with the benign-specific probe (Liu 1994).

1.2.2.2.   Ribosomal RNA genes

Bacterial ribosomes are composed of two subunits, each composed of RNA and proteins. Sedimentation coefficients of the subunits are used to identify the RNA components of each subunit. Thus, for *Escherichia coli*, the sedimentation coefficients of the two subunits are 50S and 30S and the larger (50S) subunit includes two RNA molecules (23S and 5S) , the smaller subunit (30S) includes only one (16S) RNA molecule (Dale 1998).

The genes responsible for the production of ribosomal RNA (rRNA) are present in the chromosomal DNA of bacteria and are highly conserved within prokaryotic and eukaryotic genomes. Consequently, knowledge of rRNA sequences can add to the understanding of the

evolution of bacterial genera, species and strains, and their degrees of interrelatedness. Ribosomal RNA is present in large amounts in bacterial cells and the rRNA genes are usually present in multiple copies in bacterial genomes. The abundance of rRNA or rRNA genes and its reliable species-specificity makes these nucleic acid sequences potentially useful targets for sensitive and specific molecular genetic detection (Grimont and Grimont 1986; Bingen *et al* 1992). The technique of ribotyping, using a DNA probe prepared from the *D nodosus* 16S rRNA gene, has been used in an epidemiological study of footrot by Allworth (1995) who found that the procedure could not adequately discriminate all strains but, in conjunction with other phenotypic or genetic markers, could assist identification of strains.

The rRNA of *D nodosus* has also been studied and used for the phylogenetic reclassification of the organism, formerly named *Bacteroides nodosus* to its current classification as the only species of the *Dichelobacter* genus (La Fontaine and Rood 1990; Dewhirst *et al* 1990), and the rRNA gene has been used as a PCR based method of identifying the organism without culture (La Fontaine *et al* 1993).

There are three copies of a locus, named the *rrn* locus by La Fontaine and Rood (1996), present in the genome of *D nodosus* strain A198. Each *rrn* locus is comprised of the 16S, 23S and 5S rRNA genes. Using oligonucleotides designed specifically for the *D nodosus*-specific regions of its 16SrRNA, La Fontaine *et al* (1993) were able to use PCR techniques to identify the presence of *D nodosus* in material collected from the footrot lesions of sheep. While this test has the potential to become an extremely sensitive test for the presence of *D nodosus*, in field tests it has not been reliable, principally due to substances inhibiting the PCR (JR Egerton personal communication).

1.2.2.3.   The *omp* gene

In attempting to isolate and characterise the gene or genes encoding extracellular proteases from *D nodosus* A198, Moses (1993) cloned a representative of a family of genes which encoded for the major outer membrane protein (omp). Moreover, he discovered a mechanism by which these genes can change, thus altering the antigenic structure of the major outer membrane protein.

He proposed that, at least in the strain A198, there are four genes (*omp1A*, *omp1B*, *omp1C* and *omp1D*) which are linked on one piece of genomic DNA. A 497 bp fragment containing the promoter and 5' coding sequence is included in the same *omp1* region. The promoter and 5' coding sequence are constant, whichever gene (*A*, *B*, *C* or *D*) is expressed. The four genes are structurally similar but not identical. Inversion of linked sequences of two genes (*A* and *D* or *B* and *C*) or inversion of the promoter and 5' coding sequence leads to the expression of any one of the four genes. The proteins expressed by genes *A*, *B*, *C* or *D* vary in molecular weight (70.5, 72.2, 74.4, or 74.8 kDa) and have some structural similarities. Omp1C and omp1D proteins have 66% overall identity; omp1A and D have 58% identity and the remaining pairs 45% to 46% identity.

Moses and coworkers demonstrated, with immunofluorescent labelling of *D nodosus* cells with sheep antibodies raised against recombinant omp1 proteins, that the omp1 protein was indeed a surface-exposed antigen, although he considered it unlikely that this protein functioned as a basal protein for fimbriae as suggested by Mattick *et al* (1984), because of its abundance and uniform distribution over the cell surface (Moses *et al* 1995) (see section 1.3.2.3).

Moses (1993) synthesised two oligonucleotide primers (A and C) based on nucleotide sequences which were almost completely conserved within the four genes. The only inconsistency in the sequence across the four genes was in oligo C, in which the third base was adenine (A) in *omp1D*, but thymine (T) in *omp1A*, *B* and *C*. Expected fragment sizes for each gene region are all approximately 480 bp (465, 489, 488 and 483 for *omp1A*, *B*, *C* and *D*). He demonstrated that PCR product of approximately this size was produced by PCR amplification and two bands could be distinguished - it being unlikely that the larger three products could be distinguished on the agarose gel.

Each of the four expected PCR products should produce different restriction maps after digestion with *Sau3A*. *Omp1A* was expected to produce fragments of 5, 88, 106, 120 and 146 bp, *omp1B*; 184 and 305 bp, *omp1C*; 488 and *omp1D*; 208 and 275. Moses (1993) was able to demonstrate nine bands corresponding to all fragments 88 bp and larger, indicating that all four genes were present in the genome of one isolate of *D nodosus*.

As a small extension of his work, Moses (1993) used the same primers in PCR-mediated amplification reactions on genomic DNA from 15 *D nodosus* isolates representative of the nine serogroups. Multiple DNA fragments, varying in size, were produced from all but one isolate, leading him to suggest that the likely polymorphism within the gene but the high degree of conservation at the sequences complementary to primers A and C could be used to identify *D nodosus* strains in epidemiological studies. Subsequently, this methodology has been used by Allworth (1995), Ghimire (1997), Ghimire *et al* 1999, Ghimire and Egerton (1999) and by me (see Chapter 7).

1.3. Antigenic variation between isolates of *D nodosus*

1.3.1. Serological classification

The existence of antigenic variation between strains or isolates of *D nodosus* was recognised by Beveridge (1941) who used agglutination tests with isolates from Australia and USA. Subsequently, Egerton *et al* (1972), in preliminary development of vaccines against footrot, found that groups of sheep given vaccine prepared from strains different from those present in the flock had lower levels of protection against infection than those given homologous vaccine. Heterologous vaccination also gave lower rates of healing of footrot-affected sheep than homologous strains (Egerton and Morgan, 1972). Egerton (1973) described two distinct types of antigens of *D nodosus* - first a heat labile antigen which was substantially removed by washing and, second, an antigen which persisted despite boiling for 1 hour. By analogy with systems for naming the envelope and somatic antigens of *Escherichia coli* and *Pasteurella multocida*, the first, presumably a surface antigen, was referred to as the K antigen and the second, a somatic antigen, as the O antigen.

The K antigen was subsequently shown to be associated with the pili of *D nodosus* (Stewart 1973; 1978a). Purified preparations of the pili are antigenic in rabbits and sheep and antisera produced with such preparations produce the typical K type agglutination reaction. Electron microscopy studies of *D nodosus* cells indicated that the pili reduce in number or disappear with repeated sub-culture in liquid media (Stewart 1973), consistent with earlier observations that such isolates lose the ability to stimulate K type antibody production (Egerton 1973).

Serogroup determination has been done with antiserum raised in rabbits (for example, Claxton 1981; Thorley and Day 1986; Gradin *et al* 1993) or sheep (Hindmarsh and Fraser 1985; Kingsley *et al* 1986), which is used without prior absorption or with pre-absorption (Thorley and Day 1986) with heterologous antigen. Agglutination is measured on slides, without dilution of the antiserum (Claxton *et al* 1983), or with serial dilution of the antiserum in tubes (Egerton and Laing 1979) or on plates (Day *et al* 1986).

Knowledge of the nucleotide sequences of the fimbrial genes of the prototypes of each serogroup has led to the development of a PCR-based method to determine which serogroups are represented in footrot lesions without the necessity of culture and slide agglutination procedures (John *et al* 1999).

A convention has been proposed (Ghimire *et al* 1998) of defining serogroup as the determination made by slide agglutination with unabsorbed antiserum, and serotyping for the characterisation achieved with tube agglutination, with absorbed or unabsorbed serum. Slide agglutination, particularly with unabsorbed antiserum, is more likely to lead to errors in serogroup determination, and cross-reaction, than tube agglutination tests and the use of preabsorption (Chetwin *et al* 1986).

Studies in Australia described the existence of three (Egerton 1973), eight (Claxton *et al* 1983) then nine (Claxton 1986) distinct serogroups of *D nodosus* isolates, based on the K or pilus antigen. These serogroups are labelled A to I and have been further subdivided into a total of 17 serotypes by cross-tube agglutination tests. The B serogroup is recognised as the most complex with four sub-types and frequent difficulties in confidently assigning B serogroup isolates to one serotype (Kingsley *et al* 1986). Cross reactions occur between serogroups but in general, homologous reactions are much stronger than heterologous reactions. Claxton (1986) was able to classify nearly all of over 4 000 isolates into one of the nine major serogroups.

A further nine major serotypes (J to R) have been proposed (Thorley and Day 1986; Day *et al* 1986) by workers in UK who proposed that three of the four serotypes of the B serogroup (B_2, B_3 and B_4) could be reclassified to distinctly different serogroups. In common with Claxton's

four B serotypes, their serotypes K, L and N showed a high level of cross reaction, which they were able to demonstrate with immunogold labelling of agglutination reactions arose from the presence of antigens on pili which were common across all three serogroups as well as antigens which were unique to each serogroup. Strains classified by Claxton as serotype B_1 to B_4 showed cross reactions with isolates of serotypes K, L and N but there was not a consistent pattern in homologous or heterologous reactions.

It seems likely that Claxton's B serogroup includes strains which are analogous to the K, L and N serogroups referred to by the UK workers (Stewart *et al* 1991b) and that, in both systems, these isolates form a group which differs from other serogroups by the complexity of their agglutinating pilus antigens.

A lack of cross protection within serogroup H between subtypes 1 and 2 has led Day *et al* (1986) and Stewart *et al* (1991b) to recommend that serotype H_2 be re-classified as serogroup O.

Serogroup M, identified by Day *et al* (1986) as serotype M, was found to be present on 5% and 6% of Australian and New Zealand farms surveyed by Chetwin *et al* (1986) and has been demonstrated in a large collection of isolates from Nepalese sheep and goats (Ghimire *et al* 1998). These workers found that the Nepalese serogroup M isolates did not cross react with any prototype antisera, unlike those reported by Day *et al* (1986) which cross reacted with serogroup F antiserum, but that Australian F and I prototype isolates cross reacted with M antiserum. Molecular studies of the fimbrial subunit gene showed 86% sequence homology between serogroup M isolates and prototype serogroup F isolates, more than reported for similarities between isolates from any other two serogroups. Despite the similarity, lack of cross reaction warrants the classification of serogroup M as a distinct serogroup (Ghimire *et al* 1998). At present, 19 serotypes in 10 serogroups are recognised in the Australian classification system (Table 1.1).

Table 1.1   Serogroups and serotypes of *D nodosus* currently recognised in Australia

	Serogroup									
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**M**
Number of serotypes	2	4	2	1	2	2	2	2	1	1

The existence of a relatively simple classification system based on serological reactions has allowed some preliminary epidemiological observations to be made in natural and experimental footrot outbreaks.   Serogrouping provides identification of relatively stable phenotypic characteristics of particular isolates which allows the mixed nature of many field cases of footrot to be demonstrated. The presence of mixed-serogroup infections in sheep flocks is probably normal in Australia (Claxton 1986), Great Britain (Hindmarsh and Fraser 1985; Thorley and Day 1986) and New Zealand (Kingsley *et al* 1986).   Up to six serogroups (Egerton 1983; Claxton 1986) and nine serotypes (Thorley and Day 1986) have been reported in any one flock and it is noted that the more sheep that are sampled within a flock, the greater the diversity of serogroups.

Common serogroups in Great Britain include H, D (Thorley and Day 1986), and B (Hindmarsh and Fraser 1985) which was also most common in the survey of Claxton *et al* (1983). Common serogroups in United States include B and H (Gradin *et al* 1993). These workers described 21 serotypes (I to XXI) which could be grouped into 11 serogroups, eight of which were related to the first eight (A to H) Australian serogroups.

Table 1.2   Prevalence of serogroups of *D nodosus* on farms (% of farms with each serogroup (authors 1,3,4 & 5) or % of isolates made on farms (author 2))

	Author	A	B	C	D	E	F	G	H	I*	M
1	Claxton *et al* 1983 (south eastern Australia)[b]	26	40	14	16	19	26	19	17		
2	Hindmarsh and Fraser 1985 (Britain)	2	40	2	11	2	9	8	27		
3	Kingsley *et al* 1986 (NZ)	17	82	28	20	18	11	5	22		
	Same authors (UK)	2	40	2	11	2	9	8	27		
	Same authors (Australia)	17	33	3	12	7	10	11	8		
4	Thorley and Day 1986[c] (Great Britain)	9	10	10	41	7	10	5	47		12
5	Chetwin *et al* 1991 (Australia)	17	>14	7	10	7	15	7	9	3	5
	Same authors (NZ)	19	>65	26	19	20	15	5	21	15	6

a   Serogroup I not described until Claxton (1986)

b   7% of farms had non-agglutinating isolates

c   a further 30% were J,K or L serotype; and 4% were O, P, Q or R

Thorley and Day (1986) used a system of classification in which serogroups were replaced by serotypes, and subtypes of each existing serogroup in the Australian system (Claxton *et al* 1983) became a new serotype J to R. The existing (Australian) serogroup system, however, has much to offer in vaccine usage, where cross protection between serotypes within most serogroups requires only one representative of each serogroup to be represented in a multivalent vaccine (Stewart *et al* 1991b). Rationally, however, some serotypes should be transferred to new serogroups, particularly H_2 and, possibly, some serogroup B subtypes (Day *et al* 1986; Stewart *et al* 1991b) because of a lack of cross-protection within these serogroups.

1.3.2.   Vaccination

1.3.2.1.   The first vaccines

The first use of *D nodosus* vaccines against footrot was described by Egerton and Burrell (1970), Egerton (1970) and Egerton and Roberts (1971). These early experiments involved cultures grown on solid or biphasic media containing ground hoof, oil-adjuvanted vaccines and challenge with the homologous strain. The vaccines were tested in field outbreaks (Egerton and Burrell 1970) and with artificial challenge in pens (Egerton and Roberts 1971) and were shown to be effective in both preventing footrot and accelerating the healing of footrot lesions. Similar results were obtained in New Zealand, with single strain vaccines prepared in liquid media (Skerman 1971; Skerman and Cairney 1972).

Alum precipitated (AP) vaccines were also found to produce a satisfactory immunity in British breed sheep, and their crosses (Skerman and Cairney 1972) although a shorter duration of protection, compared to oil adjuvant vaccines, was reported by Roberts *et al* (1972) in Britain. AP vaccines had the advantage of much less obvious site reactions than oil-adjuvant preparations.

Further experience with vaccines against field outbreaks showed that the protection afforded by vaccination varied between outbreaks (Egerton *et al* 1972), and the presence of heterologous strains of *D nodosus* was considered as a possible contributing factor to low efficacy, although in some studies, protection against homologous challenge was also limited (Keogh 1976). The absence of cross protection between some serogroups, identified by their K antigen agglutination reaction (Egerton 1973), was shown to be a reason for failure of protection by vaccine in specific cases (Egerton 1974).

1.3.2.2.   Identification of pili as the immunogen of *D nodosus*

The description by Egerton (1973) of two distinct types of surface antigen on *D nodosus* cells, the K and O antigens, is discussed in 1.3.1 above. Electron microscopy studies of the ultrastructure of the organisms (Stewart 1973; Walker *et al* 1973; Stewart and Egerton 1979)

showed that they possessed filamentous pili or fimbriae. Pili were most numerous in cells from cultures in the rapid growth phase and much less numerous in older cells from cultures in the stationary phase (Walker *et al* 1973). Repeated subculturing reduced the amount of piliation of cells (Stewart 1973; Short *et al* 1976). The loss of pili during sub-culture leads to changes in colony morphology such that the edges of colonies of cells with few pili appear non-fimbriate, while those of piliated cells appear fimbriate (Thorley 1976; Short *et al* 1976). The word 'fimbriate' refers here to a colony characteristic, rather than the presence of pili.

Pili were shown by electron microscopy to emerge in clusters from one end of the cell and were up to 5 μm (Walker *et al* 1973) or longer (Stewart 1973; Every 1979). Antibody prepared by immunizing rabbits and sheep were shown to specifically react with the pili, indicating that the pili were, or contained, the immunogen involved in stimulating antibody production and are the K antigens described by Egerton (1973) (Walker *et al* 1973; Short *et al* 1976). The pilus-associated immunogen is the serotype-specific antigen, and heterologous antisera do not specifically attach to pili. Low titre cross-reactivity is associated with *D nodosus* cell surface antigens other than pilus (Short *et al* 1976), which may be the surface antigen responsible for cross-reactivity between prototype A and B serogroup strains which does not cross-react with the prototype of serogroup C (Egerton 1974; Stewart 1978b)

Vaccines made from various cellular components confirmed that the protective, agglutinating immunogen is associated with pili and is a heat-labile, high molecular weight (>300 000 Da) protein distinct from the heat-stable O antigen of the cell wall (Egerton 1973; Stewart 1978a). Vaccines made from pili separated from cells produced higher agglutinating titres than piliated whole cell vaccines, even when the whole cell vaccines contained similar quantities of pili (Every and Skerman 1982; Stewart *et al* 1982b). Piliated whole cell vaccines produce higher titres than sparsely piliated cell vaccines (Stewart *et al* 1982b).

Vaccines made from pure pili conferred protection from footrot challenge either as well (Every and Skerman 1982) or more effectively than vaccines made from well piliated whole cells (Stewart *et al* 1982b).

1.3.2.3.   Structure of pili

Pili were found to consist of aggregates of a single polypeptide sub-unit (Stewart 1978b) with no associated carbohydrates, lipid or lipopolysaccharides (Every 1979). The polypeptide sub-unit, pilin, of two strains had a molecular weight of 18 400 Da and led Every (1979) to note the similarity of the pili to the type 4 pili (Ottow 1975) of *Pseudomonas aeruginosa* (Frost and Paranchych 1977). McKern *et al* (1983) subsequently demonstrated structural homology between *P aeruginosa* pili and those of *D nodosus* and determined the complete amino acid sequence of A198 pilin. A well as *P aeruginosa*, type 4 pili occur in *Moraxella bovis*, *M nonliquefaciens*, *Neisseria gonorrhoeae* and *N meningitidis* (Dalrymple and Mattick 1987). Characteristics of type 4 pili include the polar location of pili on the cell and 'twitching motility', a characteristic which may enable surface translocation of the bacterium (Henrichsen 1983; Depiazzi and Richards 1995).

Mattick *et al* (1984) found that the pilus sub-unit of A198 had a molecular weight of approximately 17 000 Da and was frequently co-purified with a polypeptide with a molecular weight of about 80 000 Da. This polypeptide was visualised by electron microscopy and appeared to be attached to one end of pili, forming a cap-like structure which suggested that it represented the attachment plate of the pilus to the bacterial cell wall. This notion was rejected by Moses *et al* (1995) (see section 1.2.2.3) who used immunofluorescent labelling with electron microscopy and considered the protein to be too abundant and uniformly distributed on the cell wall to be a basal protein for pili with a polar distribution.

The structural sub-unit of pili differ in size between the prototypes of the eight serogroups A to H (Anderson *et al* 1986). Pilin consisted of one polypeptide molecule, with molecular weights ranging from 16 500 Da (serogroup D) to 19 000 (serogroup F), except for serogroup H, where the pili were found to consist of two polypeptides of different molecular weights. The two molecules, one about 6 000 Da and one about 10 000 Da, apparently derived from a larger molecule of 16 000 Da which had been cleaved. The common epitope of serotypes H_1 and H_2 appear to be on the smaller polypeptide. Anderson *et al* (1986) reported minor differences between serotypes within the A, B, E and G serogroups, more marked differences between F_1 and F_2, and minor variations between strains within some, but not all, serotypes.

The ~80 000 Da polypeptide, the putative basal plate of pili, also showed variation in size between strains, from 77 000 Da to 88 000 Da, but the variation did not follow serogroup classifications.

1.3.2.4.   Genetics of pili

The nucleotide sequences of the pilus subunit gene (*fim*A) were determined for the prototype strains of the nine serogroups (A to I) of *D nodosus* by Mattick *et al* (1991). This work enabled the classification of *D nodosus* serogroups into two major categories on the basis of the pilin gene sequences. Class I includes A, B, C, E, F and G and class II includes serogroups D and H. These workers found that, despite genetic differences between strains, serotype and serogroups, there was absolute conservation of the 5' coding sequences, suggesting these sequences may be involved in mediating site-specific recombination. Hobbs *et al* (1991) presented evidence that recombination events involving the subunit gene *fim*A had occurred during evolution of strains of *D nodosus*. The apparent absence of *fim*A-related sequences in other parts of the *D nodosus* genome suggests that recombination involving the gene region is an inter-genomic, rather than intra-genomic phenomenon, and may occur as a device to escape fimbrial-specific bacteriophage attack (Mattick *et al* 1991) or to avoid host immune system responses (Moses *et al* 1995). This finding is of significance to the associations between *omp* gene PCR-RFLP patterns and serogroup classification discussed in Chapter 7 of this thesis.

*Fim*A sequence information has also been used by John *et al* (1999) as a way to identify the serogroup of a *D nodosus* strain by PCR amplification of parts of the gene, using serogroup-specific primers.

1.3.2.5.   Recombinant vaccines

D nodosus is difficult to grow in quantity in culture because it is inherently slow-growing and fastidiously anaerobic. The pili, which are necessary for vaccine production, are sometimes lost in cultured cells, particularly if the cultures are maintained in liquid culture. If a fast-growing, less fastidious species of bacterium could be transformed with the pilin sub-unit gene and then

express the polypeptide as assembled pili, vaccine manufacture would be greatly simplified. Moreover, if the pili were the principal immunogen, the product of the transformed cells would be free of extraneous proteins which may be unnecessarily antigenic. The gram negative bacteria which naturally possess pili are appropriate candidates to be host cells for recombination.

Anderson *et al* (1984) cloned the *D nodosus* pilus sub-unit gene into *Escherichia coli* and the cloned gene expressed in the recombinant host. In *E coli*, fimbrial sub-unit peptides of the expected size (17 500 Da) were produced, but not assembled into pili (Elleman *et al* 1986a). It is necessary for pilin to be assembled into pili to be an effective immunogen (Emery *et al* 1984; Stewart *et al* 1991a). In *P aeruginosa*, however, cloned genes express pilin and the pili are assembled at the cell surface and stimulate an effective immunity against the homologous *D nodosus* strain (Elleman *et al* 1986b; Mattick *et al* 1987; Egerton *et al* 1987). This has also been done for serogroup H, where the product of the cloned gene expressed pilin and assembled pili from polypeptides which were not cleaved, as happens in native *D nodosus*. Despite the difference from *D nodosus* pili, the cloned pili were effectively immunogenic (Elleman and Stewart 1988). These authors also linked cloned DNA from two serogroups in tandem within recombinant cells which then produced pili of two types, representing the pili of the parent serogroups.

After considerable technical difficulty in scaling up vaccine production to commercial volumes, a recombinant vaccine was released in 1993 (Schwartzkoff *et al* 1993c). It has not, however, been produced commercially.

Identification of an epitope common to all serogroups which could be used in vaccines, instead of the pili of all serogroups, would simplify vaccine production and possibly improve vaccine responses in sheep. Attempts to do so with monoclonal antibodies have so far been unsuccessful (Young *et al* 1989; Gradin *et al* 1991).

1.3.2.6.   Action of vaccines

The mode of action of the agglutinating antibodies in protecting vaccinated sheep from footrot

can be proposed from the results of a number of studies. As an infection with *D nodosus* develops in the epidermis of the interdigital skin, the inflammatory response increases and the amount of exudate moving into the infected tissues increases. The anti-pili antibodies are of the IgG_1 isotype of immunoglobulins (Egerton and Merritt 1973; Stewart 1978b; Fahey *et al* 1983; Emery and Stewart 1984) which are probably better able to diffuse through tissues to the site of infection than IgM immunoglobulins, based on inferences from studies of antibodies to *Pseudomonas* sp in mice (Bjornson and Michael 1970). Pili, which are known to be an important virulence factor (Short *et al* 1976), probably have a functional role in bacterial mobility rather than attachment to host tissue cells, so their normal function is more likely to be important in extension of a footrot lesion rather than initiation of it (Every 1979). Antibodies are known to act by interfering with pili and, in electron microscopic studies appear to hold cells together (Every 1979). Thus it seems plausible that antibody concentration will be increasing in the immediate epidermal vicinity of an infection as the exudative process increases, and will act to lessen or prevent the further development of the infection by preventing the spread of organisms through the epidermis (Walker *et al* 1973). This view is consistent with the observation of Egerton and Roberts (1971) and Stewart *et al* (1982b) that vaccines do not prevent infection of the interdigital skin but, when effective, protect against the development of severe interdigital lesions and underrunning infections.

1.3.2.7. Protective agglutinin titres

Natural infections with footrot elicit a small antibody response in infected sheep but the titres are probably insufficiently high to provide protection, for sheep can be repeatedly infected with homologous strains of *D nodosus* (Egerton and Roberts 1971). The agglutinin titres necessary to provide protection against footrot are very high and are only produced by vaccination, not natural infection. Titres below 3 000 (Thorley and Egerton 1981) or 3 200 (Stewart *et al* 1982b) were considered too low to protect sheep against homologous challenge, whereas titres above 5 000 (Thorley and Egerton 1981) or 12 800 (Stewart *et al* 1982b) were high enough to protect most sheep. The relationship between K-agglutinin titres and immunoprotection was illustrated succinctly by Egerton *et al* (1987), using recombinant pilus vaccine, who showed that there was a threshold titre of 2 560, below which animals were generally susceptible and that the relationship between titre and susceptibility was poor. At titres of 5 120 and above,

animals tended to have resistance to footrot, with little increase in resistance with higher titres. Superimposed on the general relationship are other factors influencing the presence and severity of footrot lesions in individual sheep, which lead to a relatively low correlation between titre and footrot severity in individual animals (Stewart *et al* 1982b; Egerton *et al* 1987).

Raadsma *et al* (1994b) showed that the relationship between agglutinating titre and protection from footrot varied with the severity of the challenge and, possibly, the serogroup, the two effects being confounded in their experiment. For both strains, there was a strong relationship between protection and agglutinating titre but, for serogroup A, full protection occurred at titres of 10 240 and above while for serogroup B, it occurred at 2 560. The difference in titres affording protection for the two strains could be explained by a threshold effect, in which the threshold of protection shifts with the severity of the challenge - which in turn is determined by environmental, bacterial and host characteristics in addition to agglutinating antibody titres.

These workers also noted the partial protection provided by medium range titres which was manifest by a reduction in the number of feet affected and the number of feet with underrunning lesions in groups of sheep with titres below the full-protective threshold.

1.3.2.8.   Use of adjuvant in vaccines

Commercial footrot vaccines now available in Australia contain oil adjuvants and are formulated as oil-emulsion (OE) preparations. These vaccines are recognised as causing vaccination site reactions which may become chronic, discharging granulomata (Roberts *et al* 1972). Alum precipitated (AP) vaccines have been used successfully in Europe (Roberts *et al* 1972; Kerry and Craig 1976) but their failure to stimulate prolonged antibody responses or immunity in Merinos in Australia has been reported (Egerton *et al* 1978; Egerton *et al* 1979; Egerton and Thorley 1981). Two doses of AP vaccine protected Merino sheep for eight weeks, four weeks less than OE vaccine (Egerton and Thorley 1981). Quil A, an adjuvant of the saponin type, improved the performance of AP vaccines (Egerton *et al* 1978) and can produce titres comparable with OE vaccines (Walduck and Opdebeek 1996). Granulomatous site reactions were not a feature of AP vaccines with or without Quil A (Egerton *et al* 1978) or of Quil A vaccines (Walduck and Opdebeek 1996).

1.3.2.9.   Antigenic competition in multistrain vaccines

'Antigenic competition' refers to the apparent competition for immune responses which occurs when multi-component vaccines are compared to any one component of the vaccine administered alone. To use vaccines in the control of footrot, it is necessary to use multistrain vaccines because serogroup-specific vaccination provides little cross-protection and most outbreaks include a range of serogroups (Hindmarsh and Fraser 1985; Claxton 1986; Kingsley *et al* 1986; Thorley and Day 1986) and, even if the range is restricted, normal investigations do not survey the bacterial flora exhaustively, and the range of serogroups is not known with sufficient confidence to justify the use of a vaccine with a restricted range of antigens.

Antigenic competition significantly reduces the response by sheep to vaccination with multistrain *D nodosus* vaccines, compared to the response to each component (Schwartzkoff *et al* 1993a; Egerton *et al* 1994; Raadsma *et al* 1995). Raadsma *et al* (1994b) described a linear relationship, with a negative slope, between the number of *D nodosus* serogroups represented in a vaccine and the logarithm of the agglutinating titre at three weeks after the primary vaccination and three, five and eight weeks after the secondary vaccination. As a result of the decline in titre with increasing number of antigens in the vaccine, sheep which received the decavalent vaccine had the least protection against footrot eight weeks post secondary vaccination, compared with those receiving one, two or five antigens only. By contrast, monovalent vaccination is capable of producing high and sustained agglutinating titres (O'Meara *et al* 1993).

Antigenic competition is not overcome by increasing the total antigen load in the vaccine, nor by distributing the multivalent vaccine at more than one vaccination site (Hunt *et al* 1994). The reduced response in agglutinating titres is directly associated with a reduction in the number of serogroup-specific antibody secreting cells (ASCs) in the lymph nodes draining the vaccination site (Hunt *et al* 1995). The responses in both B-cells and T-cells limit the immune response. There appears to be a limit on the number of antigen-specific B-cells which can be triggered within a lymph node and, therefore, which give can rise to ASCs. At high doses of antigen, the maximum number of cells is stimulated and, if more than one antigen is present in the vaccine, competition for B cells occurs. The limitation in T-cell response is not clearly

understood but does not appear to be associated with deficiencies in antigen presentation (Hunt *et al* 1995)

1.3.2.10. Genetic variation in vaccine responsiveness

In a study to examine the genetic basis of the variation in immunological responsiveness to footrot vaccine in Merino sheep, Raadsma *et al* (1995) found two-fold differences in the estimated breeding values between the highest and lowest ranked sires. The estimated heritability of the post-vaccination titre for serogroup A was 0.41 and for serogroup B was 0.56, indicating that a major component of the variation in agglutinating titres in the eight weeks after vaccination between sheep is under genetic control. The genetic correlation between vaccine responsiveness and innate resistance is, however, low. These workers also found that the genetic correlation between responses to each serogroup was 0.5, indicating that some of the genes involved are different for each serogroup.

1.3.2.11. Time between doses of vaccine

Chetwin *et al* (1986) used an oil-adjuvanted whole-cell vaccine containing nine strains of *D nodosus*, including eight serogroups, A - H, (Footvax, Coopers Animal Health, New Zealand) with intervals between doses of 6, 10 and 16 weeks. For all intervals tested, maximal antibody titres resulted within three to four weeks of the second dose. A different result was observed with a nine strain recombinant vaccine, including serogroups A - I (Vaxall Genesis, Arthur Webster Pty Ltd, Sydney). With seven interdose intervals between two and 53 weeks tested, Schwartzkoff *et al* (1993b) found that agglutinating titres three weeks after the second vaccination became higher as the interdose period became longer. They also observed that titres fell faster when the dose interval was greatest so that, by 12 weeks post-vaccination, titres of all groups were similar.

1.3.2.12. Duration of protection

Two immunisations with pili vaccines against one serotype produce high agglutinin titres which peak four to eight weeks after the second vaccination and remain high for at least 28 weeks

(Stewart *et al* 1982b). Other estimates of the duration of protection from mono-specific vaccination range from five months (Skerman and Cairney 1972), six months (Egerton and Burrell 1970) to four years (O'Meara *et al* 1993). Raadsma *et al* (1995) remarked on the very high group mean titre six months after the second dose of monovalent recombinant fimbrial vaccine.

Vaccines containing antigens to protect against eight to ten serogroups provide much shorter duration of protection. Lambell (1986) found that two doses, nine weeks apart, of a multivalent (eight serogroup) vaccine protected Merino ewes for at least ten weeks during an outbreak of footrot. A number of other reports suggest that multivalent vaccination can be expected to protect Merino sheep for up to 12 weeks after the second dose, although for some animals protection may be inadequate at eight weeks (Skerman *et al* 1982; Schwartzkoff *et al* 1993a; O'Meara *et al* 1993).

1.4.   Effects of footrot on sheep production

The effect that severe footrot has on the health, welfare and productivity of sheep is clear to any observer and producers have readily appreciated that an outbreak of VFR will have very significant effects on their sheep and the profitability of their sheep grazing enterprises. For this reason, it has seemed unnecessary, or of low research priority, to quantify the effects of VFR on health, welfare and productivity when the magnitude was so obvious to those whose flocks were affected.

The fact that VFR causes severe lameness and that lameness in grazing sheep will inevitably reduce feed intake and, therefore productivity, has been recognised since the earliest reports (Mohler and Washburn 1904; Gregory 1939; Beveridge 1941). Beveridge (1941) also noted the effect of footrot on bodyweight, wool quality and fleece weight and reproduction without estimating the degree of losses. One of the earliest reports to attach an economic cost to footrot infection was from Hunt (1958) who compared the weight gain of lame lambs and their flock mates in an infected flock, and estimated the financial value of the difference.

1.4.1. Methods of estimating the effect of footrot

There are no published studies of the effect of natural outbreaks of virulent footrot on the productivity of Merino sheep. The limited studies that have been performed have been done in pens and small plots with single isolates introduced artificially. The one published report on a large field trial (Marshall *et al* 1991) was also based on the introduction of one isolate of a virulent strain of *D nodosus*.

Attempts have been made to measure the effects on production of benign footrot (Glynn 1993) and intermediate footrot (Cummins *et al* 1991; Glynn 1993) in natural outbreaks. Cummins *et al* (1991) compared the liveweights and fleece weights of Merino ewes exposed to footrot to ewes protected from footrot by vaccine, footbathing or both.

Symons (1978) measured bodyweight and wool growth in six pairs of infected and uninfected sheep. The sheep were penned and competed for access to food in their pens. Wool growth was measured in tattooed patches.

Stewart *et al* (1984) compared the effects of one virulent (A198), one benign (C305) and two intermediate strains (B312 and a bovine strain, 332) on wool growth and bodyweight of Merino sheep running in small experimental plots. Sheep were run on eight plots, each divided into two blocks, giving four replicates of each strain. There were four sheep on each block (64 total) plus one donor (16 total). Wool growth was measured by dye-banding of fleece staples and comparing the rate of wool growth post-infection to the rate of wool growth pre-infection for each sheep. The productivity of the affected sheep was compared but the lack of uninfected control groups limited the interpretation of the results.

Stewart *et al* (1986a) reported two pen experiments demonstrating the different effects on production of infection with benign, intermediate and virulent strains. Groups of eight sheep were challenged with either a benign strain (337), an intermediate strain (333 and 335) or a virulent strain (334 and 336). They recorded losses of bodyweight which were greatest for the virulent strains, less for the intermediate strains and least for the benign strain. In the second experiment, strains 333, 334 and 336 were used again. Again, there was a gradation of effect

on bodyweight with the intermediate isolate allowing greater increases of bodyweight than the two virulent isolates. It would appear from the results of the study of Stewart *et al* (1986a) that pen trials allow intermediate strains to cause a higher percentage of severe lesions (≥3c) than would occur in the field because, in the first experiment, the two intermediate strains caused up to 60% of feet to be severely affected.

Marshall *et al* (1991) measured bodyweight and wool growth effects in a study covering two years, commencing in October 1985. A virulent isolate of serogroup A (VCS56) was used. Ten donor animals were allowed to naturally infect 200 wethers, of which 50 were treated at intervals to limit the development of lesions. The authors calculated an interval footrot index (IFI) for each sheep, which was calculated as the number of feet with lesion scores greater than or equal to score 3a. The index related to the interval between weighing events, usually 28 days. The effect of footrot was estimated by comparing bodyweights and wool weights of untreated sheep to sheep treated by footbathing at frequent intervals during transmission periods.

1.4.2. Estimates of the effect of footrot

Foot lesions caused by infection with virulent footrot reduce the wool production of sheep (Symons 1978; Stewart *et al* 1984; Marshall *et al* 1991). Estimates of the effect of virulent footrot on wool production range from 8% of annual wool production (Marshall *et al* 1991) to a 14.6% difference in wool production between infected and uninfected sheep during an eight week period in which the lesions in the infected sheep were moderate or severe (Symons 1978).

Virulent footrot also reduces the rate of bodyweight gain, increases the loss of bodyweight or leads to the maintenance of lower body weights in affected sheep compared to uninfected sheep (Littlejohn 1964; Symons 1978; Stewart *et al* 1984; Stewart et al 1986a; Marshall *et al* 1991). Estimates of the effect of footrot on bodyweight range from a relative loss of 6.7% of bodyweight during the eight week period of infection (Symons 1978), maintenance of bodyweight at least six kilograms lower than that of uninfected adult Merino wethers during a 16 week period of infection (Stewart *et al* 1984), to a difference of 3.5% to 7% between

infected and uninfected Downs breed-sired weaners (Littlejohn 1964). Marshall *et al* (1991) related the duration and severity of footrot lesions to the change in bodyweight and found that the more infected feet that a sheep had and the longer the period of active infection, the greater was the effect on bodyweight. They concluded that one foot continuously affected with footrot (score ≥ 3) for two years would reduce by 12.3 kg the bodyweight of Merino wethers which weighed 54 kg before infection.

It is clear from these experimental studies that the greatest changes in bodyweight occur during periods when footrot is spreading within the flock and lesions are actively developing. At times when lesions are regressing or following treatment, previously affected sheep may regain some lost weight (Stewart *et al* 1984; Marshall *et al* 1991). While it is possible for sheep to regain much of the lost bodyweight rapidly after curative treatment, with compensatory gain, lowered wool production over the period of active infection will not be fully recovered following treatment but, presumably, the rate of woolgrowth will return to normal about the same time that lesions heal. Thus the effect of virulent footrot on annual wool production will depend on the duration of the infection as well as the severity of lesions.

It is likely that the reduction of 8% of wool production found by Marshall *et al* (1991) is the best available estimate of the effect of virulent footrot on annual wool production but there are two reasons why this is probably an underestimate. First, the comparison was made between treated and untreated sheep. Footrot did occur in the treated sheep and so one can presume that these sheep would have produced more wool had they been completely free of footrot. Second, welfare concerns led to treatment of the infected sheep on two occasions during the experiment, and the withdrawal of one animal which was severely affected. It can therefore be assumed that the 'untreated' sheep would have produced even less had no treatment whatsoever been administered. Marshall *et al* (1991) observed no statistically significant difference for yield of clean wool, fibre diameter, or tensile strength between treated and untreated sheep in either year of the experiment with VFR. The observed difference in fibre diameter was 0.41μ and 0.48μ, the treated group having the broader wool.

Infection with less virulent strains of *D nodosus* causes less severe effects on wool weight and bodyweight than virulent strains (Stewart *et al* 1984; Stewart et al 1986a; Cummins *et al* 1991;

Glynn 1993). In a study of a natural outbreak, infection with a strain classified as intermediate in virulence led to differences of 5% in greasy fleece weight between untreated sheep and sheep treated by footbathing to reduce the severity and prevalence of footrot lesions (Glynn 1993). Uncontrolled intermediate footrot led to significantly lower bodyweight than that of treated sheep, although the effect of the footrot was presumably exacerbated by grass seeds penetration of the interdigital skin. There were also differences in the percentage of tender fleeces and in mean fibre diameter but the writer does not state whether these differences were statistically significant or not. Treatment did not completely prevent lesions and so, again, the effect of footrot on production is likely to be an underestimate of its full effect on the flock.

Dobson (1986), without providing specific estimates of the effects of intermediate footrot, records that the economic motivation to eradicate it is almost non-existent. Cummins *et al* (1991) did not demonstrate any difference in fleece weight between control sheep with intermediate footrot and those protected with vaccine, footbathing or both, and the difference in liveweight of 3.4 kg at the height of disease expression was of low statistical significance (P=0.07).

There are few published estimates of the effect of benign footrot on bodyweight. Glynn (1993) found that uncontrolled benign footrot decreased bodyweight of sheep in some parts of the year compared to sheep treated to reduce the effect of footrot, but that there were no significant differences at the end of the footrot spread season. Roycroft (1986) records the occurrence of pregnancy toxaemia in ewes as a consequence of benign footrot infection.

1.5.   Epidemiology of footrot

1.5.1.   The effect of environmental conditions on survival of the organism

It is generally considered that *D nodosus* is incapable of surviving on pastures for more than seven days and transmitting to unaffected sheep, as concluded by Beveridge (1941). Under some favourable conditions, survival may be enhanced. When material from lesions of footrot were mixed with mud *D nodosus* was found to be capable of surviving three days, rarely one week and never three weeks. In sheep faeces the organism could not survive two weeks

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(Beveridge 1938b). Laing and Egerton (1981) found that *D nodosus* could be cultured from lesion material after five days in the environment, but not after seven days. It is not necessary for sheep to cohabit to transmit footrot - Whittington (1995) reported the transmission of footrot from an infected flock with a very low footrot prevalence to predisposed, but uninfected, sheep through common use of wet sheepyards. The uninfected sheep moved into the yards immediately after the infected sheep left.

1.5.2.   The effect of environmental conditions on transmission and development

Footrot will not spread through a flock of sheep unless there is adequate moisture present in the pasture or in the surface on which the sheep are walking (Beveridge 1934b; Graham and Egerton 1968). The main role for moisture is probably the facilitation of a predisposing infection in the interdigital skin with *F necrophorum*, which precedes or occurs concurrently with *D nodosus* infection (see section 1.1.1). Moisture is probably also important in the survival of the organism outside the host for sufficient time for it to be transferred successfully from one sheep to another (Beveridge 1938b).

The most comprehensive study of the role of environmental factors on footrot transmission was done by Graham and Egerton (1968). They found that footrot transmission occurred in spring in those years which had a high and evenly distributed rainfall averaging about 50 mm or more per month. Outbreaks occurred less commonly in late summer and autumn and only in years when the average monthly rainfall for the four or five months preceding the outbreak had exceeded 70 mm per month and the outbreaks occurred in months when the rainfall exceeded 75 mm. Sustained rainfall was necessary for outbreaks to occur and footrot did not spread following isolated periods of heavy rain.

Graham and Egerton (1968) also observed that free moisture on the pasture was required for transmission but that, in cool months and when pastures were long and dense, heavy dew would allow pastures to stay wet all day even when rain did not fall. Under these conditions, transmission of footrot occurred during periods without rainfall. Persistent moist conditions were necessary for transmission and irrigation of pastures during a hot, dry climatic period did not lead to transmission of footrot. The important role of pastures in maintaining a suitable

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climate for the disease has been noted. Longer pastures favour the transmission of the disease (Cummins *et al* 1991) making control more difficult (Sinclair 1957).

These workers also found that temperature was important in precipitating outbreaks of footrot once there was sufficient moisture in pastures and the interdigital skin was predisposed to infection. Footrot did not spread on the southern and central Tablelands of New South Wales during winter, even when rainfall was adequate. Outbreaks began when mean daily temperatures consistently exceeded 10°C. In the regions in which their study flocks existed, Graham and Egerton (1968) determined that conditions suitable for the spread of footrot would occur in approximately one of every two years and would commence in early spring and persist only for three to four weeks, unless persistent heavy rain fell. In a similar environment, Egerton *et al* (1983) found that spread in endemically infected flocks occurs in August, September and October and the highest prevalence of infection occurs in October.

The report of Graham and Egerton (1968) was used a number of times in the studies reported in this thesis to predict footrot outbreaks and to assist with the understanding of the epidemiology of footrot. For further discussion, see Chapters 3, 4, 5 and 6.

1.5.3.   The effect of environmental conditions on lesion development

The major clinical difference between intermediate footrot and virulent footrot is in the proportions of affected sheep which develop severe lesions (see section 1.1.2). Thus, in an environment which is suitable for the expression of the disease, it is expected that virulent footrot will lead to a high percentage of score 4 lesions but that, with intermediate footrot, there will be relatively few score 4 lesions. The effect of the environment on the expression of the disease is, however, not well understood. In particular, the possibility that conditions can be suitable for transmission of footrot but not suitable for the development of severe lesions has not been fully explored. The issue is relevant to state control programs because, if the environment has a more marked effect on lesion development than on disease transmission, footrot outbreaks in 'unsuitable' districts cannot be fully evaluated, even if the prevalence of footrot is relatively high. Thus, graziers with footrot which causes only a small percentage of the flock to develop severe lesions, even when spread has occurred within the flock, should be

forced to eradicate the disease or at least restrict its spread because the disease may become more severe in more 'favourable' environments. Woolaston (1993) reported that paddock differences have an effect on footrot severity but, in his study, severity was measured as an average paddock score which included unaffected sheep, so it is not possible to dissociate the extent of the lesion and the presence of a lesion into two separate effects. Depiazzi *et al* (1998) compared the expression of footrot, probably an intermediate strain, in five different environments but presented no evidence to suggest that the severity of the lesions, was worse in any environment in which a higher prevalence of footrot occurred.

The study by Depiazzi *et al* (1998), conducted at five sites in Western Australia, used a strain of *D nodosus* which reportedly caused score 2 lesions only in 95% of affected feet in the flock of origin. The strain produced thermostable proteases, with an S1 zymogram and was classified as category 3 by gene probe (Rood *et al* 1996), a category which predominantly comprises benign strains. Under experimental conditions, the footrot outbreaks produced by this strain were of a high prevalence at two sites only. At one site, the yearly maximum prevalence of footrot-affected feet was 81%, 81% and 68% in three consecutive springs. The authors do not differentiate between score 3 and score 4 lesions. In each spring, the proportion of feet with score 3 or 4 lesions was approximately 10% at or about the peak of the outbreak, except for a short-term (one inspection) increase two weeks after the peak in prevalence in one spring. At the other site where a high prevalence occurred in one spring, the proportion of affected feet with score 3 or 4 lesions appeared to be also about 10%, although at one inspection near the peak of the outbreak, there was a short-term increase in the proportion of feet with score 3 or 4 lesions.

The clinical behaviour of this outbreak is, by the definitions used in this thesis, intermediate.

1.5.4.   Geographic distribution of footrot in New South Wales

The areas considered suited to footrot transmission and development are the areas east of the 500 mm isohyet, which runs through Wagga Wagga and Dubbo (Plate B), and in the cooler regions of the state. The area inside the 600 mm rainfall isohyet is the part of Australia which is most favourable for the expression and persistence of all forms of footrot (Egerton and

Raadsma 1993). In this region the prevalence of infected flocks is high (Scott-Orr 1986) and footrot outbreaks are characterised by a high proportion of sheep with severe foot lesions.

The prevalence of infected flocks declines between the high rainfall areas south west of Canberra and the low rainfall areas around and south of Hay (Locke and Coombes 1994). Very low temperatures may reduce the expression of footrot, such as in the district east of Cooma, in the Monaro district, in the south-east part of NSW. The prevalence is also generally higher in the areas of the state with significant winter rainfall, in the southern part, although a recent audit of the NSW Footrot Strategic Plan found that 12% and 22% of the flocks in the Armidale and Glen Innes Rural Lands Protection Board areas (northern Tablelands of NSW) had virulent footrot (Walker 1997).

Plate B

Climatic regions of NSW. Following page, left, average annual minimum temperature and, right, average annual maximum temperature. Subsequent page, average annual rainfall. Data are based on climatology records for the period 1961 - 1990. (Copyright Commonwealth of Australia, Bureau of Meteorology)

The areas considered suited to footrot transmission and development are the areas east of the 500 mm isohyett, which runs through Wagga Wagga and Dubbo, and in the cooler regions of the state (Egerton and Raadsma 1993). The prevalence of infected flocks declines between the high rainfall areas south west of Canberra and the low rainfall areas around and south of Hay (Locke and Coombes 1994). Very low temperatures may reduce the expression of footrot, such as in the district east of Cooma, in the Monaro district, in the south-east part of NSW. The prevalence is also generally higher in the areas of the state with significant winter rainfall, in the southern part.

Average Annual Minimum Temperature
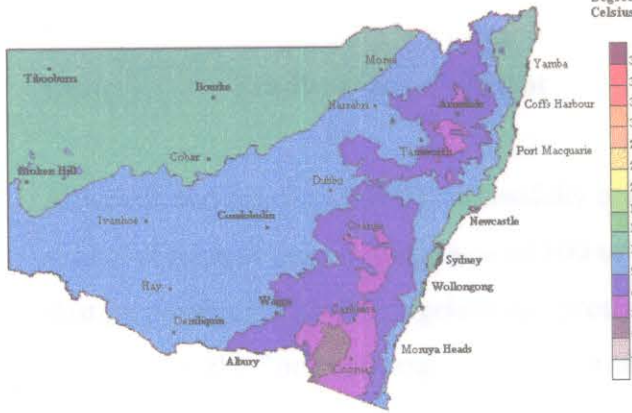
Degree Celsius

39
36
33
30
27
24
21
18
15
12
9
6
3
0
-3

Based on a standard 30 - year climatology (1961 to 1990)
© Copyright Commonwealth of Australia, Bureau of Meteorology

Average Annual Maximum Temperature

Degrees Celsius

39
36
33
30
27
24
21
18
15
12
9
6
3
0
-3

Based on a standard 30 - year climatology (1961 to 1990)
© Copyright Commonwealth of Australia, Bureau of Meteorology

Average Annual Rainfall

Millimetres

3200
2400
1800
1200
900
600
400
300
200
100
50
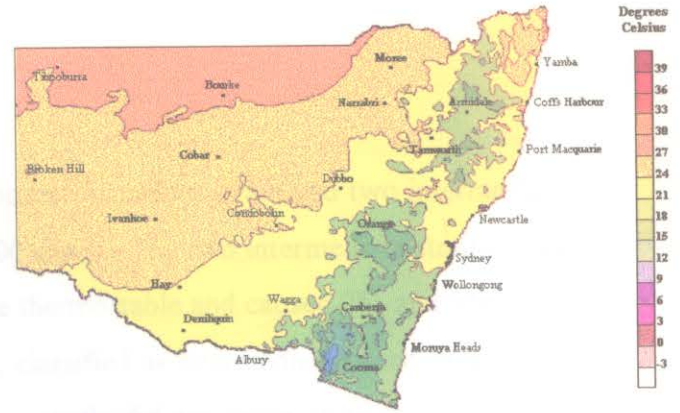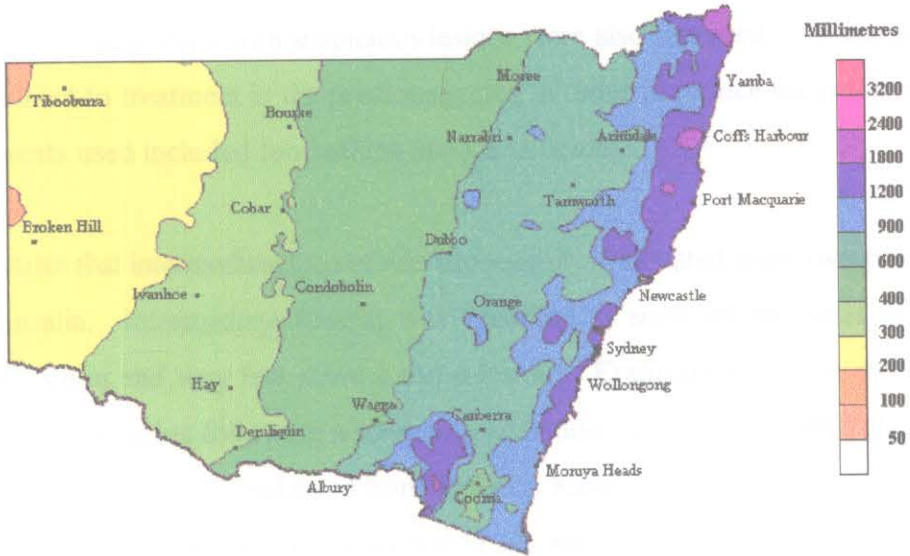
Based on a standard 30 - year climatology (1961 to 1990)
© Copyright Commonwealth of Australia, Bureau of Meteorology

1.6. Treatment and control of footrot

1.6.1. Eradicability of footrot

Allworth and Egerton (1995) successfully eradicated a virulent strain and two intermediate strains of footrot from three groups of 300 to 400 sheep. The two intermediate strains were elastase positive (at 21 days), gelatin-gel protease thermostable and caused 7% and 5% score 4 lesions in the flocks of origin. A third strain, classified as intermediate based on elastase testing and protease thermostability but which reportedly did not cause score 4 lesions in the flock of origin, was apparently eradicated from both mobs in which it had demonstrably become established before eradication commenced. One benign strain persisted in each of two mobs from which all other strains were eradicated. One of these benign strains had some characteristics of intermediate strains in that it produced thermostable proteases.

The method used to eradicate the disease was to remove, in February, all sheep which had visible lesions of footrot and to repeat inspections twice more, at approximately monthly intervals, at which time any sheep with suspicious lesions were also removed. All sheep in the trial had been subject to treatment in the previous spring in order to reduce the prevalence of lesions. Treatments used included footbathing and vaccination.

Gwynn (1986) states that intermediate footrot was successfully eradicated from two properties in Western Australia. Intermediate footrot was classified as such on the basis of a low prevalence of infection and very few score 3 and 4 lesions. Eradication was determined by inspection of all feet in spring following a summer eradication. Robinson (1986) also states that intermediate footrot has been eradicated from flocks on Kangaroo Island (South Australia) and that breakdowns have occurred when hospital flocks are retained.

Brownrigg (1986) described the appearance of footrot outbreaks caused by strains of low virulence during a statewide program aimed at reducing the prevalence of footrot in South Australia in the 1950s and 1960s. These strains persisted in flocks despite attempts to eradicate the disease. The disease produced 2% - 3% of sheep with score 3 lesions but no score 4 lesions so were benign or intermediate in virulence.

It is widely believed that benign footrot is difficult or impossible to eradicate from flocks with techniques which are successful with virulent strains (Beveridge 1941; Roycroft 1986). The number of inapparent infections is thought to be higher with mild strains of footrot than with virulent strains (Egerton and Raadsma 1993), so the chance of missing infected feet during an inspection is higher. Low virulent forms have been detected in apparently healthy skin by microscopy (Egerton and Parsonson 1969) and by culture (Glynn 1993; Allworth 1994; Depiazzi *et al* 1998)

1.6.2. Antibiotic treatment

In many reports on the efficacy of topical and parenteral treatments for footrot, the rate of spontaneous recovery in untreated animals is either not measured or not reported. To properly attribute the recovery from footrot to a particular treatment, the rate of spontaneous recovery should be known and the rate of cure attributable to the treatment should be calculated with respect to the animals which would not otherwise have recovered. In this thesis, the term *cure rate* will be used where the spontaneous recovery rate is taken into account in a report, and *recovery rate* when it is not.

1.6.2.1 In-vitro susceptibility of *D nodosus* and *F necrophorum*

D nodosus is very sensitive to penicillin *in vitro* and moderately sensitive to erythromycin, chloramphenicol and oxytetracycline. Streptomycin and dihydrostreptomycin have minimum inhibitory concentrations *in vitro* 10 to 100 times higher than other antibiotics listed (Stewart 1954a; Egerton *et al* 1968; Gradin and Schmitz 1983).

F necrophorum is sensitive to, in decreasing order, penicillin, erythromycin, tetracycline and chloramphenicol (Clarke *et al* 1989).

1.6.2.2 Topical antibiotic treatment

Chloramphenicol has been used as a topical treatment for footrot with some success. Ten percent solutions in propylene glycol (Stewart 1954a), in methanol (Stewart 1954b; Penny

1955; Sambrook 1955; Sinclair 1957) and in ethanol (Cross 1978b) were effective in pen trials and in field trials. Solutions were applied to feet after rigorous paring. Standing sheep on hard, dry surfaces after treatment improved results. Recovery rates were high but, when done during transmission periods, repeated treatments at two weekly intervals were considered necessary. At four weeks after treatment, relapse was common (Stewart 1954b; Sinclair 1957).

The relatively high expense of 10% chloramphenicol solutions created interest in the efficacy of lower strength solutions but these were found to be significantly less effective. Stewart (1954a) obtained poorer results with 2% and 5% solutions than with 10% solutions. Harriss (1955a) found that chloramphenicol in 2% and 0.5% alcoholic solutions was no more effective than 10% copper sulphate solutions applied in a footbath, but was more expensive. Baxter and Smyth (1956) obtained slightly inferior results with 5% chloramphenicol in methanol including 4:4'-diaminodiphenyl sulphone compared to 10% alcoholic solutions.

Oxytetracycline ointment (5 mg/g, with 10 000 units/g of polymixin B sulphate) applied to pared foot lesions was effective in mid-winter in England (Harriss 1955b). In the trial, the sheep were held in dry conditions for at least one hour after treatment before being returned to pasture.

1.6.2.3. Parenteral antibiotic treatment

One parenteral treatment with penicillin is effective in curing a high proportion of sheep affected with footrot, but the dose required is higher than that recommended by manufacturers for use in other disease conditions. In reported studies, doses of procaine penicillin of 300 000 units per sheep (Forsyth 1953) and 50 000 units/kg (Egerton *et al* 1968) have been unsuccessful, while dose rates of 70 000 units/kg were successful (Egerton and Parsonson 1966a; Egerton *et al* 1968). Doses of 140 000 units/kg were no better than 70 000 units/kg. Cross (1978b) found that a combination of benzathine penicillin and procaine penicillin (42 000 units/kg of each) was a moderately effective treatment.

Streptomycin, given alone at doses of 37.5 mg/kg or 70 mg/kg, was ineffective but, in combination with procaine penicillin has been shown in a number of studies to be highly

effective, and has been widely used in the field (Egerton *et al* 1968; Venning *et al* 1990; Webb Ware *et al* 1994). Egerton *et al* (1968) recommended dose rates of 70 000 units/kg plus 70 mg/kg (procaine penicillin and streptomycin respectively) on the basis of greatly improved efficacy over either drug alone. Cross (1978b) found that the combination (56 000 units per kg plus 70 mg/kg) was moderately effective. Reported cure rates vary from approximately 40% (Webb Ware *et al* 1994) to 96% (Egerton *et al* 1968) and the more successful results are achieved when sheep are returned to dry conditions after treatment.

Oxytetracycline (11 mg/kg) by IM injection is effective (Cross 1978b) and is recommended by the manufacturer of the long acting formulation at a dose rate of 20 mg/kg based on in-house and other unpublished studies (Pfizer technical information update No 378).

Erythromycin has been found to be highly effective at 20 mg/kg (Egerton *et al* 1968) and 12 mg/kg (Webb Ware *et al* 1994). Its efficacy at 12 mg/kg was similar to penicillin/streptomycin when erythromycin was used in combination with a formalin footbath (Egerton *et al* 1968). Webb Ware *et al* (1994) found that erythromycin at 12 mg/kg was more effective than penicillin/streptomycin (70 000 units/kg, 70 mg/kg) but that a lower dose of erythromycin (8 mg/kg) was similar to the penicillin/streptomycin combination.

Venning *et al* (1990) treated footrot-affected sheep with a combination of lincomycin/spectinomycin (at 5 and 10 mg/kg of each antibiotic respectively) and penicillin/streptomycin (at 70 000 units/kg and 70 mg/kg), both combinations given once, intramuscularly. Cure rates and recovery rates were high with both treatments. No significant improvement in efficacy was achieved by repeating treatment with lincomycin/spectinomycin on two following days, nor by using a three times higher dose. Jordan *et al* (1996) found that long-acting oxytetracycline (24 mk/kg) and lincomycin/spectinomycin (6 and 12 mg/kg) both were more effective than penicillin/ streptomycin at 60 000 units/kg and 60 mg/kg.

A feature of parenteral use of antibiotics is that paring of affected feet is not necessary before treatment, beyond that necessary to make a diagnosis (Egerton *et al* 1968; Venning *et al* 1990; Webb Ware *et al* 1994), resulting in less time spent preparing sheep for treatment compared to that necessary for topical treatments and less painful preparation for the affected sheep.

Most authors agree that recovery rates following parenteral antibiotics are higher when the sheep are kept in dry conditions following treatment. During transmission periods, this is achieved by standing sheep on slatted floors, usually in woolsheds, for 24 hours after treatment (Egerton *et al*, 1968; Jordan *et al* 1996), but even this strategy may be insufficient to achieve good success rates if the sheep are returned then to wet pastures (Webb Ware *et al* 1994). High humidity reduces the diffusion of penicillin/streptomycin combinations into the exudate of footrot lesions (Egerton *et al* 1968), a fact which probably accounts for the poorer recovery rates reported when sheep are returned to wet environments after treatment.

The simultaneous footbathing of sheep treated with antibiotics has resulted in improved rates of recovery when formalin was used (Egerton *et al* 1968) or zinc sulphate was used (Allworth 1995). Possibly, the footbathing improves efficacy by killing *D nodosus* organisms which are too superficial to be exposed to antibiotics given parenterally (Egerton *et al* 1968).

The rational application of antibiotics in footrot treatment and eradication programs has been discussed by Vizard (1996) who recommended that they be used only if 20% or more of a flock were affected by footrot at the beginning of summer. If the prevalence was lower than this, culling of affected sheep was a more affordable solution which avoided the risk of re-infection.

1.6.3. Footbathing

A variety of antiseptic solutions have been used to treat footrot, including formalin at strengths up to 10% (Beveridge 1938c; Fitzpatrick 1961), 10% copper sulphate (Beveridge 1941) and 10% zinc sulphate with 0.2% Teepol[R] (Skerman *et al* 1983a and b). Following the discovery that sodium lauryl sulphate (SLS) enhanced the penetration of hoof horn by zinc sulphate (Malecki and McCausland 1982), a formulation including the two compounds was patented and made commercially available as Footrite[R] (Hardman Chemicals Pty Ltd, Seven Hills, NSW, Australia) (Malecki, McCausland and Lambell 1983; Malecki and Coffey 1987). Skerman *et al* (1984) evaluated a copper 8-hydroxyquinolate solution (Defeat[R]) and a 10% solution of CHF-1020 (copper nitrate trihydrate/copper chloride dihydrate in water) has been recently released commercially as a treatment for footrot, marketed as Radicate[R] (Reed and Alley 1995; 1996).

Footbathing is practised in conjunction with paring of feet to improve the access of the solution to infected tissues, or it can be administered without prior paring. Severe paring was recommended when investigations were performed first in Australia (Beveridge 1941; Pryor 1954) but, more recently, the laborious nature of paring (Egerton 1986) and the impact of severe paring on the welfare of the sheep has led to interest in the success of topical treatments with no prior preparation of the feet (Malecki *et al* 1983; Reed and Alley 1996). When footbathing is used as a treatment for chronic infections, however, paring may be necessary to achieve acceptable cure rates (Malecki and McCausland 1982; Lambell *et al* 1986).

Recommendations for the duration of footbathing treatment range from walk-through (Beveridge 1941; Lambell *et al* 1991), where sheep may have only a few seconds of contact with the footbathing solution, 5 minutes (Skerman *et al* 1983b), 15 minutes with Radicate[R] (Reed and Alley 1996), 30 minutes in zinc sulphate (Mulvaney *et al* 1986) to one hour with zinc sulphate and Footrite[R] (Malecki and Coffey 1987).

The frequency of treatment with footbath solutions can vary from twice weekly (Skerman *et al* 1983a), every five days (Atkins 1986; Malecki and Coffey 1987), weekly (Fitzpatrick 1961; Mulvaney *et al* 1986) or every two weeks (Reed and Alley 1996).

The effectiveness of footbathing solutions is dependent on the contact between the antiseptic solution and the footrot infection. While some chemicals may have a limited ability to move into horn tissue, such as zinc sulphate when used in conjunction with sodium lauryl sulphate (Malecki and Coffey 1987), other chemicals, like formalin, have no ability to penetrate the horn and they will only be efficacious if they come in contact with the infected tissue. This fact limits the usefulness of footbathing solutions for underrun lesions without paring (Egerton 1986) although they may be useful for benign and intermediate footrot infections where most foot infections are limited to the interdigital skin or minor underrunning. Plant and Claxton (1986) found that, after severe foot paring, 5% formalin footbathing, every five days, for five minutes, repeated four times and Footrite[R] footbathing, twice at five day intervals, for 60 minutes reduced the prevalence of score 2 lesions present 28 days after treatment commenced. Solutions of zinc sulphate (10%) were not significantly better than paring alone.

Other workers have found zinc sulphate at least as effective as formalin solutions. It does not have the unpleasant and toxic characteristics of formalin and, when used on a regular basis at weekly intervals, may be able to prevent new infections during periods when conditions favour disease transmission (Skerman *et al* 1983a; Stewart 1989).

In summary, many comparisons of the efficacy of various footbathing solutions have shown that footbathing treatments generally have no long-term advantages in the control and eradication of footrot, but may reduce the prevalence of footrot in the short-term (Plant and Claxton 1986).

CHAPTER 2

CONTENTS

MATERIALS AND METHODS

2.1.   Materials

2.1.1.   Commonly used laboratory solutions

2.1.1.1.   Loading buffer (6 x)

Loading buffer used in electrophoresis of PCR products and RE digests was prepared by dissolving bromophenol blue (0.25% w/v) and sucrose (40% w/v) in sterile distilled water (Sambrook *et al* 1989). Loading buffer was prepared and stored in 1 ml aliquots at -20°C.

2.1.1.2.   Lysis buffer

Lysis buffer consisted of 50 mM Tris borate pH 8.9, 2 mM EDTA, 1% Triton X-100. It was used to assist the lysis of bacterial cells by heat in preparation of genomic DNA for PCR.

2.1.1.3.   Phosphate buffered saline (PBS)

Stock solution of PBS at twenty times (20 x) working strength consisted of 170 g sodium chloride, 41 g disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$) and 3.12 g sodium dihydrogen phospate dihydrate ($Na_2HPO_4.2H_2O$) dissolved in 1 litre of distilled water and autoclaved (Claxton 1981). The pH was adjusted to 7.4 with sodium hydroxide before use at working strength.

2.1.1.4.   Tris-borate buffer (TBE)

Stock solution of TBE was made at five times (5 x) working strength by dissolving 54 g Tris base, 27.5 g boric acid and 20 ml 0.5 M EDTA in 1 litre of deionised distilled water and autoclaving (Sambrook *et al* 1989). TBE was used at 0.5 x working strength for electrophoresis of agarose gels and at 1 x in polyacrylamide gels.

2.2.   Methods

2.2.1.   Foot inspections

For examinations of the feet, sheep were inverted in a variety of ways. When no sheep handling equipment was available, sheep were caught and turned over into a sitting position on the floor. On other occasions, sheep were pushed onto an electric-powered V-belt handler and inverted into dorsal recumbency on the handler for examination, then returned to an upright position and ejected from the handler (Plate C).

Feet were examined by separating the toes, inspecting the interdigital skin and the skin-horn junction for evidence of separation and underrunning. Paring of horn was avoided in most cases to prevent any iatrogenic influence on the course of lesion development.

2.2.2.   Footscoring

Footrot lesions were 'scored' using a system based on that described by Egerton and Roberts (1971). Each foot of the sheep is examined and allocated a score from 0 to 4, reflecting the extent of the footrot lesion. A normal foot has a score of 0; score 1 indicates mild interdigital dermatitis; 2, interdigital dermatitis with evidence of skin necrosis characteristic of footrot; 3, underrunning of the horn of the heel and sole but restricted to the soft horn of the posterior part of the foot; 4, underrunning of the sole extending to the hard horn of the abaxial wall or the anterior part of the sole (Plate A, Chapter 1).

Scores of the four feet were recorded as a series of four numbers for each sheep, always in the same order, starting from left fore, then right fore, left hind and right hind. After reading the sheep's ear tag, tag number and footscores were called out to an assistant who recorded the scores on pre-printed forms (Plate C). A foot was considered to be 'affected' with footrot if it had a score of 2, 3 or 4 (see 2.2.3)

Plate C

TOP

Examination of sheep for footscoring. The feet of sheep were examined by inverting the sheep on a V-belt handler. Each foot was examined in turn and the ear tag number and footscore was called out to a recorder. The sheep was then ejected from the handler onto a wool-filled cushion and simultaneously rolled back into an upright position by the person inspecting the feet. The forward and backward motion of the V-belts was controlled by a pedal bar under the foot of the inspector. Illustrated is the author examining sheep at property K, near Tarcutta, NSW (see Chapter 5).

BOTTOM

Collection of specimens for bacteriology. Specimens were taken for bacterial culture by scraping a stick over the affected tissues of the interdigital skin, skin-horn junction or underrun horn of the hoof. This photograph shows how necrotic material is collected from the skin horn junction onto the end of a specimen stick, prior to applying the material to a 4% hoof agar plate.

2.2.3.   Classification of sheep by maximum footscore

For most analyses, each sheep was given one number, from 0 to 4, based on the maximum score of all four feet. In this thesis, the expression 'sheep with score n lesions', refers to sheep with a maximum footscore of n. Sheep were considered to be 'affected' by footrot if they had at least one foot with a score 2, 3 or 4 lesion.

2.2.4.   Classification of virulence in outbreaks

In the study flocks reported in this thesis, it was determined before selecting a flock that the form of footrot present was intermediate in virulence, based largely on clinical assessment but, in every case, the diagnosis was supported by laboratory assessments of protease activity.

The method used was in accordance with the approach discussed in section 1.1.2.3.

- At one inspection of at least 100 sheep selected at random from the flock, between 2% and 10% of the affected sheep had at least one score 4 lesion.

- In the same sample, more than 10% of the affected sheep had at least one score 3 lesion underrunning at least half of the soft horn of the sole.

- At the height of an outbreak, following a transmission period, the percentage of sheep with at least one score 4 lesion did not exceed 10%.

- Outbreaks were characterised by a mixture of score 2 and score 3 affected sheep, rather than a predominance of score 2 lesions (as in benign footrot) or a predominance of score 4 lesions (as in virulent footrot).

2.2.5. Collection of lesion material

Lesion material was collected for bacterial culture using a wooden swab stick to scrape material from the interdigital skin, skin-horn junction or from an area of underrun horn on the sole or wall of the hoof (Plate C). The material was then applied to the surface of a 4% hoof agar (HA) plate as a streak across the centre of the plate. The same swab stick was streaked across one end of the first inoculum streak at right angles, and a third and fourth streak were made across each end of the second streak. Plates were placed in three litre anaerobic jars (Le Parfait Supreme preserving jars) and made anaerobic with the BBL GasPak Plus Anaerobic System (Becton Dickinson and Company, Cockeysville MD USA). As soon as possible, usually within eight hours, the jars were placed in an incubator at 37°C for four days before examination of the plates.

2.2.6. Culture procedures

After four days incubation, the lesion plates were examined for the characteristic colonies of *D nodosus*. If none was detected, plates were replaced in anaerobic jars and re-incubated for up to three days. If colonies were detected, these were picked off the agar with a sterile loop and applied to a new 4% plate. The first sub-culture plates were often divided into two or four and one colony, as free of contaminants as possible, was streaked onto one half or one quadrant of the secondary plate. Plates were then incubated anarobically at 37°C for three to five days. For secondary sub-culture, single colonies were removed from the first sub-culture plates and streaked onto 2% HA plates, then incubated.

2.2.7. Elastase test for virulence

Elastin-agar plates were made as described by Stewart (1979) and Stewart and Claxton (1993). Plates were divided into quadrants by drawing two lines on the bottom of the plate. A pure culture of a *D nodosus* strain to be tested was applied to the centre of one quadrant in a 25 mm radial streak. A second test strain was applied in the opposite quadrant and a virulent reference strain was applied in a third quadrant and, usually, an intermediate reference strain was applied opposite the virulent control.

Plates were incubated anaerobically at 37°C and examined after 4, 7, 11, 14, 18, 21 24 and 28 days. A positive test result was clearing of the elastin particles around the streaked inoculum. The first day at which clearing occurred for each control and each test strain was recorded for each plate tested.

2.2.8. Serogroup and serotype determination

Serogroup was determined by two methods, a slide agglutination test and a tube agglutination test. Sub-types of the serogroups (serotypes) were determined by tube agglutination tests. Slide agglutination is quicker and simpler than tube agglutination and was performed generally as a screening test. Cross reactions between serogroups, however, occurred in some instances with slide agglutination which prevented positive identification of some isolates. In these cases, tube agglutination was used to confirm the serogroup of particular isolates.

2.2.8.1. Slide agglutination

Antisera to reference isolates representing each of the nine major serogroups A to I were prepared by vaccination of adult New Zealand white rabbits with formadehyde-killed cells in Freund's incomplete adjuvant, using a method described by Claxton *et al* (1983). *D nodosus* cell suspension was prepared by scraping bacterial colonies with a sterile scalpel blade from 2% HA plates into PBS and diluting to a concentration of approximately 5×10^9 cells per ml. For slide agglutination, a loop was used to place a drop of cell suspension onto a glass microscope slide and a loopful of undiluted antiserum was added and mixed with the cell suspension by stirring. The slide was then rocked gently from end to end by hand for up to 30 seconds. Coarse, flocculent agglutination was considered a positive reaction.

2.2.8.2. Tube agglutination

Bacterial cell suspension and antiserum were prepared as for slide agglutination. The method used was similar to that described by Egerton and Laing (1978). Aliquots of 0.5 ml of cell suspension were placed in a series of tubes and 0.5 ml of serial dilutions of antiserum in PBS, starting with a 1:10 dilution, were added to each of 12 tubes. The tubes were incubated at

37°C for four hours then left at room temperature for 12 to 18 hours. The reciprocal of the highest dilution at which flocculation clearly occurred was recorded. The possible results ranged from 20 (reaction only with 1:10 dilution of antiserum) to 40 960.

2.2.9. PCR - RFLP procedures

2.2.9.1. DNA preparation

Isolates of *D nodosus* collected and purified in the course of these studies were stored as lyophilized samples under vacuum at 4°C in glass ampoules. To extract and prepare genomic DNA from lyophilised samples for PCR-RFLP procedures, the bacteria were suspended in 50 µl of sterile water, the suspension then transferred to a 0.5 ml Eppendorf tube containing 50 µl of lysis buffer and vortexed for 10 seconds to form a lysate. The lysates were cooled on ice before heat treatment at 100°C for 10 minutes in a waterbath. The tubes were cooled on ice prior to centrifugation for two minutes at 10 000g. DNA extraction from the supernatant was performed via the ethanol precipitation method described by Sambrook *et al* (1989).

In short, the supernatant (90 µl) was transferred to a sterile Eppendorf tube containing 10 µl of 3M sodium acetate, pH 5.2. Two and half volumes of ice-cold absolute ethanol (250 µl) was added and the samples stored at -20°C overnight. The samples were centrifuged at 12 000 g for 30 min at 4°C in a microfuge. The supernatant was carefully removed and 0.75 ml cold 70% (v/v) ethanol added. The tubes were centrifuged at 12 000 g for two minutes at 4°C. The supernatant was removed and washed again with 70% (v/v) ethanol. The supernatant was removed and the pellet dried for 15 minutes on the bench at room temperature. Once dried the pellets were resuspended in 50 µl of sterile deionised water and stored at 4°C.

2.2.9.2. Polymerase chain reaction (PCR) procedures

Two primers, A (5' AAT CAA GGA ACT GAA GAA 3') and C (5' AAT GCC GTA CAT TAA AGC A 3') (Moses 1993) were used for the amplification of the multiple variant *omp1* genes within the genome of *D nodosus*. The reaction mixture consisted of PCR buffer supplied by the manufacturer with the polymerase, one unit of *Taq* DNA polymerase (Life

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Technologies, USA) and final concentrations of 3 mM $MgCl_2$, 0.4 µM of each primer and 200 µM of each of the four deoxynucleotide triphosphates. Sterile deionised water was used to make a final volume of 20 µl after the addition of DNA template, the volume of which was optimised for each bacterial sample. Each PCR reaction was performed in 20 µl capillary tubes supplied by Corbett Research (Mortlake, Australia).

A touchdown PCR procedure (Don *et al* 1991) was used to amplify the *omp1* gene region in *D nodosus*. The amplification cycle commenced with denaturation at 94°C for two minutes followed by two cycles of 94°C for five seconds, 56°C for five seconds and 72°C for 30 seconds. The annealing temperature was decreased by 1°C every two cycles. At 50°C the cycle was repeated 20 times before a final extension at 72°C for two minutes. The solution containing PCR products was transferred to a sterile Eppendorf tube before 3 µl was removed for electrophoresis on an agarose gel. The remaining solution of PCR product was stored at 4°C for later restriction digest.

2.2.9.3. Visualisation of PCR product by electrophoresis and staining

The success of the PCR was checked by electrophoresis of a small portion of the solution of PCR products in a 1.2% agarose gel. The gels were stained with ethidium bromide and visualised under ultraviolet light.

2.2.9.4. Restriction digests

Restriction endonuclease digests were carried out in solutions containing the buffer solution supplied with the restiction enzyme, 2 µg of acetylated bovine serum albumin (BSA) and 1µl (10 units) of *Hpa*II or *Sau*3AI (Promega Corporation, Madison, Wisconsin). Fifteen µl of solution containing the PCR products (aproximately 1 µg of DNA) was added and sterile deionised water was used to make a final volume of 20 µl. All reactions were carried out in sterile 0.5 ml Eppendorf tubes at 37°C overnight.

2.2.9.5. **Visualisation of digested PCR products by electrophoresis and staining**

The products of each restriction endonuclease digest were analysed using either 2.5% agarose gels or 4-20% gradient TBE minigels (Novex, San Diego, California). Five μl of loading buffer (6 x) was added to the digest product and loaded onto the gel. The molecular weight marker pUC19/*Hpa*II (Progen, Ipswich, Queensland) was loaded into the first lane of each gel. Electrophoresis was carried out using submarine gel tank systems (Owl Scientific Plastics Incorporated, Cambridge, Massachusetts) at 90-100 volts for 90 minutes. Gels were stained in a 5 ng/μl TBE-ethidium bromide solution for 20-30 minutes with regular agitation. The size of DNA fragments appearing as bands on the gel was estimated by comparison to the molecular weight marker.

2.2.10. **Collection of blood samples**

Blood samples were collected from the jugular vein into 10 ml Vacutainer tubes (Becton Dickinson, Lincoln Park USA). Tubes were kept at moderate temperatures, in shade or at room temperature, for up to 12 hours before refrigeration. Serum was separated by centrifugation and poured into 5 ml plastic vials which were strored at -20°C until serology was performed.

2.2.11. **Serology procedures**

The technique used for serology is essentially similar to that described by Stewart and Claxton (1993). To measure agglutinating titres for a specific serogroup, the prototype strain of the serogroup was cultured on 4% HA. After five days incubation at 37°C under anaerobic conditions the plate was subcultured onto six 2% HA plates and incubated under the same conditions. Similarly each plate was then subcultured onto five 2% HA plates and incubated. Each plate was washed with 750 μl of formol PBS, the suspensions pooled and the optical density (A_{260}) determined. The concentration of antigen required for serology (10^8 cells per ml) was produced by diluting with formal PBS (FPBS).

Agglutinating tests were performed in 96-well microtitre plates. The serum sample (20 μl) to be tested was added to 180 μl of PBS in the first well, mixed, 100 μl withdrawn and added to 100 μl PBS in the next well. The process of serial dilution was continued to a total of 12 wells. A multichannel pipette was used to transfer aliquots in eight rows simultaneously. Antigen suspension (100 μl) was added to each well in columns 2 to 12. The plates were agitated gently for 10 min and incubated at 37°C overnight. The lowest concentration at which agglutination occurred is the titre recorded. As the initial dilution of serum in column 2 was 1:20, serial dilutions progressed by a factor of two to 1:40 960.

2.2.12.   Artificial infection

The technique of artificial challenge was similar to that described by Egerton and Roberts (1971). Briefly, sheep were held in pens on floors covered in rubber mats overlaid with foam and kept saturated by frequent wetting from a hose. Faeces was allowed to accumulate. Heavy growth of cultures of *D nodosus* on 2% hoof agar plates were scraped so that the bacteria were collected at one point at the edge of the plate. A plug of agar covered in the bacterial colonies was cut from the plates with a scalpel blade and placed on a moistenned cotton wool swab, which was then applied to the interdigital skin of the sheep and bandaged into place (Plate D). The sheep were returned to the wet mats for a further three days then the bandages removed. Sheep were maintained on wet mats for at least one further week before moving to field sites to act as donors.

2.3.   Time of studies

The dates at which the various studies reported in this thesis were carried out are reported in Table 2.1. Six properties were involved in the studies and five are these are indentified by letter to retain the anonymity of the flock owners. The final field study was performed at the University of Sydney's JB Pye farm at Bringelly, NSW.

Plate D

Artificial infection of sheep with footrot. Sheep were held in concrete-floored pens covered in rubber mats overlaid with foam and kept saturated by frequent wetting from a hose. After one week, cultures of *D nodosus* on 2% hoof agar plates were scraped so that the bacterial cultures were collected at one point at the edge of the plate (lower photograph). A plug of agar covered in the bacterial colonies was cut from the plates with a scalpel blade and placed on a moistenned cotton wool swab, which was then applied to the interdigital skin of the sheep and bandaged into place.

The sheep in the top photograph are held in a small pen on a wet rubber mat immediately prior to the application of bacteria. These sheep were donors for the artificial challenge experiment described in Chapter 6.

Table 2.1   **Dates of studies**

Property :	F	B and W	K and T	JB Pye
Location :	Yass	Cooma	Tarcutta/Holbrook	Bringelly
Chapter :	3	4	5	6
Jan-June 1992		Preliminary inspection Trial commenced Property B		
July-Dec 1992	3 preliminary examinations.	Trial at Property B completed. Study at Property W commenced.	Field site located	
Jan-June 1993	Preliminary eradication trial commenced	Study at Property W completed.	Study commenced.	
July-Dec 1993	Trial completed		Study continued.	
Jan-June 1994	Eradication of whole flock commenced		Study continued.	
July-Dec 1994	Eradication complete		Flock transferred to Holbrook.	
Jan-June 1995			Study completed.	Site constructed
July-Dec 1995				Study commenced.
Jan-June 1996				Study completed.
July-Dec 1997			PCR-RFLP studies carried out	
Jan-June 1998	Final inspection			

CHAPTER 3

CONTENTS

CHAPTER 3

THE ERADICATION OF INTERMEDIATE FOOTROT

3.1. Introduction

The variation which is observed in the expression or presentation of footrot in different flocks has led to the notion of a continuous spectrum of disease, from mild forms, where almost all affected sheep have lesions confined to the interdigital skin, to highly virulent forms, where nearly all affected sheep have severe underrunning of the hooves (Stewart 1989; Egerton 1989). This spectrum of disease has been simplified by categorising outbreaks into one of three categories which have been called scald, mild footrot and severe footrot (Alexander 1962) or, more commonly in recent times, benign, intermediate and virulent footrot (Egerton and Parsonson 1969; Stewart 1984). Strains of *Dichelobacter nodosus* isolated from outbreaks of each of these three forms of footrot demonstrate differences in a number of virulence-associated factors, particularly including protease activity, protease thermostability and electrophoretic mobility (Thomas 1962; Egerton and Parsonson 1969; Depiazzi and Richards 1985; Stewart *et al* 1986; Stewart 1986). A more detailed discussion of the clinical and microbiological differences between forms of footrot is provided in the following chapter (section 4.1).

Methods for the eradication of virulent footrot from sheep flocks without the destruction or disposal of the entire flock have been clearly described (Beveridge 1938c; Gregory 1939; Beveridge 1941; Pryor 1956; Thomas 1957; Toop 1957; Littlejohn 1961; Hayman and Triffit 1964; Egerton 1986; Stewart 1989; Allworth 1990). These methods were based on the identification of all infected sheep during a non-transmission period, usually summer, and the removal, or treatment, of affected sheep. The success of eradication programmes was improved if control measures were applied during the previous transmission period, usually spring, to ensure a low prevalence of infection at the start of the non-transmission period. Control measures have included footbathing in antibacterial solutions (Beveridge 1938c) and vaccination (Egerton 1986).

Inspection of sheep in the non-transmission period requires that every sheep in the flock be inverted and every foot carefully examined, with paring of any excess horn if necessary (Pryor 1956; Toop 1957; Lambell *et al* 1991).

Where affected sheep are not culled from the flock but treated, local treatment with severe paring and topical application of antibacterial solutions (Beveridge 1938c; Thomas 1957) or parenteral antibiotics (Egerton and Parsonson 1966) have been used and recommended. Egerton (1989) suggested that antibiotic treatment could be selective, after identification of cases by foot inspections, or whole-flock. The latter approach, while using more antibiotic, would have the potential benefit of eliminating footrot from sheep with unrecognisable infections. The subject of antibiotic treatment is discussed further in section 1.6.2.3.

Vaccines, particularly multivalent vaccines, are recommended for control of footrot and are known to reduce the prevalence and severity of footrot. Multivalent vaccines will not eliminate footrot from a flock although they can contribute substantially to the success of eradication programs based on inspection in non-transmission periods (Egerton 1986). The problem of antigenic competition in multivalent vaccines is discussed in 1.3.2.9 and 1.3.2.12. Vaccines made with more limited ranges of serogroups of *D nodosus*, however, offer much more hope as tools which might alone lead to eradication of the disease from flocks, based on the higher titres achieved (Raadsma *et al* 1995) and persistence of titres (O'Meara *et al* 1993). A bivalent vaccine has been used successfully to eliminate footrot from sheep and goat flocks in Nepal (Egerton et al 1996; Ghimire 1997).

There are no documented cases of the eradication of benign footrot and there have long been suspicions that the disease cannot be eradicated by methods based on the identification of infected sheep. Benign footrot has been noted to reappear in individual sheep after clinical recovery (Alexander 1962), organisms have been identified in histological sections of clinically normal skin (Egerton and Parsonson 1969), *D nodosus* has been isolated from asymptomatic sheep in footrot-affected flocks (Glynn 1993; Allworth 1994; Depiazzi *et al* 1998) and strains of low virulence have recurred in flocks following the apparently successful eradication of more virulent strains (Brownrigg 1986; Egerton and Raadsma 1993; Allworth and Egerton 1995).

The eradicability of intermediate footrot is unclear. It may, like benign footrot, be difficult to eradicate, or it may be like virulent footrot and be eradicable. Without precise clinical descriptions of the form of the disease, Gwynn (1986) and Robinson (1986) claim that intermediate footrot has been eradicated from flocks in Western Australia and South Australia although it is not stated what surveillance, if any, was performed to confirm eradication. Allworth (1995) successfully eradicated one virulent and two intermediate strains of *D nodosus* from three groups of 300 to 400 sheep. The two intermediate strains were considered capable of causing 5% and 7% of score 4 lesions respectively in the flocks of origin. In his experiment, a third strain, classified as intermediate by the elastase test and protease thermostability test, but which did not cause score 4 lesions in the flock of origin, was also eradicated from the two mobs in which it had become established.

The field trial described here was designed to test three methods of eradicating intermediate footrot from a sheep flock and to apply one of those methods to a whole-flock eradication program. In the first part of the trial (the *preliminary eradication trial*), three methods (foot inspection, either alone, combined with monospecific vaccination or combined with antibiotic treatment) were compared as strategies for eradicating footrot from 10 experimental flocks. In the second part of the trial, eradication from the entire flock (*whole flock eradication*) was attempted using a method shown to be effective in the preliminary eradication trial.

3.2.   Materials and methods

3.2.1.   The flock

The flock of sheep in which footrot was studied consisted of approximately 3 000 Merino sheep of a medium wool type running on property F. The flock included approximately 600 ewes which formed a ram-breeding nucleus. Other flock owners with similar breeding objectives contributed ewes to the ram breeding nucleus. Rams bred on this property were sent to another property operated by the same grazier and were also dispersed to contributing flocks. The association with other graziers ceased with the outbreak of footrot but ram breeding for the owner's commercial flock continued.

3.2.2.   The environment

Property F, near Yass in the southern Tablelands of New South Wales, is 803 hectares in area. It is subdivided into 26 paddocks, the largest being 89 hectares. Introduced pastures including _Phalaris tuberosa, Lolium perenne_ and _Trifolium subterraneum_ were established in 1962 and have been fertilised on an annual basis at the rate of 125 kg of superphosphate (9.1% phosphorus) per hectare per year.

3.2.3.   Characteristics of the footrot outbreak

Footrot was detected in the flock in early January 1992. Attempts by the owner to eradicate the disease by footbathing the flock in 5% formalin solution within a few weeks of the diagnosis were unsuccessful. Infection was still present on 13 April 1992 when 1 717 sheep from 13 mobs were examined. At that time, 181 sheep were found to be affected with footrot and of those, 31% had score 3 lesions and 2% had score 4 lesions. This clinical evidence suggested that the footrot was of intermediate virulence.

In March 1992, samples were collected for bacteriological examination from three sheep with score 4 lesions and submitted to the NSW Agriculture regional laboratory at Wagga Wagga. Two primary isolations were made, sub-cultured and five secondary isolations were made. Protease thermostability testing classified four of these isolates as thermostable and one as unstable. Three thermostable isolates were also elastase positive at 14 or 21 days. The isolates were considered to be of two virulence types, one benign and one intermediate.

Footrot was controlled in the flock during spring 1992 by vaccination (Norot, Fort Dodge Australia Pty Limited, Castle Hill, NSW, Australia) on 2 June 1992 and 27 July 1992. Footbathing in 10% zinc sulphate solution was also performed on 27 July. Footbathing of the entire flock with the same treatment was repeated on 29 December 1992.

A further inspection carried out in February 1993 (see 3.2.6) revealed seven sheep with score 4 lesions. The presence of about 1% of sheep with a maximum footscore of 4, despite the vaccination and footbathing administered in June, July and December, provided further

evidence that the form of footrot in the outbreak was intermediate rather than benign.

3.2.4. Experimental objectives

The owner agreed to participate in a trial in a proportion of the flock to determine if eradication were possible and, if so, which of three methods was preferable. This trial, nominated the *preliminary eradication trial*, involved approximately 600 ewes and ran from February to October 1993. If eradication was successful in the first trial, an attempt would be made to eradicate footrot from the whole flock (*whole flock eradication*) in the following year and this attempt would be monitored and evaluated against an infected control flock.

3.2.5. Site for the preliminary eradication trial

The experimental site consisted of a set of twelve 10 hectare paddocks set in a low lying, flat area of the property. The paddocks were normally used for single-sire mating of stud ewes. For this experiment, each replicate of 62 or 63 ewes was allocated at random to one 10 hectare paddock. Two paddocks were ungrazed. A plan of the site appears in Plate E.

3.2.6. Preliminary eradication trial

On 1 February 1993, the preliminary eradication trial began. Six hundred and forty nine ewes aged 3, 4 and 5 years were inspected and 20 (3%) affected ewes were culled. Seven of these ewes had chronic score 4 lesions (Table 3.1). Six hundred and twenty nine ewes which were free of clinical evidence of footrot were eartagged and allocated to one of three treatment groups and, within treatment groups, one of three or four replicates. Ewes were allocated to treatments and to replicates within treatments at random after stratification on age. Each replicate was held in a separate paddock. Sheep in group VAC were given one ml of a monovalent (serogroup A) vaccine subcutaneously and allocated to one of three replicates. Sheep in group AB were injected intramuscularly with a penicillin/streptomycin mixture (2.5 g of procaine penicillin and 2.5 g of dihydrostreptomycin) (Penstrep, Ilium Veterinary Products, Troy Laboratories, Smithfield, NSW, Australia) and allocated to one of three replicates. The nil-treatment group sheep (group CON) received no treatment and were

allocated to one of four replicates. In summary, there were three replications of group VAC, three of group AB and four of group CON. Each replicate initially consisted of 62 or 63 ewes.

The ewes weighed approximately 50 kg. Joining to rams commenced in March and the ewes lambed in August. Lamb marking was done on 27 September.

Vaccination of group VAC sheep was repeated once, on 1 March 1993.

3.2.7.   Monitoring of serum antibody titres

Blood samples were taken by venipuncture from 10 ewes in each of the three replicates of group VAC and 10 ewes in three of the four CON replicates on five occasions commencing 1 February 1993 and up to and including 27 September 1993, as shown in Table 3.2. Agglutination titres to serogroup A were measured as described in Chapter 2.

Table 3.1   Footscores of affected ewes removed on 1 February 1993 at the commencement of preliminary eradication trial

	Score 0/1	Score 2	Score 3	Score 4
	Toe abscess	0002	0030	4000
		2200	0032	0040
		0002	0030	4000
		0002		0400
		0020		0400
		2000		4444
		0020		0004
		0002		
		2000		
Total	**1**	**9**	**3**	**7**

Table 3.2   Plan of experimental activities on property F

Date	Prelimipary eradication trial	Whole flock eradication
1 February 1993	Trial began	
	All ewes inspected	
	V1 to 3 groups*	
	Blood sampling 1	
1 March 1993	V2 to 3 groups*	
	All ewes inspected	
	Blood sampling 2	
22 March 1993	All ewes inspected	
27 April 1993	All ewes inspected	
	Blood sampling 3	
18 June 1993	All ewes inspected	
4 August 1993	All ewes inspected	
	Blood sampling 4	
27 September 1993	All ewes inspected	
	Blood sampling 5	
25 October 1993	All ewes inspected	
1 December 1993		All sheep inspected
		Control flock formed
7 February 1994		All sheep inspected
3 March 1994		All sheep inspected
26 July 1994		All sheep inspected
19 October 1994		All sheep inspected
20 December 1994		All sheep inspected
		Control flock disbanded and sold
12 May 1995		All sheep inspected
3 February 1998		All sheep inspected

*   V1, V2 first and second vaccination
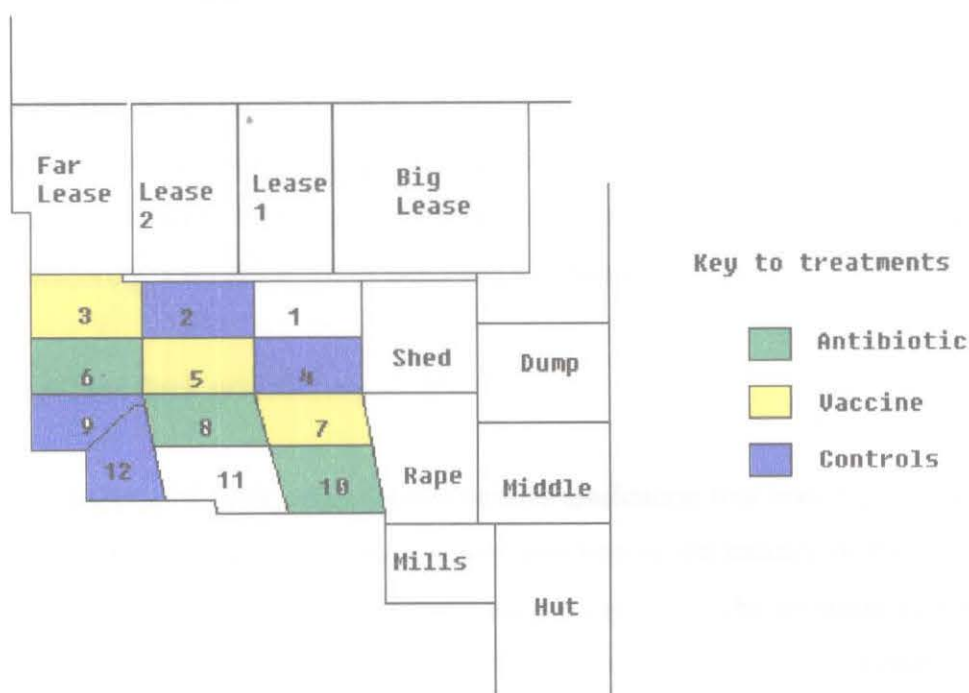
Plate E

The experimental site on property F. The experimental plots were a set of twelve 10 hectare paddocks set in a low lying, flat area of the property. The paddocks were normally used for single-sire mating of stud ewes. For this experiment, each replicate of 62 or 63 ewes was allocated at random to one 10 hectare paddock. Two paddocks were ungrazed.

3.2.8.   Inspections during the preliminary eradication trial

Inspection of the feet of all experimental ewes was done on seven occasions at intervals of approximately four to six weeks during autumn, winter and spring of 1993 (Table 3.2). Inspections were carried out by inverting each sheep on a V-belt sheep handler and examining each foot. Footscoring was performed as described in Chapter 2.

3.2.9.   Whole flock eradication

During winter and spring of 1993, while the preliminary eradication trial was in progress, the owner of the flock maintained a low prevalence of infection in the balance of the flock by footbathing with 10% zinc sulphate solution on eight occasions. The program of footrot eradication from the entire flock commenced on 1 December 1993. All sheep, approximately 2 300 adults and 1 400 weaners, were inspected six times before the end of 1994. Only sheep free of evidence of footrot infection were retained, except for an infected control flock.

3.2.10.   The control flock

One hundred sheep with evidence of footrot were identified on 3 December 1993 and managed in isolation from the rest of the flock to form an infected control flock. This flock was derived from the ewes which had been treated with antibiotics in the preliminary eradication trial. Sheep with score 4 lesions were not retained; all sheep retained had score 2 or score 3 lesions. Specimens for bacteriology were collected from these sheep.

3.2.11.   Footrot inspections during the whole flock eradication

Following the first inspection on 1 December 1994, all sheep including those in the control flock were inspected on 7 February, 3 March, 26 July, 19 October and 20 December 1994 (Table 3.2). No further inspections were carried out until 12 May 1995 when 2 990 sheep were inspected. Finally, on 3 February 1998, I examined all 3 170 sheep present on the property.

3.2.12. Climatic records

Daily rainfall was recorded at Rye Park, an official recording site for the Bureau of Meteorology which is situated on the adjacent property to the experimental site. The site is situated at 34°35'54"S, 148°56'06"E at an elevation of 585 m. Daily maximum and minimum temperatures were recorded at the Yass Linton Hostel official recording station, situated at 34°49'57"S, 148°54'40"E at an elevation of 520 m and approximately 18 kilometres from the experimental property.

3.2.13. Bacterial isolations during the trial periods.

On 15 December 1992, 1 340 weaner sheep (four months of age) were examined and specimens for bacteriological examination were collected from a sample of these. Further isolations were made in September 1993, March 1994 and October 1994. Specimens were collected as described in Chapter 2, *D nodosus* isolated when possible, and the serogroup of isolates identified by slide agglutination. Selected isolates were lyophilised and stored for further examination.

PCR-RFLP of the *omp* gene was performed on stored isolates using the method described in Chapter 2.

3.2.14. Statistical methods

Chi-squared tests for independence were used to examine the statistical significance of differences in footrot prevalence between treatment groups.

3.3.   Results

3.3.1.   Footrot detected in the preliminary eradication trial

The trial commenced on 1 February 1993, when sheep, apparently free of infection, were allocated to groups and paddocks, and received antibiotic, vaccine or nil supporting treatment. At each of the three subsequent inspections in March and April, any sheep which had inflammation of the interdigital skin were removed from their groups and returned to the main (non-experimental) flock. The footscores of affected sheep are shown in Table 3.3. At the second inspection on 1 March 1993, nine sheep were removed from the four inspection-only (CON) replicates, one sheep was removed from the three vaccinated replicates (VAC) and one was removed from the antibiotic-treated replicates (AB). The differences in numbers of affected sheep between the CON group and either the VAC group or AB group are statistically significant ($\chi^2 = 8.20$ P<0.025) On the third inspection (22 March 1993), only four sheep were removed, all from two of the three AB replicates. On the fourth inspection (27 April 1993), three were removed from CON replicates, four from VAC replicates and none from AB replicates. On the fifth and sixth inspections (18 June and 4 August 1993), no lesions were detected in any sheep and none was removed.

Overall, from 1 March to 4 August, 12 (4.8%), 5 (2.6%) and 4 (2.1%) sheep were identified with footrot and removed from the CON, VAC and AB replicates respectively. The differences were not statistically significant ($\chi^2 = 2.77$).

On 27 September 1993, all ewes were inspected at lamb marking. No lesions were detected in any of the four CON replicates or the three VAC replicates, however all three AB replicates had substantial numbers of footrot-affected sheep. Across all three AB replicates, 64 ewes (36%) had score 2 lesions, 20 ewes (11%) had score 3 lesions and 2 ewes (1%) had score 4 lesions (Table 3.4.). The lambs from these ewes were also affected and were footbathed after marking (Plate F). The prevalence of footrot in the lambs was not determined but the presence of footrot in them demonstrated that transmission of footrot had occurred.

Plate F

The lambs from the AB ewes were also affected with footrot and were footbathed after marking on 27 September 1993.

Table 3.3   Footscores of sheep removed during trial

(a)   1 March 1993

Replicate	Treatment Group		
	CON	**VAC**	**AB**
1	1000	-	-
	2200		
	0200		
	0200		
	2000		
2	0200	2020	0200
	0001		
	0020		
3	-	-	-
4	1200		
Total	9	1	1

(b)   22 March 1993

Replicate	Treatment Group		
	CON	**VAC**	**AB**
1	-	-	0100
			1100
2	-	-	0022
			Injured
3	-	-	-
4	-		
Total	0	0	4

78

(c)   27 April 1993

Replicate	Treatment Group		
	· CON	VAC	AB
1	0020	-	-
2	2000	0022	-
		2200	
3	-	0022	-
		0100	
4	0002		
Total	3	4	0

(d)   27 September 1993 (number of affected sheep, not footscores)

Replicate	Treatment Group		
	CON	VAC	AB
1	-	-	20
2	-	-	34
3	-	-	31
4	-		
Total	0	0	85

Table 3.4 **Prevalence of sheep of each maximum footscore category in the three AB replicates, 27 September 1993**

Replicate	Maximum footscore				Total sheep
	0/1	2	3	4	
1	67%	25%	7%	2%	60
2	45%	40%	13%	2%	62
3	45%	41%	9%	0%	56
Overall	52%	36%	11%	1%	178

3.3.2.   Footrot detected during the whole flock eradication

At the first inspection in the eradication phase on 1 December 1993, 2 690 sheep which had not been in the AB group in the preliminary eradication trial were examined and 24 sheep (1%) with footscores of 1 or 2, or with foot abscess, were culled. Of the AB group, 174 were present and 110 were culled because of footrot, of which 100 formed the control flock and 10 were sold for slaughter.

No infected sheep were detected at the inspection of the flock (excluding controls) on 7 February 1994.   Two weaners which had been born in the AB group in 1993 were removed at the March inspection. At further inspections on 26 July, 19 October and 20 December 1994 no further infected sheep were detected, except for a flock of ram weaners, in which two were found with footrot on 26 July. The ram weaners were born to ewes in the preliminary eradication trial and included sheep born to AB group ewes. The flock of ram weaners was kept in isolation from the remainder of the flock for the duration of 1994.

At each inspection, the control flock was infected (Table 3.5).   On 3 March 1994, the prevalence of infection in the control flock had declined to 15%, and all footrot lesions were either score 2 or score 3. The prevalence rose at the mid-winter inspection but fell to 6% (October) and 5% (December) as dry, warm conditions prevailed.

Following the December 1994 inspection, the control flock and the ram weaner flock were sold for slaughter. The property was then believed to be free of footrot.

Further whole flock inspections were conducted on 12 May 1995 and 3 February 1998. No *footrot was detected at either inspection.*

Table 3.5   **Prevalence of footrot in the control flock (100 sheep) during 1994 whole flock eradication**

Date	Prevalence
1 December 1993	100%
3 March 1994	15%
26 July 1994	19%
19 October 1994	6%
20 December 1994	5%

3.3.3.   Serogroup and reference numbers of isolates of *D nodosus*

From 20 sheep sampled on 15 December 1992, 23 isolates were obtained (Table 3.6). Of these, 22 were serogroup A, one was I. Four isolates were lyophilized for future reference. Three of these were of serogroup A (reference numbers 59, 61 and 62) and one was of serogroup I (reference number 60).

On 27 September 1993, isolations were made from seven sheep in the three AB replicates which had developed footrot (Table 3.6). All isolates were of serogroup A. Lesions sampled were score 2 in all cases but one, which had score 3 lesions in all four feet. One CON group sheep with a toe abscess, one VAC group sheep with a cavity underlying the sole and one VAC group sheep with a score 1 lesion were sampled but *D nodosus* was not isolated from any of these.

On 3 March and 26 July 1994, 23 sheep were sampled, three of them on both occasions. Of these 23, 21 were derived from the infected control flock. Eighteen sheep had score 2 lesions, four had at least one score 3 lesions and one sheep had a score 1 lesion only. Two were ram weaners, as described in 3.3.2. Specimens from six sheep were culture-negative, including the sheep with a score 1 lesion, and 18 isolates, all serogroup A, were made from the remainder (Table 3.6). Three isolates were lyophilized (reference numbers 91, 92 and 93).

On 19 October, 12 isolations were made from three sheep in the control flock or the affected ram weaner flock. All were serogroup A and 4 were lyophilized (reference numbers 100, 101, 102 and 103).

Table 3.6 **Classification and identification of isolates collected during eradication studies at property F**

Date sampled	Number of sheep sampled	Number of isolates		Isolates retained and ref. numbers
		Serogroup A	Serogroup I	
15 December 1992	20	22	1	3 A, 1 I (59 - 62)
27 September 1993	10	9	0	
3 March 1994	11	7	0	3 A (91, 92, 93)
26 July 1994	12	11	0	
19 October 1994	3	12	0	4 A (100 - 103)
Total	56	61	1	

3.3.4.   Elastase testing

Three serogroup A isolates (reference numbers 59, 90 and 101) and the one serogroup I isolate (reference number 60) were tested for elastase activity and showed similar activity to the intermediate reference isolate used as a control in this test (VCS1718/26). All four isolates were positive at seven days, the same as an intermediate reference isolate, and were therefore classified as intermediate. The virulent control (VCS1001) was elastase positive at four days.

3.3.5.   PCR-RFLP pattern

The RFLP patterns produced by *Hpa*II and *Sau*3AI digestion of *omp* gene PCR product derived from the 11 lyophilized isolates are illustrated in Figures 7.3 and 7.4. With *Hpa*II, there were two distinct RFLP patterns, nominated D1 and D2. Isolate numbers 59, 61 and 62 were all of pattern D1, all other isolates including number 60 were of pattern D2. Isolates numbered 59 to 62 were the only samples retained from those collected in December 1992, before the eradication trial began. Isolate number 60 was pattern D2, in common with the isolates collected in September 1993 from infected sheep in the AB group, and in March and October 1994 from the control flock. Of interest is the fact that number 60 was of serogroup I while all other isolates were of serogroup A. Of the samples taken before the eradication trial began, three were pattern D1 and serogroup A, one was pattern D2 and serogroup I. Of the samples taken after the eradication trial, all seven were pattern D2 but serogroup A.

3.3.6.   Serological responses to vaccination

Homologous titres in sheep in the three vaccinated replicates rose to high levels following the first vaccination on 1 February 1993 and were still elevated, relative to unvaccinated controls, at the last sampling on 27 September 1993, by which time footrot had occurred in some replicates (Figure 3.1, Table 3.7).

Table 3.7 **Serological titres to serogroup A (geometric mean agglutinin titres from 10 sheep per group)**

	Vaccinates			Controls		
	Pad 3	**Pad 5**	**Pad 7**	**Pad 2**	**Pad 9**	**Pad 12**
1 Feb 1993	190	175	181	121	80	75
1 Mar 1993	26651	4305	8492	106	148	113
27 Apr 1993	6436	15202	12347	80	93	65
4 Aug 1993	4396	4744	4246	57	80	49
27 Sep 1993	4711	10240	4810	69	59	92

3.3.7. Climatic data

Mean annual rainfall at the site is 678 mm with a relatively uniform seasonal pattern. The wettest month, on average, is July (79 mm) and the driest is February (28 mm) but mean rainfall in all other months lies between 50.1 mm and 65.3 mm. Mean monthly rainfall and mean monthly mean daily temperature records are shown in Figure 3.2 for the seven year period over which the flock was under surveillance.
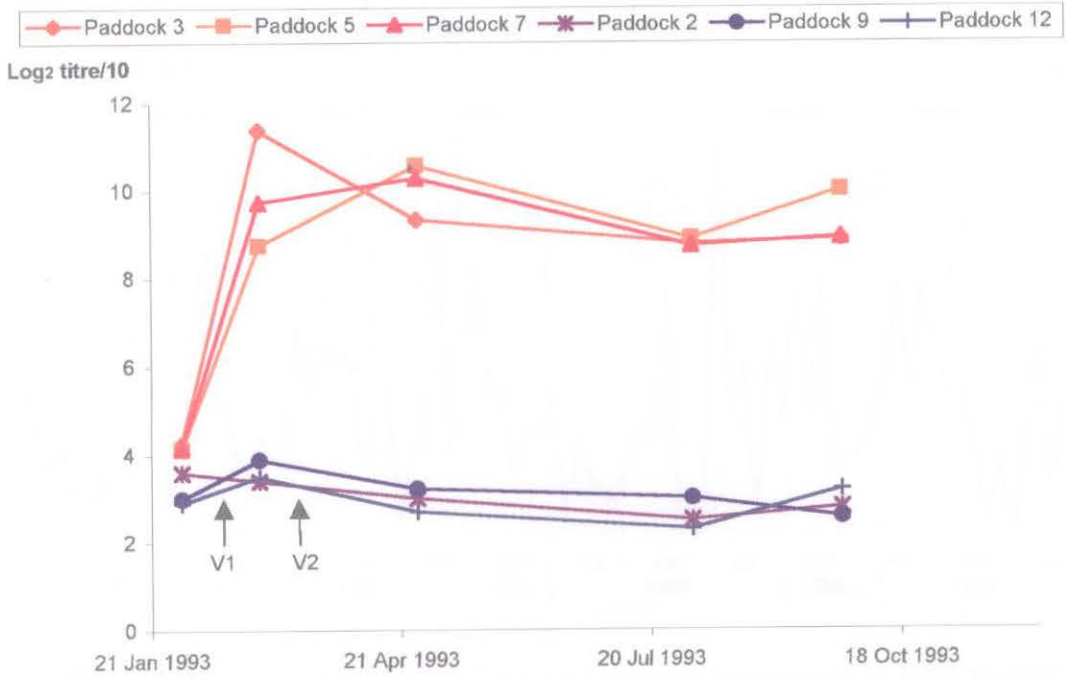
Figure 3.1   Mean agglutinating titres (log base 2 / 10) to serogroup A in controls and vaccinated sheep, property F, preliminary eradication trial. Each sampling group consisted of 10 sheep. First vaccination (V1) was given on 1 February 1993, the second (V2) was given 1 March 1993. Vaccinates were in paddocks 3, 5 and 7. Controls were in paddocks 2, 9 and 12.
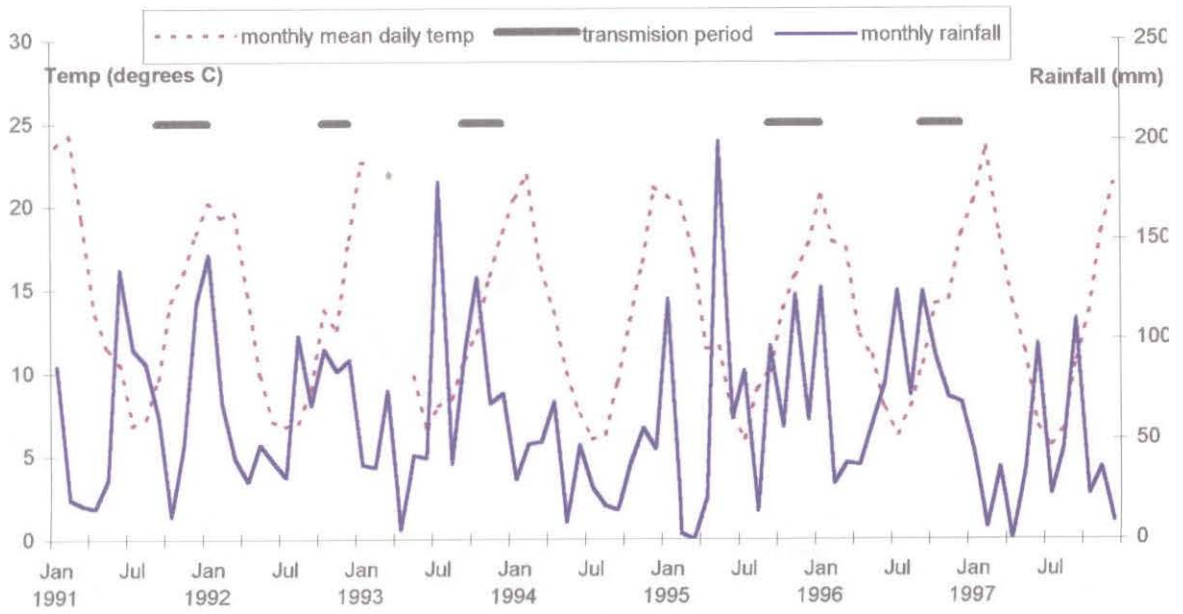
Figure 3.2   Climatic data recorded at Yass and adjacent to property F, 1991 to 1997 inclusive.   Transmission periods are predicted from the report of Graham and Egerton (1968) who found that footrot outbreaks occurred in the spring following wet winters once daily mean temperatures exceeded 10°C.
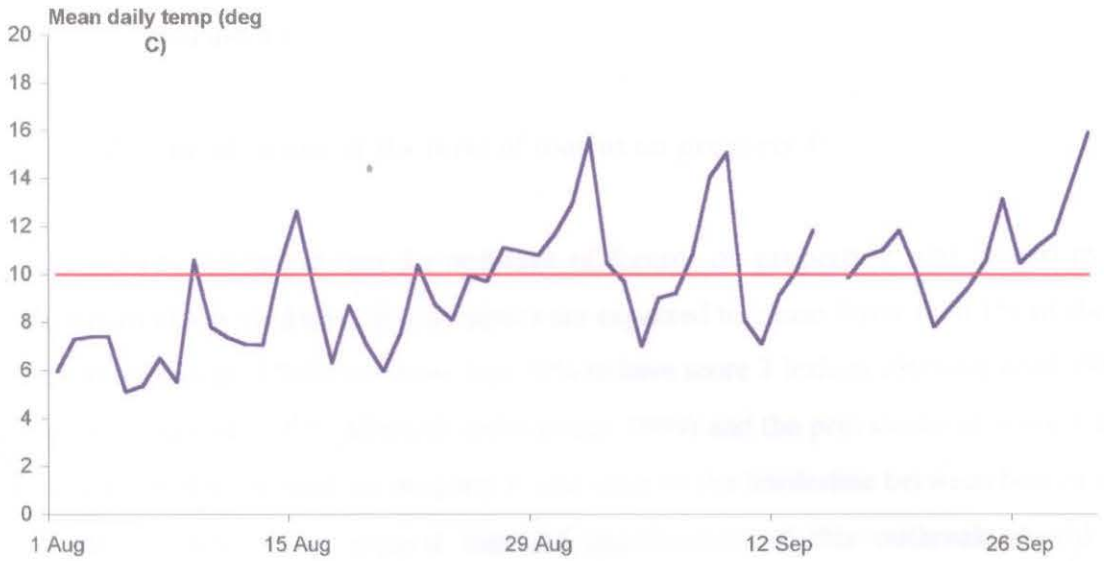
Figure 3.3   Mean daily temperature for the period of August and September 1993
preceding the footrot outbreak detected in AB group sheep on 27 September, at Yass
and property F. No footrot was detectable on 4 August but by 27 September 48% of
the ewes in the three replicates of AB group (antibiotic treated) had footrot lesions,
25% of which (12% of the total number of ewes) had score 3 or score 4 lesions. It is
presumed that transmission commenced in late August, when mean daily temperature
exceeded 10°C for five days.

3.4.   Discussion

3.4.1.   Classification of the form of footrot on property F

Clinical evidence suggested that the outbreak of footrot on property F was caused by *D nodosus* strains of low virulence. Benign strains are expected to cause fewer than 1% of sheep in a flock to have score 4 lesions and less than 10% to have score 3 lesions (Stewart *et al* 1984; Egerton 1989; Allworth 1995; Allworth and Egerton 1999) and the prevalence of score 3 and score 4 lesions in the outbreak on property F was close to the borderline between benign and intermediate. Two factors suggest that the classification of this outbreak should be intermediate, rather than benign. First, the low prevalence of severe lesions on property F when the flock was first inspected was almost certainly influenced by prior control measures. Had the disease been benign footrot, one would have expected the topical treatment and summer conditions to have led to the resolution of almost all cases by April 1992. Similarly, in February 1993, 1% of the ewe flock had score 4 lesions despite the application of control measures in the previous spring. This view is further supported by the description of the outbreak in September 1993, after a period when control measures had not been applied. The footscore profile of the AB group ewes is very similar to the footscore profile during the transmission period in the outbreaks described in Chapters 4 and 5. In those outbreaks, where control was not applied, the proportion of the flock with score 3 and score 4 lesions continued to rise after pastures started to dry, reaching levels in summer which clearly classified the outbreak as intermediate, rather than benign. If control measures had not been applied in late September 1993 on property F, it is expected that more feet with score 4 lesions would have developed.

Second, the laboratory tests showed protease thermostability and moderate elastase clearing ability are characteristics of isolates with more virulence than benign strains. There was not close agreement between the tests in different laboratories and this is likely to be due to laboratory-specific differences in the test procedure. In such cases, interpretation should be made by the respective laboratory staff. The U strain detected in the NSW Agriculture testing could be the occurrence of a benign strain which was possibly present in the flock before the intermediate strain was introduced. Alternatively, it could reflect an aberrant test.

3.4.2.   Climatic conditions and footrot outbreaks

Conditions are considered suitable for transmission of footrot when there is sufficient soil and pasture moisture over a sustained period to predispose the interdigital skin to infection and when environmental temperatures are sufficiently high. Graham and Egerton (1968) found that outbreaks of footrot occurred in spring in those years having a high and evenly distributed winter rainfall averaging about 50 mm or more per month. Rainfall at that level resulted in saturated soil conditions and sufficient moisture to produce abundant pasture growth in spring. Conditions were equivalent to, or wetter than these in 1991, 1993 and 1996 on property F. Graham and Egerton (1968) also found that outbreaks occurred in the spring following these wet winters, but not until daily mean temperatures were above 10°C. At Yass, mean daily temperatures above 10°C were consistently recorded in those three years after 25 September 1991, 24 September 1993 and 15 September 1996 (data not shown). In 1991, total rainfall for the winter period was 318 mm and for the following spring period was 120 mm. Rainfall in December (118 mm) and January (142 mm) was well above average (53 mm and 65 mm respectively).   These conditions are highly favourable for both transmission of footrot and sustained pasture growth. The relatively high and dense pasture sward encouraged moisture to persist at the base of the sward despite the high temperatures. Given that footrot was present, it is not surprising, therefore, that it was detected by the flock owner in January 1992.

In 1992, June and July rainfall was less than 50 mm per month (39 and 31 mm respectively) but August rainfall was 102 mm and was followed by a sustained wet spring (67, 84 and 90 mm in September, October and November respectively. Mean daily temperatures above 10°C were consistently recorded after 1 October 1992. These conditions were expected to be conducive to an outbreak of footrot in the spring-summer period.

In 1995, rainfall in May was very high (199 mm) and was followed by a period of eight months when rainfall was over 50 mm in every month except August (61, 84, 14, 97, 56, 122, 60 and 126 mm for June to January 1996 inclusive.) Mean daily temperatures exceeded 10°C consistently after 13 September in that year.

In 1997, conditions were barely suitable for footrot transmission, based on the criteria described above. Rainfall in the June to September period was 98, 23, 46 and 110 mm per month.

The suitability of conditions for the spread of footrot during spring 1993 is supported by the outbreak of footrot in the three replicates of group AB ewes at the latter part of the preliminary eradication trial. On the 27 September of that year almost half of the ewes had footrot. Although mean daily temperatures did not consistently exceed 10°C until 24 September, there had been several sustained periods of warm days starting on 25 August (Figure 3.3). When the trial ewes were inspected on 4 August there was no evidence of footrot in any sheep. Inspections could not be carried out after that date until 27 September because lambing was underway. It seems likely that the 'spikes' of warm weather in late August and early September precipitated the outbreak of footrot in three of the paddocks. Mean daily temperatures exceeded 10°C on 19 of the 30 days between 27 August and 27 September (records were not available on one day). These climatic conditions were apparently sufficient to precipitate a footrot outbreak given the high rainfall in the previous months.

That conditions were not favourable for transmission during 1994 was demonstrated by the decline in prevalence and severity of the footrot lesions in the control flock during 1994, to 15% in early March, a slight increase to 19% in July, then a decline to 6% in October and 5% in December, at which time the control mob was sold.

3.4.3.   Comparison of methods of footrot eradication

The first descriptions of a method to eradicate footrot from infected flocks was given by Beveridge (1938c; 1941) and Gregory (1939). The method required the control of footrot during the transmission period so that the prevalence of disease was relatively low when transmission ceased, followed by the inspection of all feet of all sheep in the flock in the subsequent hot, dry non-transmission period and the removal or treatment of all affected sheep. These basic recommendations have been used successfully since their first publication (Stewart 1954a; Toop 1957; Egerton 1986; Stewart 1989).

Recommended treatments to reduce the transmission of disease during spread periods have included footbathing (Beveridge 1941; Fitzpatrick 1961), topical antibiotics (Hayman and Triffit 1964) and vaccination (Egerton 1986). Treatment for infected animals at the time of inspection in the non-transmission period included footparing and topical antiseptic solutions (Beveridge 1941; Pryor 1956; Littlejohn 1961), topical antibiotics (Stewart 1954a; 1954b) and parenteral antibiotics (Egerton *et al* 1968; Egerton 1986).

While these methods are well accepted for the control and eradication of virulent footrot, uncertainty existed at the start of these studies about the possibility of eradicating milder forms of footrot with the same methods. The preliminary eradication trial was designed to test the success of eradication programs based on inspection and culling alone but there was concern that intermediate footrot, unlike virulent footrot, may persist in a 'carrier' state and escape detection at the summer inspections and lead to outbreaks in the following spring (Egerton and Parsonson 1969; Glynn 1993; Allworth 1995; Depiazzi *et al* 1998). For this reason, two additional treatments were introduced in order to determine if they increased the chances of eliminating all *D nodosus* organisms (at least those of significant virulence) over visual inspection and culling alone. It was reasoned that high serum antibody titres and high serum antibiotic concentrations may reduce the chance of survival of footrot organisms in the skin or horn tissue of the hoof, given the knowledge of the efficacy of the treatments discussed in 3.1.

In the spring of 1992, preceding the commencement of the preliminary eradication trial, conditions were favourable for footrot transmission on the property. Control was achieved by vaccination with a multistrain vaccine in early June and late July 1992, the second vaccination being combined with footbathing in 10% zinc sulphate solution. On 1 February 1993, when the preliminary eradication trial began, the prevalence of footrot was low (3%). Antibody titres at this time were low. Mean titres to A antigen were between 90 and 175 (Table 3.7) which are not considered protective (Thorley and Egerton 1981). Six months had elapsed since the second multistrain vaccination had been given, so it was expected that protection would have waned completely (Egerton and Thorley 1981; Skerman *et al* 1982) and vaccination in the previous year would not be a factor contributing to the success or failure to eradicate in groups CON and AB. The CON group sheep, which received no vaccine in the preliminary eradication trial, maintained low titres throughout the study period (Figure 3.1), as expected.

At the second inspection of the sheep classed as free of footrot, four weeks after the first, 11 sheep (2%) were found to have footrot lesions, of which nine came from the four CON replicates, one from one paddock of each of the VAC and AB replicates. Both vaccination and antibiotic treatment had a significant effect on the number of cases of footrot appearing between the first and second inspections. The two treatments either cured or suppressed the expression of latent infections which had been undetectable at the first inspection. It is very unlikely that the new cases developed as a result of transmission, despite 36mm of rain, because, during February 1993, daily temperatures were very high and the average monthly mean daily temperature was 22.8°C. Conditions on the pasture were very dry for most days of the month. The small number of rainy days, while not creating conditions suitable for transmission, may have been sufficient to trigger the development of the few lesions which were then detected. This provided strong evidence that the new cases arose as a result of the relapse of latent infections.

At the subsequent two inspections, seven and twelve weeks after the first, ten sheep were detected with footrot, at similar rates from all treatments (Table 3.3, b and c). The effect of the antibiotic treatment and vaccination on the number of replapses was, therefore, evident only in the inspection four weeks after administration of the one antibiotic injection and the first vaccination.

The significance of the difference in numbers of sheep detected with footrot must be considered in relation to the subsequent breakdown with footrot in all three paddocks of AB group sheep, but not in the CON and VAC groups. A hypothesis to explain the outcome is as follows. In the controls, all latent infections developed to detectable lesions during February, March and April and were removed before transmission could occur. Thus, no sheep in the CON group remained infected by the time transmission commenced in late August. In the VAC group, all latent infections either broke down, as in the CON group, or were cured by the high titres which developed in most sheep. It is proposed that the sheep with latent infections which failed to develop protective titres following vaccination developed lesions by the end of April, and were removed. In the AB group, however, antibiotic treatment suppressed latent infections but did not remove all *D nodosus* organisms from the interdigital skin or horn of the hoof.

The presence of a carrier state has been observed before, although the lesions under discussion in those reports were probably underrunning, rather than interdigital. Thomas (1957) reported sheep relapsing with footrot up to 21 days after formalin treatment, despite the absence of signs of footrot after treatment. He noted also that topical application of oxytetracycline could lead to relapse into obvious footrot after a period apparently free from the disease and he considered these to be 'carriers'.

Footrot infections of the interdigital skin involve the epidermis and are remote from the blood vessels of the dermis. Nevertheless, the ability of antibiotics, including penicillin/streptomycin combinations, to cure footrot was demonstrated by Egerton *et al* (1968). Roberts (1967) showed that antibiotics diffuse into the epidermis of inflamed skin after parenteral injection and penicillin diffuses into wound exudates when the serum level is high (Lambert and O'Grady 1992). Less is known about dihydrostreptomycin or streptomycin, but it is distributed in the extracellular fluid in humans at concentrations about one quarter of plasma levels (Lambert and O'Grady 1992).

The site at which *D nodosus* organisms persist in the interdigital skin in undetectable infections is not known, but Egerton and Parsonson (1969) demonstrated organisms associated with benign footrot persisting in the epidermis of the interdigital skin, in the absence of a visible footrot lesion.

It follows that, if the organisms can persist in interdigital skin without evidence of a lesion and, if antibiotics are unable to diffuse into epidermal tissues unless an inflammatory process is occurring, latent infections may be refractory to antibiotic treatment.

In the study reported here, compared to no treatment, parenteral antibiotics resulted in a reduction of the number of sheep which relapsed four weeks after the first 'clean' inspection but appeared not to eliminate all *D nodosus* organisms from the sheep's feet. Neither penicillin nor streptomycin are likely to persist at therapeutic levels in plasma, even following the relatively high doses administered, for more than 48 hours (Lambert and O'Grady 1992). The drugs would not, therefore, be exerting any antibacterial effect for more than two or three days at most. Why, then, would antibiotics cause latent infections to revert to a state which requires

much more environmental stimulation before developing patency, compared to the labile carrier state seen in the CON group sheep?

One possible explanation is that the antibiotic treatment, while failing to remove all *D nodosus* organisms from all sheep, may have eliminated or reduced the population of other bacteria, like *F necrophorum*, involved in the development of a footrot lesion. A reduction in the numbers of these bacteria and a reduction in the sub-clinical inflammation accompanying them may have allowed the skin to become relatively refractory to footrot. Persistent wetting of the foot may have then been necessary to provide conditions suitable for the reintroduction of *F necrophorum* and the development of a clinical footrot infection.

A similar suggestion was advanced by Egerton and Parsonson (1969), for the activity of topical formalin solutions in eliminating bacteria other than *D nodosus* from the causal flora of the footrot lesion, where they remain in a quiescent state until environmental conditions permit the renewal of an active infectious process. These authors were considering benign strains of *D nodosus* in particular and it may be that the low virulent strains are more prone to this behaviour than virulent strains.

The preliminary trial demonstrated that intermediate footrot could be eradicated from relatively small groups of sheep by identification of infected sheep in a non-spread period and their removal from the flock. Vaccination against the most commonly isolated serogroup (A) responsible for the outbreak had no negative effects on the attempt to eradicate the disease and reduced the number of sheep which relapsed with footrot during the non-transmission period without inducing a carrier state. Antibiotic treatment, however, appeared to inhibit eradication in that it seemed to induce carriers, some of which relapsed and transmitted footrot in the following spring. The probability of failure in all three replicates of the antibiotic-treated group by chance alone is 0.8%[1]. The case against antibiotic usage in management of intermediate footrot seems compelling but the reasons for its failure are unclear.

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[1]   The probability of all three failures being in the one group is the product of the three probabilities of consecutive failures being in the same group; ie, 3/10 x 2/9 x 1/8.

3.4.4.   Eradication from the whole flock

Following the determination that inspection alone was sufficient to eradicate this form of footrot from a flock, the same principles were applied to the whole flock. The success of the methods used to achieve eradication was confirmed by the continuing presence of footrot in the control flock during 1994, its elimination from the main flock over the same time period and its continued absence in February 1998, three years later.

The difficulties associated with the eradication of benign footrot from flocks has long been recognised (Alexander 1962). The benign strains appear to be able to maintain a presence in the feet of sheep without detectable lesions and may remain in flocks from which virulent footrot has been eradicated (Egerton and Parsonson 1969; Allworth 1995)

The eradicability of intermediate strains has, therefore, been uncertain. Should they behave as virulent strains and be incapable of surviving in sheep's feet except in obvious lesions, then eradication by conventional means is possible. But if they share the covert infection characteristics of benign strains, eradication may be much more difficult by methods based on visual detection of lesions.

Allworth (1995) successfully eradicated four strains of *D nodosus* from experimental flocks, using methods dependent on visual detection of affected sheep, but was unable to eradicate two strains from the same flock. Three of the four eradicated strains were clinically virulent or in the more virulent range of the intermediate category, based on their history in affected flocks, elastase test result, protease thermostability test result and gene probe test result (see section 1.2.2.1). One of the four was similarly classified by the laboratory tests, but had a history of causing no score 4 lesions in the flock from which it was isolated (Allworth 1995). The two strains which defied eradication were of low virulence. Both had a history of no score 4 lesions in the flock of first isolation. One was classified as benign by all three laboratory tests used and the other, although classified as benign by the gene probe, was elastase positive and had thermostable protease. This last strain was considered to be of low grade intermediate virulence.

In that experiment, Allworth (1995) inspected the infected flocks first in February 1993, when he removed 10.5% of the sheep because they had footrot lesions and 1.8% for other foot abnormalities. He inspected the sheep twice more while environmental conditions were dry (in March and April) and removed 1.1% more of the flock although none of the foot abnormalities leading to culling appeared to be footrot lesions. In the following spring, two of three of his experimental flocks developed footrot caused by the benign and low grade intermediate strain described above.

In the whole flock eradication described here, a strain which was of intermediate virulence (a higher grade of virulence than the strain that Allworth (1995) could not eradicate) was eliminated from the flock using methods based on inspection and culling. Inspections were carried out in December 1994, when conditions were no longer suitable for footrot transmission, and repeated in February and March 1995. These inspections, combined with the control measures practised in 1993, successfully removed all sheep carrying *D nodosus* organisms, or at least those organisms capable of causing overt footrot, from all but a flock of ram weaners which were subsequently sold for slaughter.

The findings of the preliminary eradication trial, plus the few sheep found with footrot in the March and July 1994 inspections of the whole flock, suggest that *D nodosus* organisms of this level of virulence are capable of persisting in sheep's feet without causing visible lesions for a period of time, possibly several weeks in duration, before leading to the development of lesions. This latency, or carrier state, will allow organisms of low virulence to escape detection in summer inspection programs. This was also the view formed by Allworth (1995) following his eradication trial.

Repeated inspections, particularly during climatic conditions which may induce some expression of the infection but which are not suited to a high risk of transmission, will detect all or most of these infections and allow these sheep to be identified and removed. Repeated inspections into the autumn were a characteristic of the property F trial and of Allworth's (1995) trial. Antibiotic treatment, as used in the preliminary eradication trial, may reduce the likelihood of expression of the latent infection and increase the risk that infected feet remain undetected during the autumn.

Other workers, discussing virulent footrot, have commended the value of repeated inspection of sheep judged to be free of infection (Pryor 1954; Toop 1957; Hayman and Triffit 1964; Egerton 1986; Allworth 1990). In the case of virulent footrot, repeated inspections detect cases of footrot which were not observed at first. In the case of intermediate footrot, repeated inspections may detect cases which were not observable at the earlier examinations.

An issue which has not been clarified in this trial is the length of time that *D nodosus* populations in a foot can remain in a carrier state without either precipitating a fulminating infection or becoming extinct. Evidence from this trial suggests that persistence can be more than six months, given the detection of infected weaners in the whole flock eradication in July, after several clean inspections commencing in December, and the breakdown in the antibiotic treated ewes in September after clean inspections commencing in February.

The permanence of the disappearance of the strains, however, suggests that, if infection is not apparent in a transmission period following the clean inspections, elimination from the flock has been achieved. No evidence of footrot has been seen in this flock since the end of 1994, despite the vigilance of the flock owner and whole flock inspections by me in 1995 and 1998. In that period, certainly one year (1996) and probably another (1995) provided seasonal conditions favourable for footrot. Absence of footrot during a transmission period is considered to be sufficient grounds to claim eradication of virulent footrot (Egerton 1986). With benign strains, the ability of the organism to survive for years without detection has been suspected (Glynn 1993), although the theory has not been tested rigorously. It seems likely, from the work reported here, that intermediate strains may be intermediate between virulent and benign strains in this respect as well as in virulence - the bacteria can survive months, but not years in the carrier state.

In summary, it is considered that key factors contributing to the success of the eradication on property F were

- successful control methods applied during 1993, which reduced the prevalence of infection to very low levels by December 1993

- frequent inspections during 1994 which, given the conditions unsuitable for transmission, allowed the removal of cases before transmission could occur

- a season in 1994 which was unsuitable for high rates of transmission of footrot

3.4.5. Demonstration that footrot persisted in controls

The claim that eradication was achieved by the methods described here (repeated inspection and culling) and was not brought about by the unfavourable environment alone is supported by the continuing presence of clinical cases in an isolated control flock throughout the year of the eradication program. Furthermore, it was demonstrated that the strains of *D nodosus* which persisted in the controls were the ones present in the flock before the eradication program began.

Two techniques were used to identify the persistence of the strains responsible for the outbreak. One technique was serogrouping, the other was the genetic fingerprinting of isolates. Both techniques confirmed that the isolates recovered from the control flock in spring 1994 were of the same strain as the isolates present in late spring 1992. The two 'types' identified in 1992 were pattern D1, serogroup I, and pattern D2, serogroup A. Isolates collected from March 1994 and later were all of pattern D1, serogroup A. The serogroup A, pattern D2 strains present in 1992 were not identified again among the seven isolates tested in 1994. The interaction of serogroup and PCR-RFLP pattern are discussed in greater depth in Chapter 7, but it is clear from the results presented here that the pattern D1 strain persisted from spring 1992 to spring 1994, even though the serogroup with which it was associated changed from I to A during the period of investigation.

3.5.   Summary

This chapter reports the study of an outbreak of footrot on a property near Yass, in the southern Tablelands district of New South Wales, between 1991 and 1998.   Conditions were suitable for the expression and transmission of footrot on the property under study in 1991, 1992, 1993, 1995 and 1996. Conditions were not suitable in 1994 and 1997. In 1993 and 1994, eradication strategies were evaluated and carried out with success. Reviews of the flock in 1995 and 1998 demonstrated that footrot remained absent from the flock.

The form of footrot reported in the study was intermediate in virulence, based on clinical and laboratory evidence. Control measures were rarely relaxed so it was not possible to make observations of the disease at its worst. Nevertheless, despite control measures, up to 1% of the sheep at risk developed score 4 lesions on some occasions.

During 1993, eradication was attempted from a flock of 600 sheep with methods which have been used successfully to eradicate VFR. It was shown that inspection and culling alone was effective in eradicating intermediate footrot from the flock. Further, the results demonstrated that antibiotic treatment of apparently footrot-free sheep was contra-indicated, because all three replicates of a group which were treated in this way developed footrot in the subsequent spring transmission period.

It was hypothesised that antibiotics are ineffective in treating latent infections and may actually delay the transition of latent infections into clinical cases beyond the period when repeated inspections are carried out as part of an eradication program.

Footrot was successfully eradicated from the flock of approximately 3 000 sheep during 1994 by inspection and culling of affected sheep. Footrot was not seen again on the property despite seasons suitable for the disease in 1995 and 1996. Whole-flock inspections, in conjunction with the flock owner's on-going vigilance, demonstrated that the disease had been eradicated.

This study has shown that intermediate footrot can be eradicated using strategies which have been used successfully for more virulent strains of *D nodosus*. It is likely that effective control

during the previous transmission period, using vaccine and footbathing, combined with seasonal conditions which were unfavourable for footrot in 1994, assisted the eradication program. The findings of the study support the view that latent infections with intermediate strains of *D nodosus* do occur and footrot eradication programs with intermediate strains should involve repeated inspections of the whole flock during non-transmission periods. The carrier state did not seem to persist indefinitely in this flock. Management of sheep during the summer and autumn inspection period should encourage the development of latent infections, rather than suppress them, so the infected sheep can be culled. Vaccination, which appeared to cure some latent infections and did not lead to a carrier state, should be considered a useful control measure in eradication programs.

3.6.   Addendum at time of writing

At the time of writing (February 2000), the owner of property F has informed me that footrot is still absent from the flock, despite conditions in spring and summer 1999/2000 which were highly suited to the transmission and expression of the disease.

CHAPTER 4

CONTENTS

CHAPTER 4

STUDY OF ENDEMIC INTERMEDIATE FOOTROT IN THE FLOCK OF ORIGIN

4.1.   Introduction

The variation in the severity of outbreaks of necrotizing dermatitis of the feet of sheep associated with the presence of *D nodosus* has been recognised for several decades (Thomas 1957; 1962; Alexander 1962). Differences in severity are manifest as variation in the proportion of the flock affected with footrot, the number of affected feet of each sheep and the degree of damage done by the disease to the affected foot. Factors which contribute to the variation can be grouped as bacterial virulence factors, host susceptibility factors and environmental factors (Egerton and Raadsma, 1993).

The variation in virulence-associated factors, particularly protease production, between isolates of *D nodosus* has been demonstrated (Thomas 1962; Egerton and Parsonson 1969). While the existence of differences in virulence between field isolates of *D nodosus* is widely accepted the method of categorisation is not. Field and laboratory studies suggest that there is a range of virulence from benign to highly virulent which could be described as a spectrum of virulence (Stewart *et al* 1986), or with a five level (Anon 1993) or three level classification system (Depiazzi and Richards 1985; Claxton 1986). The first reported use of the term 'intermediate' to describe the innate virulence of some isolates of *D nodosus* was by Stewart *et al* (1982). Isolates of intermediate virulence are distinguished from virulent isolates in field outbreaks by a relatively low proportion of advanced lesions, score 4 lesions in particular. They are distinguished in some laboratory assays by differences in the amount or character of protease produced (Depiazzi *et al* 1990) and by differences in genes likely to be related to virulence expression (Liu and Yong 1993b).

Host factors which influence the expression of footrot include breed (Skerman *et al* 1982; Emery *et al* 1984), strain (Egerton and Raadsma 1993), age (Beveridge 1941; Littlejohn 1966/67) and sex (Littlejohn 1961; Skerman 1986). In addition, within flocks there is variation between sheep in apparent susceptibility to footrot with a genetic component estimated by Raadsma *et al* (1994) to account for around 30% of the variation in footrot expression.

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Environmental conditions have an important influence on the expression of footrot within a flock. Most reports associate environmental factors with the transmission of disease (Beveridge 1941; Alexander 1962; Graham and Egerton 1968; Egerton *et al* 1969) but some note an effect of environmental conditions on the frequency of severe lesions amongst affected sheep (Stewart *et al* 1984; Woolaston 1993). The relative contribution of these two factors (innate virulence and environment) is important when decisions about control and eradication strategies are made.

Recently, in an attempt to attain national uniformity in diagnosis, regulatory authorities have adopted a two level classification of footrot; benign or virulent (Stewart and Claxton 1993; Anon 1995), based principally on the protease thermostability of isolates of *D nodosus* obtained from different outbreaks (Depiazzi and Richards 1979; Palmer 1993). To support this view it is argued that isolates of intermediate virulence occur at a low frequency in field outbreaks (Links and Morris 1995). With a two level classification system, the limited severity displayed in field outbreaks by some isolates classified as virulent by the protease thermostability test is believed to be associated with limitations on disease expression imposed by the environment rather than the inherent virulence of the bacteria (Richards and Depiazzi 1995).

Despite the availability of laboratory tests, importance is still placed on clinical inspections of affected flocks as a method of determining the virulence of infecting *D nodosus* strains and, consequently, the need for action to control or eradicate the disease. Veterinarians and footrot advisory officers are advised to arrive at a diagnosis with one or more inspections of a sample of the affected flock and to estimate the relative proportions of lesions of different severity (Egerton 1989; Anon 1994). There is little information published, however, on how these proportions change in the course of an outbreak and what indices of lesion severity are useful in correctly classifying outbreaks. Egerton *et al* (1983) described some features of an outbreak of virulent footrot. Glynn (1993) described two natural outbreaks in Victorian flocks; one associated with benign strain or strains and one associated with strains of low to mid-range virulence (intermediate). Marshall *et al* (1991) have described an experimental outbreak of footrot associated with a single isolate of *D nodosus* of high virulence over a two year period. The clinical features of single strain infections with virulent, intermediate or benign isolates have been described on irrigated field plots (Stewart *et al* 1982; Stewart *et al* 1984) and in pens

(Stewart 1986). These reports demonstrated that there are differences between isolates in virulence which can be used to classify outbreaks into at least three virulence categories based on clinical and epidemiological characteristics.

The spectrum of disease severity in ovine footrot is most clearly demonstrated by inspection of a large sample of an affected flock, rather than inspection of a few severely affected animals (Stewart 1989; Egerton 1989). The severity of disease can be described by using the lesion scoring system proposed by Egerton and Roberts (1971). With this system, score 1 lesions are those with mild interdigital dermatitis, score 2 lesions have moderate or severe interdigital dermatitis, score 3 lesions have underrunning of the soft horn of the heel and sole with or without interdigital skin lesions and score 4 lesions have extensive underrunning involving the hard horn of the anterior sole and abaxial wall of the hoof. For the purposes of field diagnosis and in the absence of individual bacteriological confirmation, sheep with score 2, 3 or 4 lesions are assumed to be affected with footrot and sheep with score 1 lesions may not be.

Benign footrot has been defined as an infection which produces only score 2 lesions in most sheep in an affected flock. Although some sheep may develop score 3 lesions no more than 1% of an affected flock are likely to develop score 4 lesions. Intermediate footrot usually produces a much higher proportion of score 3 lesions in a flock and up to 10% of sheep may have score 4 lesions. Outbreaks of virulent footrot are characterised by a much higher proportion of severe lesions of the hoof, in which more than 10% of sheep develop score 4 lesions (Dobson 1986; Egerton 1989; Stewart 1989).

In addition to differentiation on the proportion of sheep with score 4 lesions, differentiation can also be made between benign and intermediate footrot on the basis of the proportion with score 3 lesions. The relatively high proportion of score 3 lesions caused by intermediate footrot distinguishes this form from benign footrot (Allworth 1995).

Environmental conditions affect the transmission of footrot within a flock and therefore the proportion of sheep which become affected with the disease. It has been proposed that a correct differentiation of the form of footrot in a flock can still be made under less suitable environmental conditions if the proportion of sheep with score 4 lesions is calculated with the

number of affected sheep (those with score 2, 3 and 4 lesions) as the denominator, rather than the number of sheep in the flock or the number examined in the sample (Allworth 1995, Allworth and Egerton 1999).

There are very few descriptions of field outbreaks of intermediate footrot. Some information on the outbreaks described in detail in this thesis have been published (Abbott 1994) and Glynn (1993) and Depiazzi *et al* (1998) have described outbreaks of footrot which match the description given here for intermediate footrot. In the study reported in this and subsequent chapters, outbreaks of intermediate footrot are described in clinical and epidemiological terms, first in the flock of origin and then at other sites which were expected to be more environmentally suited to the development of footrot lesions. One aim of this study was to clarify the clinical and epidemiological differences between benign and intermediate and between virulent and intermediate footrot, and to make initial observations about the strain or strains of *D nodosus* isolated from the outbreak. Further studies would be necessary to demonstrate that the characterisation of intermediate footrot would still be accurate when the outbreak occurred in a region considered to be more suited to the transmission and development of footrot (Chapter 5).

A second objective of this study was to investigate control methods for intermediate footrot. Although methods for control and eradication of virulent footrot have been described (Beveridge 1941; Thomas 1957; Egerton 1986; Stewart 1989), it is unclear if forms of footrot of lesser virulence can be managed in similar ways (Alexander 1962). In the case of benign footrot, the eradicability of the disease is questioned (Brownrigg 1986; Allworth 1995; Allworth and Egerton 1995).

A third objective was to explore the possibility that latent infections develop with intermediate footrot, which might help to explain the observed rapid increase in footrot cases seen in outbreaks when climatic conditions become suitable for disease expression. The possibility of latent infections with strains of low virulence had been raised by earlier workers (Morgan *et al* 1972) and has since been proposed by Glynn (1993).

In order to identify flocks suitable for this study, seven properties in south-eastern New South Wales were visited during March and April 1992 in order to examine outbreaks of footrot which had, at least on initial examination, characteristics of the intermediate form. These flocks had been identified by the District Veterinarian and the names of the owners of the flocks had been passed to me with permission of the flock owner in each case. On each property, the feet of at least 100 sheep were examined. The flock selected for the study was considered suitable because there were sheep in the flock with advanced score 3 lesions. An inspection on 16 April 1992 of 229 sheep selected at random found that 84% of sheep had score 2 (70%) or score 3 (14%) lesions. None had a score 4 lesion. In addition, the flock owners reported that sheep from their flock, when sold to a different district of NSW, had reportedly developed virulent footrot. The method used in this study was serial observation of two subsets of the owners' flocks over a winter, spring and summer period. In part of the study, three different therapeutic and preventive treatments were applied to one part of the flock to determine the effect on prevalence and incidence. The owners of the flock alerted me in December to an outbreak of footrot in another part of the flock on improved pastures which appeared, at that time, to be more severe than the one currently under study. This flock was then included in the study as flock 2. As well as collecting epidemiological data from flock 2, an experiment was conducted to investigate the protective role of heterologous vaccine, with a view to conducting further experiments which are reported in Chapter 5.

4.2.   Materials and methods

4.2.1.   The flocks

The flock owners managed sheep on two separated properties, property B and property W, which are approximately 15 kilometres apart, 40 km east north east of Cooma, north east of Numeralla (Plate G). The approximate latitude and longitude is 36°5'S, 149°30'E. There were exchanges of sheep between the two sites. The two flocks (flocks 1 and 2) were subject to separate study in overlapping time periods. Flock 1 was a flock of adult Merino wethers of medium wool type. This flock was run at property B. A preliminary inspection in April 1992 confirmed the presence of footrot and the prevalence of score 3 and score 4 lesions indicated the outbreak was of intermediate type.

Flock 2 was a flock of adult breeding ewes, of the same genotype as property B wethers. This flock was run at property W.

4.2.2. The environment

The Monaro is a region of southern NSW consisting of a rolling grassland plain in the southern part of the southern Tablelands of NSW. Cooma, the principal town in the region, lies at 36°2' S, 149°1'E at an altitude of 778 m. The Monaro is elevated and includes part of the Snowy Mountains. Rainfall is summer dominant with an annual mean at Cooma of 550 mm; on average 12% of annual rainfall occurs in the winter months of June, July and August; 29% falls in December, January and February. Winters are cold. July is the coldest month with a mean daily maximum of 11.4°C and minimum of -2.9°C. There are on average 106 frosts per year, some severe, and frequent snow falls down to 1000 m. Summers are mild with a mean daily maximum for January of 26.7°C. The environment had been considered unsuitable for footrot expression and transmission because of the moderately low rainfall and low temperatures, compared to some other areas of NSW where footrot is endemic.

The area of study was in the eastern part of the Monaro, where the plains meet the mountains of the Great Divide, on the western side of Wadbilliga National Park. Rainfall in this region is generally higher than occurs at Cooma. Property B is on the Big Badja River, south of Big Badja Hill. The terrain is mountainous and lies between 975 and 1200 m elevation. The pastures were unimproved native grasses. Average annual rainfall was high, 1829 mm had fallen in the 12 months to April 1992.

Property W is south of property B. Although at a similar elevation, the topography of property W is rolling, rather than mountainous. Pastures were improved, regularly fertilised and included *Phalaris aquatica* and *Trifolium subterraneum*.

4.2.3. The climate

Rainfall was measured at property W for the duration of the studies; daily temperature data were recorded at Cooma, approximately 30 km from the study sites.

4.2.4.   Dates of the studies

Flock 1 was studied from 26 June 1992 until 3 February 1993. Flock 2 was studied from 15 December 1992 until 14 April 1993. Flock 1 was visited on a total of eight occasions over 32 weeks but, because the first two occasions were six days apart and involved initial procedures with different parts of the experimental flock, these two dates are considered as part of the first inspection visit.

Table 4.1   Dates of inspection visits to flocks 1 and 2

Week	Inspection number (Flock 1)	Inspection number (Flock 2)	Date of inspection
0	1		26 June, 2 July 1992
5	2		6 August 1992
10	3		8 September 1992
13	4		28 September 1992
16	5		22 October 1992
22	6		4 December 1992
24		1	15 December 1992
28		2	11 January 1993
31	7	3	3 February 1993
37		4	17 March 1993
41		5	14 April 1993

Plate G

South-eastern New South Wales. The Monaro district is centred on Cooma and part of the southern Tablelands and southwest Slopes lie at the top and top left of the map. The area of the field study of a natural outbreak of footrot is shown in the box at right (properties B and W). The area for the field study in the south west Slopes (Property K) is shown in the box upper left.
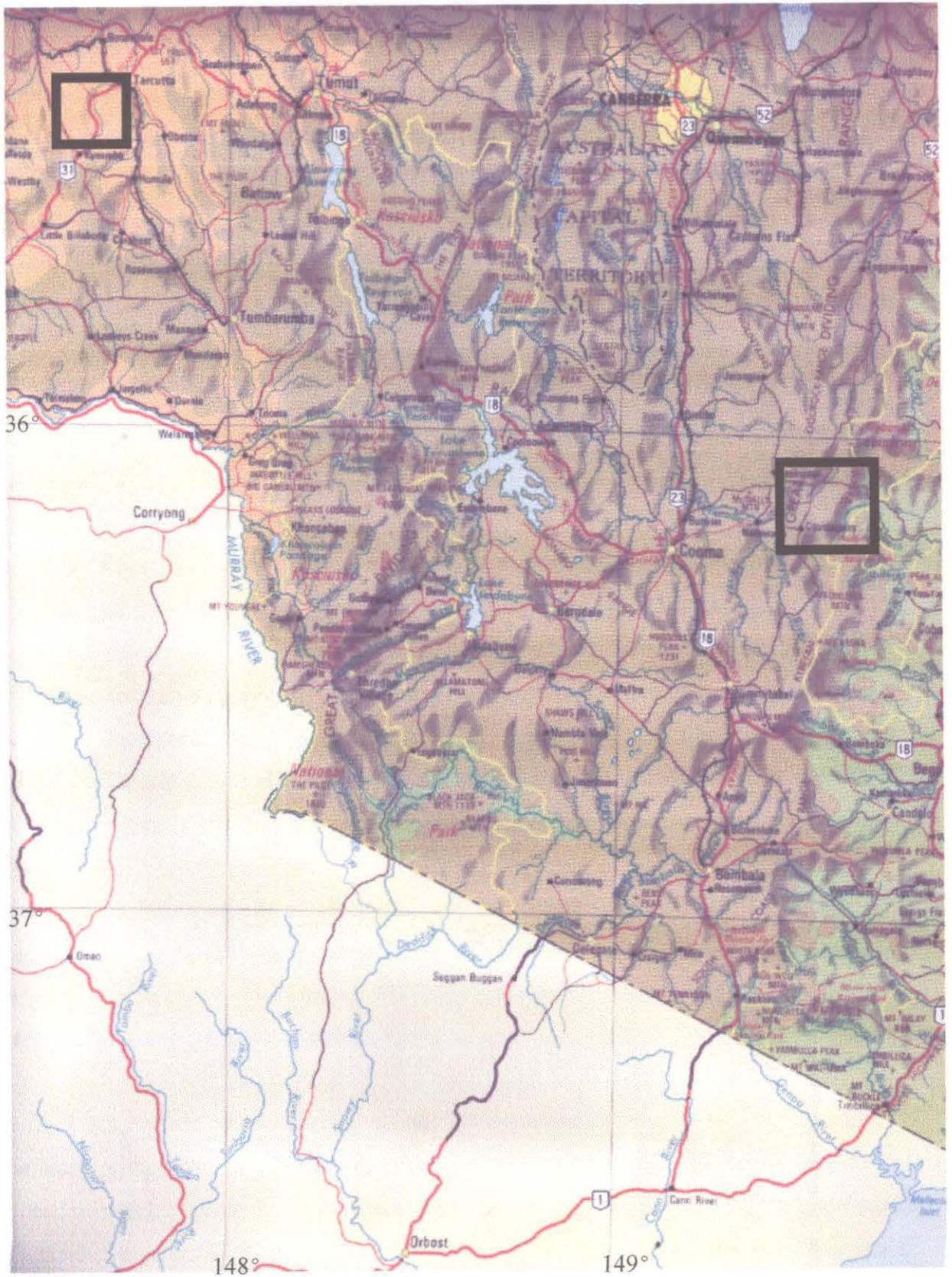
4.2.5.   The experimental sheep

In flock 1, 403 adult wethers were studied. At the first inspection, on 26 June and 2 July 1992, these sheep were eartagged and divided at random into four groups. On 26 June, 100 two year old wethers which were run as one mob in one paddock (paddock 1) were tagged (1 to 100) and allocated to one of the four treatment groups Randomness was achieved by allocating sheep in groups of eight, as they came through the drafting race, to one of the four treatment groups. The groups were Control (CON), Footbath (BATH), Vaccine (VAC), and Antibiotic (AB). Footscores of all sheep were recorded. Treatments were administered as described in 4.2.6 below. Only 100 sheep were tagged, inspected and allocated to their respective groups on 26 June before heavy rain made accurate inspections impossible, and the remainder of the inspections was postponed.

Initial activities for all sheep were completed on 2 July 1992. Three hundred and three more wethers aged three, four or five years which ran in five different paddocks (paddocks 2 to 6) were examined, tagged and allocated to treatment groups, this time in groups of six sheep or four sheep to ensure random allocation and similar numbers of each treatment group in each paddock. Numbers of wethers in each paddock, numbered one to six, were 100, 49, 141, 48, 36 and 29 respectively. Within each paddock there were, as close as possible, equal numbers of each treatment group represented. Treatments were administered as described in 4.2.6.

There were 101 sheep in all groups except the Controls, which had 100 sheep. All experimental sheep remained in the paddock mob from which they originated until the third inspection in week 10, when footrot affected sheep were removed from all mobs and run together in another paddock (paddock 7).

In flock 2, 300 ewes were observed. At the first inspection in week 22 (4 December 1992), the study group was selected from a mob of approximately 400 ewes by a random selection procedure which involved removing, at a drafting gate, every fourth sheep in the race. Selected sheep were eartagged with consecutively numbered tags. The ewes were three and four years of age, and approximately 70% of the ewes were nursing lambs. In weeks 31 and 37, 100 ewes, selected at random after stratification on footscore in week 28, were vaccinated.

4.2.6.   Experimental procedures

In flock 1, the BATH group were held for three minutes in a 50 mm to 75 mm deep footbath solution of 10% w/v zinc sulphate first in week 0 and again in week 5. The VAC group received a one ml subcutaneous injection of a commercial nine-strain multivalent footrot vaccine in week 0 and a one ml subcutaneous injection of a monovalent vaccine against serotype H_1 in week 5. The AB group received an intramuscular injection of 3125 mg procaine penicillin plus 3125 mg dihydrostreptomycin in week 0 and again in week 5, followed on each occasion by 24 hours confinement on battens in a shearing shed. Group CON received no treatment.

In weeks 10 and 13, all cases of footrot were removed and run together in a separate mob. This separate mob of cases was re-examined in week 16.

Blood was collected from a representative sample of the CON group and the VAC group in each paddock at each visit from week 0 to week 16 inclusive. These sheep were identified with an additional tag in the ear opposite to the identification number tag. In all, 51 sheep were identified for repeated blood sampling, 26 from the VAC group and 25 from the CON group. After the blood sample was collected into ten ml vacutainer tubes, the sample was transported back to the laboratory at Camden, serum was separated and frozen for later serological examination.

At each visit, samples of material from the interdigital skin or underrun footrot lesions were taken for bacteriological examination. These samples were collected with clean sticks and applied immediately to hoof agar plates. These plates were placed in anaerobic gas jars at the end of each day and placed in a 38°C incubator on return to the laboratory.

Feet were inspected in the woolshed on property B. Wethers were driven into catching pens where they were caught, tipped onto the rumps, and dragged to the shearing board. Each foot was then inspected and scored as described in Chapter 2. Blood was collected from those sheep nominated for sampling. Foot paring was performed if necessary to diagnose the footscore. Footscores were recorded for every sheep at each visit by one of the flock owners

onto prepared forms. Within a few days these records were transcribed onto a computerised database, in a commercial database management program (dBase IV, Borland International Incorporated, California).

In the study of flock 2, 150 ewes were vaccinated on two occasions (weeks 31 and 37) with a monovalent vaccine against serotype A_1, by subcutaneous injection behind the ear. All ewes were footscored and scores recorded individually in weeks 22, 28 and 41. Foot lesions from 50 ewes were sampled for bacteriology in week 22.

4.2.7.   Definitions

The presence of footrot was determined by examining all four feet of every experimental sheep and scoring the severity of the footrot lesion according to the method of Egerton and Roberts (1971). Scores of 0 or 1 were not considered footrot lesions. A case of footrot (an affected animal) was defined as a sheep with at least one foot with a score of 2, 3 or 4. A sheep was considered to have recovered from footrot if, after being affected, it no longer had a foot with a score of 2, 3 or 4 at the next inspection.

In calculating the incidence of infection at each visit, the number of new cases was divided by the number of sheep present at the visit which had been free of footrot at all previous inspections, and this proportion was divided by the number of days elapsed since the previous inspection. Incidence is expressed as a percentage of sheep per day.

Cure rates and protection rates are calculated by mathematical comparison to the change in prevalence in the control group. The expression used to calculate the cure rate was

$$\frac{P_T - P_C}{1 - P_C}$$

where P_C is the proportion recovering spontaneously (in control groups), P_T is the proportion recovering in treatment groups. Thus, the cure rate attributable to the treatment is calculated with respect to the proportion which did not recover spontaneously $(1 - P_C)$ in the controls.

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Protection rates were calculated as the proportion of sheep which were unaffected at one inspection when the treatment was administered and remained unaffected at the subsequent inspection. As with cure rate (above), protection rate is calculated with $(1 - P_c)$ as the denominator, in order to attribute protection to the treatment only for the proportion of animals which were expected to become affected. In calculating protection rates, P_T and P_C refer to the proportion of unaffected sheep remaining unaffected.

Cure rates and protection rates were calculated for the 13 week period from when treatment was first given (week 0) to week 13, a period during which transmission of footrot was not expected to occur (July, August and September). The practical application of using effective treatments in the non-transmission period is to improve the health and welfare of sheep in a footrot affected flock during the stressful conditions of winter. These treatments are not expected to cure all sheep and further measures are likely to be necessary if and when conditions become conducive for transmission in spring or summer. If a simple treatment could be given at the start of the non-transmission period which leads to a significant reduction in footrot prevalence the resultant improvement in health, welfare and productivity of the animals may make treatment cost-effective, even if further action is necessary to control the disease when transmission resumes.

4.2.8.   Bacteriology/Serology

Bacterial culture, identification and serogroup determination were performed as described in Chapter 2. Serology of sheep was performed as described in Chapter 2.

4.2.9.   PCR-RFLP of the *omp* gene

Restriction fragment length polymorphism (RFLP) with the restriction enzyme *Hpa*II and PCR product from the *omp* gene region was used as an epidemiological marker as described in Chapter 2 and discussed further in Chapter 7.

4.2.10.   Statistical methods

Prevalence data was analysed by logistic regression. As some sheep were removed from the flock from time to time the repeated measurement aspect of the data was ignored and separate analyses were done for the data at each week. For incidence data, the rate was calculated for each of the four treatment groups for each of six inspections by summing the full interval length for unaffected sheep and half that length for cases, on the assumption that cases occurred on average mid-way between inspections. For the counts of cases in the 24 group-inspection combinations (assumed to have a Poisson distribution) and with the corresponding total exposure time as an offset to adjust for differences in group-inspection exposure time, log-linear modelling was used to assess the effects on case rates of groups, linear trend over days, the interaction of linear trend with groups and a common curvature among groups, using a smoothing spline approach (Table 4.2).

Data on recoveries (Table 4.3) were treated in a way similar to the incidence data, with recoveries assumed to have occurred on average mid-way through each interval. Log-linear modelling was used to analyse the data for the 12 group-inspection combinations with total interval as an offset. Relapse data (Table 4.4) were examined in a similar way.

Logistic regression was also used to examine the significance of cure rates and protection rates (Table 4.6).

Data on the relationship between the isolation of serogroup and treatment group (Table 4.7) were also tested for independence in a 2 x 2 contingency table using the chi-square distribution. Analysis of variance was used to examine serology results, after titres were transformed by using base two logarithms to normalise the data.

4.3.   **Results**

4.3.1.   **Flock 1**

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4.3.1.1.   **Climate**

Rainfall for the period was not recorded at property B so rainfall for property W, 15 km south
of property B, is reported. The total annual rainfall for 1992 was 842 mm. Rainfall in June was
high (145 mm) but in July and August was low.   In the period from winter 1992 to autumn
1993 inclusive, monthly rainfall exceeded 90 mm at property W in all months from and
including September to March, except October (48 mm) and January (37 mm) (Figure 4.1).
 Mean monthly minimum temperatures at Cooma were -3.9°C, -3.1°C, -1.7°C in June, July
and August. Daily mean temperatures exceeded 10°C on a monthly average basis, from and
including October to April. In October, daily mean temperatures consistently exceeded 10°C
from 2 October. At property B, low overnight temperatures and shading caused by trees, the
mountainous terrain and the low aspect of the sun in winter led to the pastures remaining damp
with dew or frost for a large part of each day, even when rainfall did not occur. The period
of July, August, September and early October 1993 could be considered a non-transmission
period using the guide proposed by Graham and Egerton (1968) because the daily mean
temperature was below 10°C and rainfall for July and August was below 50 mm per month.
It was to be expected that spread would commence in late October or early November
following 48 mm of rain in October, 142 mm in November and daily mean temperatures
consistently exceeding 10°C, and continue up to and including March 1993 (Figure 4.1).

4.3.1.2.   **Incidence of footrot**

The occurrence of new cases for the six inspections in weeks 5 to 31 are shown in Table 4.2
and illustrated in Figure 4.2. The incidence ranged from zero to 0.4% of sheep per day in each
group until the last inspection in week 31, when the average incidence rose to 1.1% (CON and
VAC) to 1.3% (BATH and AB).

The data were fitted to a log-linear model and the incidence rates for all groups predicted by the model are illustrated in Figure 4.3. This analysis indicated that there was a highly significant average curvilinear response over time (P<0.001) and the curvature was predominantly quadratic. An average linear component of the response was also highly significant (P<0.001) and there were significant differences among groups in the slope of the linear component (P<0.01). There were significantly higher slopes for AB and VAC compared to CON and for VAC compared to BATH.

4.3.1.3. Recoveries

A number of sheep recovered from footrot in the first 16 week period (Table 4.3). The greatest number of recoveries occurred in the first five weeks during which 35 of 66 affected sheep recovered. Over the 16 week non-transmission period, 61 sheep recovered from footrot once and three recovered twice. In the CON group, 13 of 31 affected sheep (42%) recovered; 22 of 41 in the BATH group (54%), 9 of 13 in the AB group (69%) and 14 of 30 (47%) in the VAC group.

The log-linear model used to compare treatment groups comprised the effects of days, including a contrast between week 5 and the average of weeks 10 and 16, the effects of groups including a contrast between group CON and the average of groups BATH, AB and VAC and the interaction of the two contrasts. Both contrasts were significant (P<0.05) and their interaction was significant (P<0.05) but no other effects were detected as significant. The analysis infers that the mean rates of recovery for BATH, AB and VAC were greater than for CON at week 5, but not thereafter.

The cure rate for any of the treated groups was defined as the number of affected sheep which recovered from infection and remained free of infection at week 13, relative to the control group. The cure rates for each treatment are shown in Table 4.6. The highest response was in the antibiotic group, in which 73% of treated sheep recovered compared to 31% in the controls, a cure rate of 61%. Consistent with the analysis of recovery rates discussed above however, the cure rate was not statistically different from zero in this or any other group.

Table 4.2   New cases of footrot, flock 1 at property B

Week	. Occurrence of new cases				
	Number of sheep eligible to become cases shown in brackets¶				
	Control	Footbath	Antibiotic	Vaccine	Total
5	12 (85)	6 (80)	3 (90)	3 (81)	24 (336)
10	4 (72)	3 (74)	1 (86)	0 (76)	8 (308)
13	2 (68)	3 (70)	1 (84)	0 (72)	6 (294)
16	3 (64)	1 (64)	4 (84)	0 (68)	8 (280)
22	4 (61)	7 (62)	10 (78)	9 (66)	30 (267)
31	30 (58)	28 (55)	37 (66)	32 (57)	127 (236)

¶ The number of sheep available to become new cases was defined as the number of sheep presented for inspection which had not been classified as a case at any previous inspection.

Table 4.3   Sheep recovering from footrot, flock 1

Week	Resolution of footrot cases			
	(Number of cases at previous inspection in brackets)			
	Control	Footbath	Antibiotic	Vaccine
5	2 (14)[a]	12 (20)[b]	9 (11)[b]	12 (19)[b]
10	3 (23)	3 (14)	3 (5)	0 (9)
16	9 (27)	7 (19)	0 (5)	4 (13)

a,b Values in rows with different superscripts are significantly different at the 95% confidence level. Values in week 10 and week 16 were not significantly different.

4.3.1.4.   Relapses

Some of the sheep which recovered from footrot relapsed (Table 4.4). There were 32 relapses recorded at five inspections between weeks 10 and 31. No sheep relapsed more than once.

Three sheep recovered from a relapse. Two of these were in the VAC group, one in the CON group. These three recoveries were detected at week 16. None of the differences between groups was significant.

Table 4.4   Sheep relapsing with footrot, flock 1

Week	Number of relapses			
	Number of sheep eligible for relapse in brackets			
	Control	Footbath	Antibiotic	Vaccine
10	0 (2)	2 (12)	1 (9)	4 (12)
13	1 (5)	0 (13)	0 (11)	1 (8)
16	0 (4)	1 (13)	2 (11)	2 (7)
22	0 (13)	3 (19)	2 (9)	2 (9)
31	2 (13)	3 (16)	6 (7)	0 (7)

4.3.1.5.   Protection from new infection

In week 5, 24 new cases of footrot occurred. In week 10 eight new cases and in week 13 six new cases were diagnosed (Table 4.2). These cases represented 21%, 15%, 6% and 4% of the unaffected sheep in CON, BATH, AB and VAC groups respectively. The rate of new cases in the AB and VAC groups was significantly less than in the controls (P<0.05) and in VAC was less than in BATH (P<0.05). Expressed as protection rates, defined in section 4.2.7, the rates of 74% and 82% for AB and VAC were significantly greater than zero (P<0.05) (Table 4.6).

4.3.1.6.   Prevalence

Data for prevalence in the entire study flock were available for weeks 0, 5, 10 and 16 (Table 4.5, Figure 4.4). There were no significant differences between groups at week 0 or week 16. The prevalence in groups AB and VAC were significantly less than in CON at weeks 5 and 10 (P<0.05). The prevalence in BATH was not significantly different from CON at any inspection. Prevalence in weeks 0 and 16 did not differ significantly between groups.

The prevalence of footrot in the flock was recorded on four occasions at 5 to 6 week intervals over the winter-spring period (Table 4.5). In the controls, prevalence was between 15% and 25% during that period. The steady prevalence over the winter-early spring period was a result of a low incidence, a low but steady rate of resolution of cases and an even lower rate of relapse to affected status. For this reason, and from interpretation of climatic data, the first 16 weeks of the study which included July, August, September and most of October can be considered a non-transmission period for footrot. It is justified to examine the behaviour of the disease in the flock over this time period separately from the behaviour in the following months.

Although the entire flock was not examined at the inspections of week 22 and 31, the incidence was markedly higher on these two occasions than at any time over winter and early spring. At week 31, when only the sheep which had been free of footrot from week 5 to 16 inclusive were examined, 175 of the 309 sheep (57%) were affected with footrot. There was no significant difference in the incidence (Figure 4.2) or prevalence of footrot between any of the four treatment groups at that inspection so the four groups are considered together for the purpose of discussing the prevalence of footrot at week 31. The estimate of footrot prevalence (57%) is almost certainly an underestimate of the true prevalence in the entire flock. The expected prevalence in the 78 sheep which had been removed at weeks 10, 13 and 16 was probably at least 80%, because they had all been affected on at least one occasion between weeks 5 and 16 and had shown limited propensity to resolve spontaneously when conditions were relatively unsuitable for footrot development.

Table 4.5   Prevalence of footrot, flock 1

(see Appendix 1 for these data with details of missing sheep)

Week	Prevalence of footrot cases (% of sheep present)			
	Control	Footbath	Antibiotic	Vaccine
0	15.0	19.8	10.9	19.8
5	24.2^a	14.0^{ab}	5.0^c	10.0^{cb}
10	24.7^a	15.8^{ab}	4.0^c	13.4^b
16	21.9	15.3	10.9	11.8

a,b Values in rows with different superscripts are significantly different at the 95% confidence level. Values in week 0 and week 16 were not significantly different.

Table 4.6   Cure rates and protection rates achieved by each treatment, flock 1

		Week 0	Week 13		Cure rate	Protection rate
		Total	Unaffected	Affected		
Controls	Unaffected	84	66	18		
(CON)	Affected	13	4	9		
Footbath	Unaffected	81	69	12	42%	$31\%^a$
(BATH)	Affected	20	12	8		
Antibiotic	Unaffected	90	85	5	61%	$74\%^{ab}$
(AB)	Affected	11	8	3		
Vaccine	Unaffected	79	76	3	12%	$82\%^b$
(VAC)	Affected	18	7	11		

a, b Protection rates with different superscripts are significantly different (P<0.05). The rate for BATH was not significantly different from zero. There was no significant difference in cure rates between groups and none was significantly different from the controls.

4.3.1.7.   Distribution of maximum footscores

The relative proportions of sheep of each maximum footscore classification are shown in Figure 4.5. While the prevalence of footrot remained low in the July to October period (weeks 0 to 16), most affected sheep had score 2 lesions, 1% to 4% of sheep had score 3 lesions and only one score 4 lesion (0.3% of sheep examined) was seen in July and September. Expressed as percentages of affected sheep rather than of the whole group, sheep with score 2 lesions ranged from 70% in September to 95% in October, those with score 3 lesions ranged from 5% in October to 28% in September and those with score 4 lesions ranged from 0 to 2% in July and September.

In February (week 31), only sheep which had not been affected in weeks 5 to 16 were inspected. Compared to earlier inspections, the prevalence of footrot and the relative proportions of severely affected sheep (scores 3 and 4) had increased. Of the sheep examined (n=309), 57% were affected, 29% had a maximum footscore of 2, 20% had a maximum footscore of 3 and 7% had at least one score 4. Of affected sheep, 35% had score 3 lesions, 13% had score 4 lesions - these proportions had risen at the expense of score 2 lesions which made up only 52% of affected sheep.
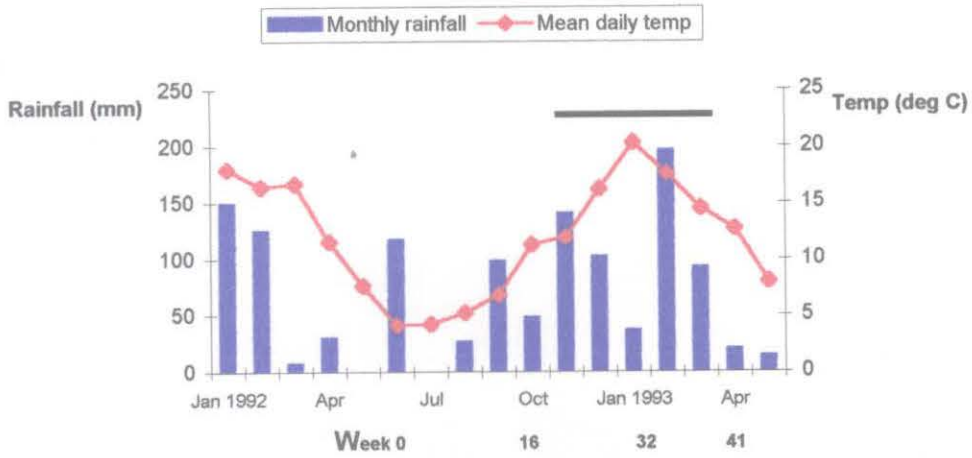
Figure 4.1   Monthly rainfall (property W) and mean daily temperature (Cooma), 1992-93. Transmission period (solid black line) is predicted from the report of Graham and Egerton (1968). The period of the study at properties B and W (weeks 0 to 41) was from June 1992 to April 1993.
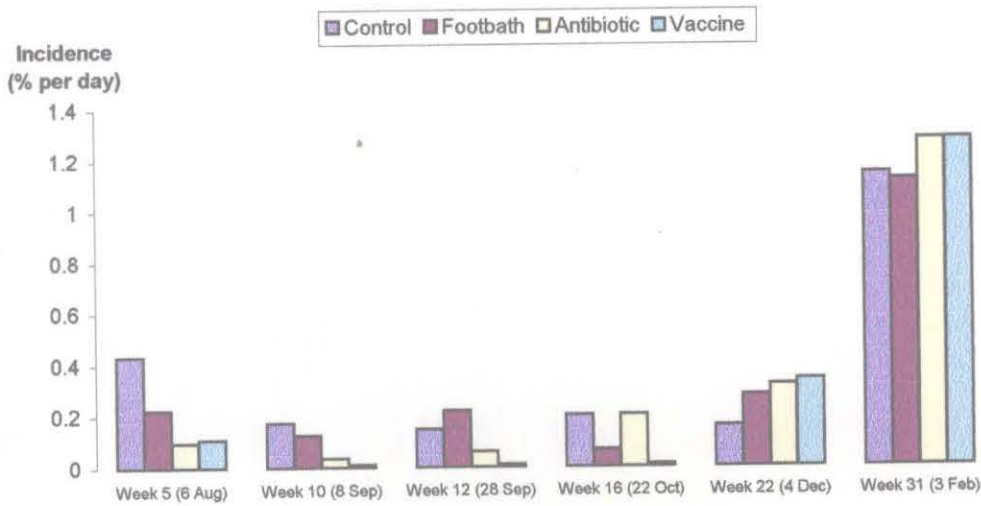
Figure 4.2 Daily incidence of footrot cases by treatment group, flock 1, property B. Sheep in groups BATH, AB and VAC were treated with zinc sulphate footbath, parenteral antibiotics or vaccine, at weeks 0 and 5. The vaccine was multivalent in week 0 and monovalent against H_1 at week 5. Incidence was calculated by dividing the number of new footrot cases at each inspection by the number of previously unaffected sheep present then dividing by half the number of days since the previous inspection. See also Table 4.2.
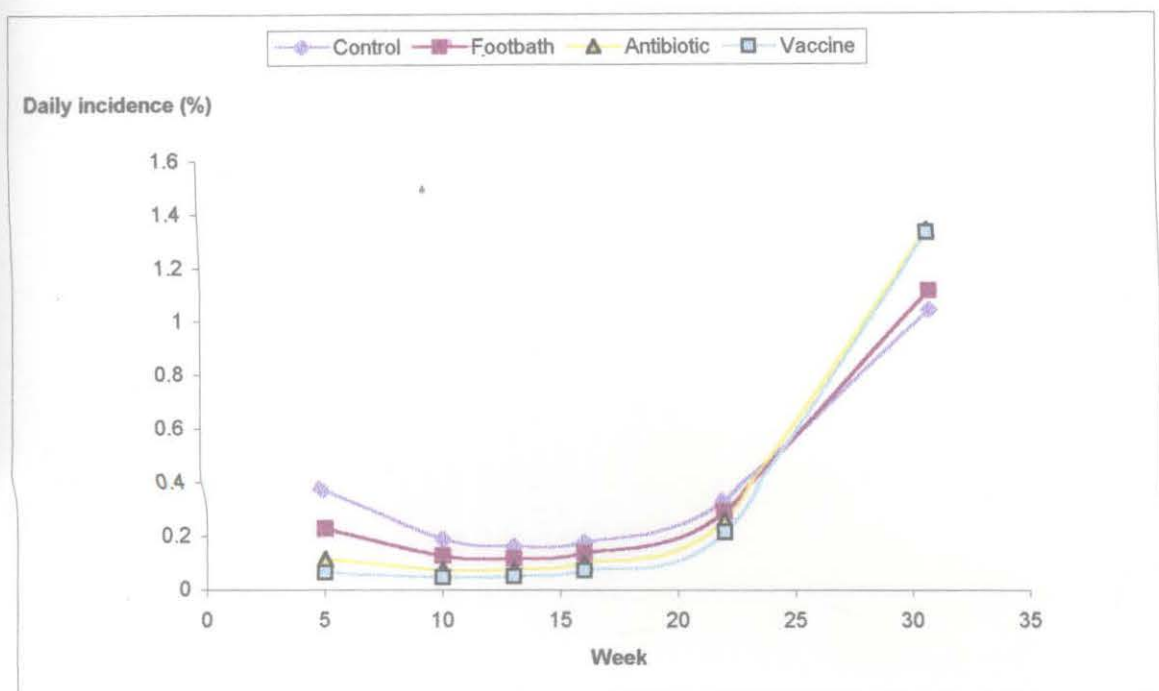
Figure 4.3 Predicted incidence of footrot cases by treatment group, flock 1, property B. Sheep in groups BATH, AB and VAC were treated with footbath, antibiotics or vaccine in weeks 0 and 5. Log-linear modelling was used to assess the effects on case rates of groups, linear trend over days, the interaction of linear trend with groups and a common curvature among groups, using a smoothing spline approach. There was a highly significant average curvilinear response over time (P<0.001). The average linear component of the response was also highly significant (P<0.001) with significant differences among groups in the slope of the linear component (P<0.01). There were significantly higher slopes for AB and VAC compared to controls and for VAC compared to BATH.
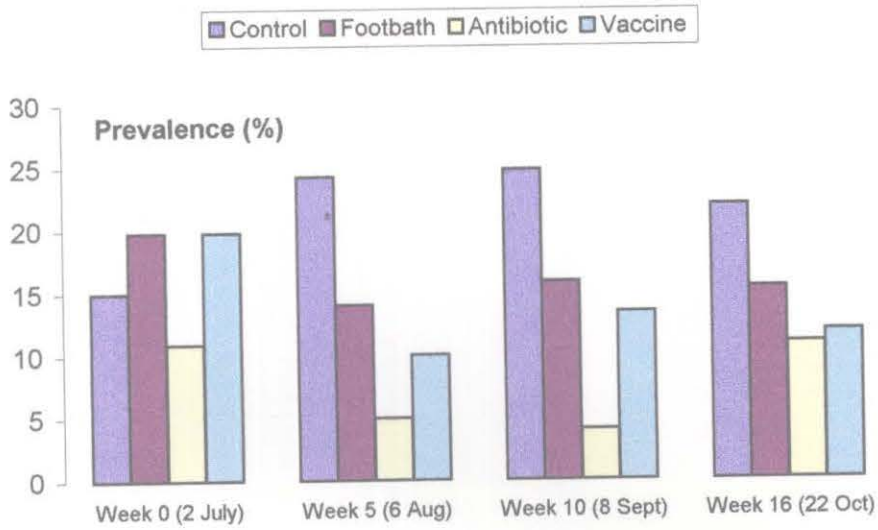
Figure 4.4 Prevalence of footrot cases by treatment group, flock 1, property B. Sheep in groups BATH, AB and VAC were treated with zinc sulphate footbath, parenteral antibiotics or vaccine, at weeks 0 and 5. The vaccine was multivalent in week 0 and monovalent against H_1 at week 5. See also Table 4.5.
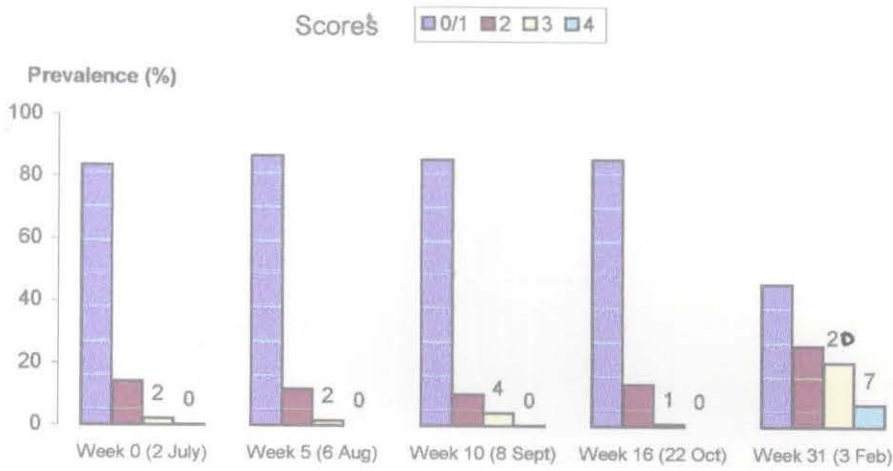
Figure 4.5 Prevalence of sheep classified by worst foot score, flock 1, property B. Prevalence data for week 31 calculated for 309 sheep which had not been affected in weeks 5 to 16. Data labels shown for scores 3 and 4. There was one sheep with a score 4 lesion in July and one in September (0.3%).
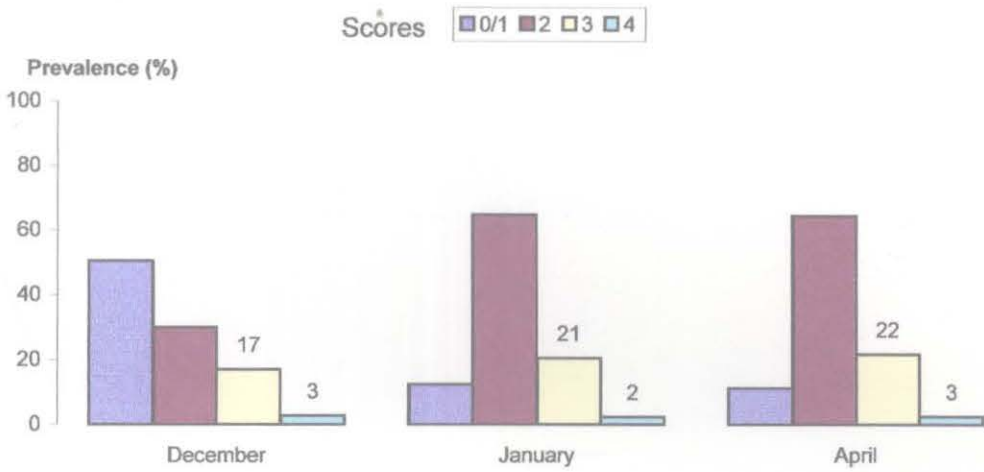
Figure 4.6 Prevalence of sheep classified by worst foot score, flock 2, property W.
The outbreak in flock 2 was an uncontrolled outbreak which occurred on improved pastures
on property W. Flock 1 and flock 2 have sufficient contact for transmission of footrot to
occur and molecular epidemiology supports the view that the outbreaks in both flocks are
caused by the same group of strains of *D nodosus*. Data labels shown for scores 3 and 4.
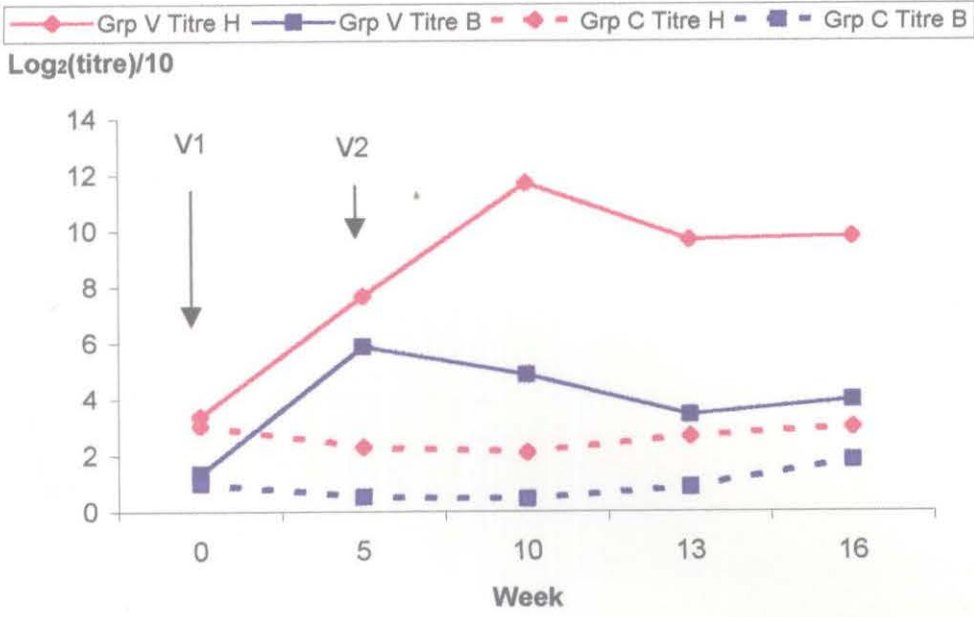
Figure 4.7 Serological responses in group VAC sheep, compared to unvacc-inated group CON sheep. Nonavalent vaccine was given at V1, monovalent (H_1) given at V2.

4.3.1.8. Clinical appearance of lesions

At inspections in weeks 13 and 16 (September and October), those lesions which were score 2 were characterised by hairlessness and a glabrous, greasy sheen to the interdigital skin, with mild erythema. In some cases, the web of skin at the posterior aspect of the interdigital space was disrupted and, in some cases, the skin and hoof wall at the skin-horn junction was roughened, ridged or broken, with small, loose pieces of necrotic tissue attached as tags. There was rarely any evidence of necrotic exudate on the skin surface, or the marked, painful inflammation which characterised score 2 lesions later in the year. Despite the very mild inflammatory changes, the presence of _D nodosus_ in cultures taken from these lesions in week 16 supported the view that these were footrot lesions, although the possibility that the organisms were surface contaminants originating from other sheep cannot be excluded.

On some occasions, the departures from normality in the IDS were so mild that the scores were 0 or 1. In week 16, four of these lesions were sampled and _D nodosus_ was grown from one. This lesion was described at the time as being a 'torn and sweaty web'.

The few score 4 lesions observed were usually limited in the amount of underrun, hoof damage and necrotic material in the underrun sole compared to the lesions which would be expected in VFR. Underrunning of hard horn was frequently not advanced, even in long standing cases. There were exceptions, however, and a small proportion of the sheep with score 4 lesions had very severely affected feet, indistinguishable from those of VFR. These sheep were usually in very light condition. Although they were not weighed, their relatively low bodyweight was readily apparent when they were tipped over in the catching pen, prior to inspection of their feet.

4.3.1.9. Isolation of _D nodosus_ from affected sheep

At the first inspection (26 June and 2 July 1992), samples were collected for bacteriology from nine sheep. Only one of these yielded _D nodosus_. At the inspection in week 13, eight affected sheep were removed from the study flocks to paddock 7. Samples for culture were collected from four of these sheep and, in one case, two feet were sampled from one sheep. All five

lesions sampled yielded *D nodosus*. Four of the lesions were score 2; one was score 1.

In week 16, 13 new cases were removed. All 13 affected sheep had lesions sampled for culture. Nine of them yielded *D nodosus*. Of the sheep which had previously been removed because lesions had been detected, 44 were sampled and 29 of these yielded *D nodosus*. Over all affected sheep sampled, 38 (67%) were positive for *D nodosus* on culture. Four sheep with feet of score 0 were sampled for culture. One of these cultures was positive for *D nodosus*.

In December 1992 (week 22), samples were collected from 16 affected sheep; 14 of which yielded *D nodosus*. Success in isolating *D nodosus* from the feet of affected sheep improved through the study period but, over the entire observation period with flock 1, was 66%.

4.3.1.10. Serogroup of isolates

A total of 60 isolates were serogrouped with the slide agglutination test. Only one isolate collected in week 0 was tested and it was serogroup H. In week 13, at least one lesion from one sheep in each treatment group was sampled and cultured. Five isolates were obtained; three were serogroup B, two were serogroup H. In week 16, 40 isolates from 39 sheep were identified. After collection in week 22, 14 isolates were tested. The combined results for the 54 isolates collected in weeks 16 and 22 which were serogrouped are shown in Table 4.7.

Table 4.7   Serogroup of isolates collected in weeks 16 and 22

	Serogroup					
	A	B	C	E	H	Total
Vaccinates	1	14	1	1	0	17
Footbath	1	8	1	0	7	17
Antibiotic	1	3	3	0	3	10
Controls	1	5	2	0	2	10
Total	4	30	7	1	12	54

The zero frequency of isolation of serogroup H strains in vaccinated sheep (0/17) was significantly different from the frequency (12/37) in the other three groups combined ($\chi^2 =$ 5.09, P<0.05).

As it appeared that serogroup B and serogroup H predominated in isolations, an isolate of each was tested in serial dilutions of antiserum to determine its serotype. The serogroup B isolate tested (from sheep 354) was identified as B_2. The serogroup H tested was serotype H_1 (sheep 225). In addition, an isolate provisionally identified as belonging to serogroup C was tested further because, in slide agglutination, it had reacted also with E antiserum. In the tube agglutination test this isolate (sheep 158) reacted to a titre of 160 with C_1 but to less than 20 with antiserum to E.

4.3.1.11.   Elastase testing of isolates

Isolates collected from flock 1 were tested for elastase activity on two occasions, 12 November 1992 and 3 March 1993. On the first occasion, six isolates were tested (Table 4.8), three of which were serogroup H and three were serogroup B. Elastase plates were examined for clearing at four, eight, and twelve days after inoculation. A virulent control (VCS1001) was elastase positive at four days. All six test isolates were negative at eight days but positive at twelve days. No other control isolate was used. It was considered that variations in incubation conditions and medium characteristics could result in variability in elastase test results in future tests, so the isolate reference number 26 was selected as an 'intermediate standard' for future elastase testing. A large number of sub-cultures from this isolate was grown and lyophilised and placed in the collection with reference number 26B.

The elastase testing done in March 1993 repeated the testing of a B serogroup isolate (reference number 25), an H isolate (27), a C isolate (34) and an A isolate (41). Isolate 26B was used as the intermediate reference isolate. Clearing of elastase was evident earlier with the B and H serogroup isolates than had occurred in November but, consistent with the first results, the B and H serogroup isolates showed delayed elastase clearing (seven days) compared to the virulent reference isolates (four days) but similar results to each other. The serogroup A isolate showed more delayed clearing (10 to 14 days), suggesting low grade

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intermediate virulence, and the C isolate failed to clear elastase up to 21 days, suggesting that it was benign.

4.3.1.12. PCR-RFLP typing of isolates

There were eight different *Hpa*II PCR-RFLP (*omp* gene) types identified from 12 of the isolates collected from sheep at property B (Tables 4.8 and 4.9a). There appeared to be no association between serogroup of isolates and PCR-RFLP type, with up to three serogroups being identified with the same RFLP type, and up to five different RFLP types within the same serogroup.

The PCR-RFLP results are discussed further in Chapter 7.

Table 4.8 Characteristics of isolates collected from flock 1 and retained in freeze-dry collection

Isolate reference number	Sheep # and group	Sero-group	Nov 1992 (days) **	March 93 (days) **	RFLP pattern
			Elastase test result		
25	155 CON	B	12	7	
26	387 BATH	H	12	7 *	5
27	387 BATH	H	12	7	5
28	332 VAC	B	12		
29	248 AB	B	12		
30	238 VAC	H	12		2
34	34 AB	C		-ve (21d)	
35	122 BATH	H			1
36	158 CON	C			1
37	160 CON	B			2
38	191 BATH	H			1c
39	200 AB	C			4
40	225 AB	H_1			7
41	346 AB	A		10-14	1a
42	354 CON	B_2			5
43	393 AB	B			1b

* intermediate control

** virulent control positive at 4 days at both November and March tests

Table 4.9

(a)   **RFLP pattern and serogroups collected from flock 1**

	Serogroup				
RFLP pattern	**A**	**B**	**C**	**H**	**Total**
1			1	1	**2**
1a	1				**1**
1b		1			**1**
1c				1	**1**
2		1		1	**2**
4			1		**1**
5		1		2	**3**
7				1	**1**
Total	1	3	2	6	**12**

(b)   **RFLP pattern and serogroups collected from flock 2**

	Serogroup		
RFLP pattern	**H**	**G**	**Total**
1	2	1	**3**
4	1		**1**
Total	3	1	**4**

4.3.1.13.   Serology

The geometric mean titres of the groups of sheep sampled during the period of observation up to and including week 16 (22 October 1992) are shown in Figure 4.7 (data in Appendix 2).

There was no significant difference between the titres of the CON group and the VAC group at week 0. Titres to both B and H antigens rose significantly following the first, multivalent vaccination in week 0 and titres to H antigen rose to very high levels following monovalent vaccination (against H_1) in week 5. Titres to B did not rise after V2 but remained significantly higher in the vaccinates than in the controls. Differences between control and vaccinate titres within serogroup were highly significant (P<0.005) at all times excepted week 0.

4.3.2.   Flock 2

4.3.2.1.   Footrot prevalence

The prevalence of footrot lesions in the ewes at property W over four months of summer and early autumn is tabulated below (Table 4.10). The prevalence of affected sheep rose from 50% in week 24 (15 December 1992) to 88% in week 28 and 89% in week 41. Despite conditions clearly suitable for a high level of transmission, the prevalence of severe lesions (score 3 or 4) remained below 23% and the prevalence of score 4 lesions remained below 4% (Figure 4.6) (data in Appendix 3).

Table 4.10   Prevalence of affected sheep and feet, flock 2

Week	Number present	Affected sheep		Affected feet	
		Number	Prevalence	Number	Prevalence
24	300	149	50%	316	26%
28	298	262	88%	704	59%
41	274	244	89%	644	59%

4.3.2.2.   Clinical aspects of lesions

Although score 4 lesions were few, it was still clear that most of them were limited in severity
compared to those normally seen with VFR. The same observation had been made for flock
1 wethers on property B. The interdigital skin associated with score 2 and 3 lesions, however,
was usually very inflamed and, in many cases, the sheep resented inspection which required
separating the digits, presumably because of pain. The ewes were, on average, in very good
condition but those affected with the painful, active lesions were noticeably lighter in condition
and bodyweight. Photographs of two affected feet (Plate H) from ewes in flock 2 demonstrate
the variation in interdigital lesions within the score 2 classification.

4.3.2.3.   Isolation and characterisation of *D nodosus*

From the 50 sheep sampled, 26 produced *D nodosus* on primary lesion plates and one isolate
from each of these plates was grown, sub-cultured and serogrouped. Of the 26 isolates, 25
were serogroup H and one was serogroup G. Three isolates were added to the freeze-dry
collection,
reference numbers 54, 55 (both serogroup H) and 63 (serogroup G). This latter isolate was
later tested by tube agglutination, which determined it to be serotype G_1.

4.3.2.4.   PCR-RFLP of isolates

Four isolates from flock 2, at property W, were of two patterns (Table 4.9). Two of the three
serogroup H isolates were of pattern 1, and one was pattern 4. The serogroup G isolate was
of pattern 1 also. Although isolates of serogroup G had not been identified from sheep at
property B, both RFLP patterns had been previously identified in isolates from there (see also
Chapter 7).

4.3.2.5.   Effect of vaccination on footrot prevalence

At the time of these activities on properties B and W (late 1992) a further study of intermediate
footrot was planned. As part of the intended study, a group of sheep were to be vaccinated

against a virulent strain of *D nodosus*, serotype A_1, while exposed to infection from sheep from the property B flock. There were concerns, however, that cross protection from vaccination may change the expression of footrot caused by heterologous strains. While making observations of the flock of ewes at property W, the opportunity was taken to vaccinate some ewes against serotype A_1 and compare the prevalence and severity of footrot between vaccinates and non-vaccinates.

In weeks 31 and 37, 98 ewes were vaccinated with monovalent vaccine against A_1. Of the 98, 85% had footrot lesions and 65% had at least one score 2 lesion as its worst lesion. The prevalence of footrot and the distribution of lesions across scores was essentially the same in the 194 sheep which were not vaccinated (data not shown).

Two months after the first vaccination (week 41) the prevalence of footrot and the footscore profile was not significantly different from that in week 28, nor was the frequency of lesions of any score different between vaccinates and non-vaccinates.

Plate H

Photographs of interdigital lesions from two ewes from flock 2, taken on 15 April 1993. The top photograph shows mild erythema in the interdigital skin and some loss of hair. The skin-horn junction shows the main lesion, with 2 or 3 ridges parallel to the coronary band, a yellow, greasy appearance of the skin immediately dorsal to the SHJ, and necrotic skin in the general area of the SHJ. The lesion was considered a score 2.

The bottom photograph shows a more obvious score 2 lesion, with pronounced inflammation and hairlessness of the IDS, ridging and pitting of the horn ventral to the SHJ, and similar but more marked changes to the skin dorsal to the SHJ to those described for the foot in the top photograph.

4.4.   Discussion

4.4.1.   Epidemiology

The environment at property B was apparently unsuitable for the transmission of footrot during July, August and September 1992 because of low rainfall in July and August and low temperatures; daily mean temperatures were consistently below 10°C from late April to early October. Graham and Egerton (1968) found that outbreaks of footrot occurred when daily mean temperatures exceeded 10°C following a period of sustained high rainfall, averaging 50 mm per month or more. These conditions did not occur at property B in 1992 until the November - December period.

Nevertheless, footrot clearly persisted in the sheep flock during the winter period. It did so in a mild form, with most affected sheep having only score 2 lesions and these were often of such a mild, inactive nature that diagnosis was difficult. Frequently, lesions were classified as score 2 lesions based only on hairlessness and mild erythema, rather than evidence of necrosis, and the distinction between score 1 lesions and score 2 lesions was, at times, arbitrary. In a number of sheep examined over winter the only lesions evident, apart from hairlessness, was a disruption of the web of cornified skin at the posterior part of the IDS, possibly caused by trauma after predisposition from an infectious process.

Two factors led me to believe that these were in fact primarily footrot lesions; one being that the lesions developed to a more obvious footrot infection at a subsequent inspection, the other being the occasional success in isolating *D nodosus* from the IDS. The possibility remains, however, that the organisms isolated were surface contaminants, rather than agents involved in the infection.

Neither of these factors prove that the diagnosis was correct, but these mild departures from normality seemed to lie at the end of a spectrum of IDS disorders, from obvious score 2 lesions with disfigurement at the SHJ and an inflamed, hairless and painful IDS, to very mild abnormalities.

Further evidence that the mild IDS derangements were footrot infections was provided by the response to vaccination and to antibiotic treatment. All sheep from each treatment group were examined at random at each visit and inspected by me or by my assistant without knowledge of the group to which they belonged. The curative and preventive effect of vaccination and antibiotic treatment evident in the prevalence data throughout winter (see discussion in 4.4.2) implies that at least a high proportion of the lesions described as score 2 were truly footrot lesions, because they were evidently cured or prevented from expression by vaccination.

Few severe lesions (score 3 or 4) occurred in the winter - early spring period. The greatest number of severe lesions during that period occurred at the first September inspection when 28% of affected sheep (16 of 57) had score 3 lesions and one had a score 4 lesion (Figure 4.5). This 'burst' of severity occurred with no increase in the number of affected sheep, but as a result of the development of lesions which had been recorded as score 2 lesions at the previous visit. Further, the burst of severity did not persist for, by 22 October, the number of score 3 and 4 lesions had fallen below that of the July and August visits.

Presumably, this increase in severity resulted from a few days of damp, relatively warm weather in the last few days of August and first few days of September. Rain fell on seven of the eleven days before the 8 September inspection and the mean daily temperatures on the first three days of September were 8°C, 6.5°C and 12°C, several degrees above temperatures in the preceding month. (Unfortunately, daily temperatures were not recorded in Cooma on 31 August or 4 and 5 September.) The low rainfall in October presumably accounted for the decline in the severity of lesions observed in week 16. The change in lesion severity profile within the flock without an increase in the number of affected sheep is consistent with the hypothesis that conditions were not suitable for footrot transmission, but that an increase in moisture levels and temperature in the environment encouraged existing lesions to extend temporarily. Low ambient temperatures such as those common in the Monaro in winter lead to a fall in the temperature of the extremities of sheep (Graham and Egerton 1968). Lower temperatures of the IDS are likely to reduce the rate of multiplication of *D nodosus* in the feet of infected sheep and, if so, fewer organisms will be shed into the environment from whence transmission to other sheep could occur. In addition, the cold IDS is likely to be an inhospitable environment for *D nodosus* to establish in an uninfected foot. These two factors, reduced shedding and

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more difficult establishment, probably account for the absence of transmission of footrot observed by Graham and Egerton (1968) in the southern Tablelands of NSW in winter. To my knowledge, this is the first recorded observation of lesions becoming more severe in response to a short-term improvement in climatic conditions at a time when ambient temperatures are generally too low for transmission to occur.

The observation also supports the view that footrot lesions in this cold, low winter-rainfall environment persist through the winter months as mild or even sub-clinical lesions of the IDS. These mild lesions would be easily overlooked and not considered to be footrot lesions unless the inspector was aware that footrot was present in the flock. Nevertheless, given an improvement in the suitability of the climatic conditions, the mild lesions can rapidly develop to more obvious forms. This behaviour may be a feature of intermediate and benign footrot, rather than of virulent footrot.

The stable prevalence of footrot cases over winter was not simply a result of persistent clinical lesions. There was a steady rate of regression virtually matched by the incidence of new cases, some of which were recurrences of earlier lesions. In the controls, 14 cases were found to have resolved while 21 new cases occurred in weeks 5, 10, 13 and 16 (early August to late October). The normal behaviour of footrot in a flock in this environment appears to be the persistence of low grade lesions, some of which alternate between a visually detectable state and sub-clinical, undetectable lesions in response to a range of host, environmental and other factors. These are not latent infections in the sense meant by Glynn (1993) or Morgan *et al* (1972). Rather, these are inapparent infections which persist due to the level of moisture maintained by the cold, damp pasture but kept at mild or sub-clinical levels by the low temperatures and lack of sufficient water to stimulate or allow further lesion development.

Flock 2, running on improved pasture with a high clover content and with a denser pasture sward than existed at property B, was not examined until 4 December 1992, when the flock owners reported an outbreak of lameness in their ewes. By that time, 49% of the 300 ewes examined had footrot lesions (Figure 4.6). This coincided with an inspection of flock 1 at property B which showed the first significant increase in incidence of footrot since inspection began in June/July (Figure 4.2).

The outbreak in flock 2 was relatively more advanced than that of flock 1, with the profile of footscores in flock 2 in December 1992 being very similar to the profile in flock 1 in February 1993 (Figures 4.5 and 4.6). This supports the view that conditions at property W were more suitable for the transmission of footrot, in that a high proportion of flock 2 were affected earlier in the season (early summer rather than late summer). Footrot is known to transmit faster in lush, clover-dominant pastures than on grass-dominant or low density pastures (Beveridge 1941, Stewart _et al_ 1984) and in long pasture transmission rates will be higher than in short pastures (Sinclair 1957; Graham and Egerton 1968; Cummins _et al_ 1991).

By mid January, 88% of flock 2 had footrot, a much higher proportion than were affected in flock 1 by early February. Also by this time, the relative proportion of sheep in each footscore class was different with a relatively high ratio of score 2 and relatively low ratio of score 4 in flock 2 compared to flock 1.

In flock 2, where an April inspection occurred as well, the nature of the outbreak remained unchanged despite, or because of, persistent moist, warm conditions during February and March. Given the excellent pasture conditions for these sheep and the apparently ideal conditions for footrot transmission, it is likely that the expression of the disease in flock 2 in April was near maximum for field conditions for this form of footrot (Figure 4.6). Egerton _et al_ (1983) found that, with VFR in footrot-endemic areas of southern NSW, the proportion of sheep which developed footrot at least once in an outbreak was 66% to 79% (three different flocks), lower than was observed in flock 2. Their definition of a footrot case was more restricted than used in this study; they required a sheep to have at least two score 2 lesions or at least one score 3 lesion before it was considered a case. Some sheep in a flock demonstrate a resistance to infection with footrot which is manifest by resistance to infection, late development of infection, (Egerton _et al_ 1983), less severe lesions (Stewart _et al_ 1984, Skerman 1986, Egerton and Raadsma 1993) and earlier resolution at the end of an outbreak (Egerton _et al_ 1983). Possibly, the relatively higher proportion of score 2 lesions in flock 2 occurred because the sheep which were most refractory to infection required more suitable conditions, for longer, before they became affected and, when they did, developed less severe (score 2 rather than score 3 and 4) lesions.

Hence, considering flock 1 in February (Figure 4.5) if conditions had been more suitable for transmission, it is probable that more than 54% of the flock would have developed footrot, but most of the new cases would have been only score 2 lesions. If so, the profile of footscores would have become more like that of flock 2 in January and April. The relatively high proportion of score 3 lesions (21%) and score 4 lesions (7%) in flock 1, compared to all previous inspections, supports the view that conditions for the worsening of lesions are less stringent than conditions for transmission. While 46% of the flock remain unaffected, the proportion of the flock which developed score 3 and score 4 lesions was similar to the proportion in flock 2, where conditions were clearly more suitable for footrot transmission.

The slightly higher proportion of score 4 lesions in flock 1 than in flock 2, in conditions less suitable for transmission than in flock 2, is of interest. Possibly, in the long, dense and wet pastures, water moves through the interdigital space and into underrunning lesions, effectively irrigating the infected tissue. This effect would be less marked in shorter and less dense pasture. It is possible that, without this irrigating effect, *D nodosus* and its proteolytic enzymes may persist at higher concentrations in the footrot lesions and the advance of the lesions may be enhanced. This may explain why, towards the end of a period of high transmission, there is a short time period when lesions extend, before regressing in the face of persistent dryness. Similar observations are reported in Chapter 3 and Chapter 5 and, from other workers, can be seen in data presented by Depiazzi *et al* 1998.

The difference in lesion profiles of the two flocks has implications for the diagnosis of footrot based on lesion prevalence. Characterising the form of footrot by dividing the number of sheep with score 4 lesions by the number of affected sheep (as suggested by Allworth and Egerton 1999) may overestimate the severity of the outbreak when conditions are less than optimal for transmission. For these two flocks, 22/166 (13%) of affected sheep had score 4 lesions in flock 1, but only 7/245 (3%) had score 4 lesions in flock 2. Thus, as the two outbreaks were caused by effectively the same strains of *D nodosus* (see discussion below), the effect of the environment or the host (wethers in flock 1, ewes in flock 2) had a significant effect on the way the outbreak would have been reported. In fact, by these definitions, the outbreak would appear to be less severe in the more favourable environment.

Both outbreaks fit the description of intermediate footrot given by a number of authors (Dobson 1986; Stewart *et al* 1984; Stewart 1989; Egerton 1989; Allworth and Egerton 1989). Fewer than 10% of the flock developed score 4 lesions, and the score 4 lesions were generally mild and less destructive than those typical of virulent footrot. The disease is clearly worse than benign footrot because significantly more than 1% of the sheep have score 4 lesions and more than 5% have score 3 lesions (Allworth 1995; Allworth and Egerton 1999).

There is strong evidence that the two outbreaks were caused by the same strains of *D nodosus*, but the attempts to determine this have highlighted the high level of complexity in characterising the bacterial flora, even of just the one species of bacterium, in naturally occurring outbreaks of footrot. At the start of the study, it was known that a strain of serogroup H *D nodosus* was involved in the outbreak. (This information was provided by the regional Government laboratory, through the District Veterinarian.) The first isolation made, in week 0, was also of serogroup H.

In the course of the next six months, 60 isolates of *D nodosus* were serogrouped by the slide agglutination test. Five serogroups were identified in total (Table 4.7) with two (B and H) predominant. Isolates of these two serogroups produced elastase test results typical of strains of intermediate virulence (Table 4.8). Testing of isolates of the next most frequently identified serogroups (C and A) suggested that they were benign and low grade intermediate respectively. The results suggest that the outbreak at property B was 'caused' by two intermediate strains, one serogroup H, one serogroup B and both of similar virulence.

Twenty six isolates were made from ewes in flock 2. Serogroup H predominated, one serogroup G isolate was found; no isolates of serogroup B were identified.

PCR-RFLP typing of the isolates from flocks 1 and 2 supported the consistency of the bacterial flora between the two outbreaks but also introduced some additional information.

Serogroup H, RFLP pattern 1 isolates occurred in both outbreaks. Across both flocks, serogroup H and pattern 1 were the most frequently identified epidemiological marker in their respective methodologies but they were not consistently related together. Pattern 1 was also

found in serogroup C and G isolates, serogroup H occurred with four other RFLP patterns (Table 4.10).

Both systems of identifying _D nodosus_ strains provided evidence of strains in common between the two outbreaks and, while it seems likely that the serogroup H, pattern 1 isolate was a common isolate in both outbreaks, the evidence is not complete. There were 11 RFLP-serogroup combinations detected in flock 1. It would require characterisation of many more isolates to determine the relative frequency of each strain and, undoubtedly, more RFLP-serogroup combinations would be detected. The 12 isolates serogrouped and subject to RFLP typing from flock 1 (Table 4.9) is clearly insufficient to fully explain the relationships between the RFLP pattern and the serogroup.

The discovery of a total of five serogroups from flock 1 is not surprising, given the results of Claxton _et al_ (1983) who found that the number of serogroups recovered from a flock increased as the number of sheep included in the sample increased. They found up to six serogroups represented in one flock; Schmitz and Gradin (1980) found up to seven serotypes in one flock in Oregon and Thorley and Day (1986) reported nine serotypes on one farm in Sussex.

There are other compelling reasons to believe that the bacterial genotypes present in the flock 1 outbreak are also present in the flock 2 outbreak, beyond the evidence given. The wethers run at property B are born at property W, from the same ewe flock as that involved in the outbreak in flock 2. Young wethers (18 months old) are transported from property W to property B each year so the prospects of a movement of _D nodosus_ strains from property W to property B are high.

Sheep did move from property B to property W on some occasions but in small numbers and less often than in the opposite direction. While it is possible that strains of _D nodosus_ present at property B could be absent at property W, it seems unlikely.

4.4.2.   Effect of vaccination on bacterial isolations

In flock 1, 101 sheep were vaccinated; first with a multivalent vaccine in week 0 then, in week five, with a monovalent vaccine specifically against serotype H_1 because, at that time, serotype H_1 was believed to be the principal, if not only, antigenic type involved in the outbreak. Two vaccinations produced agglutinin titres to H which peaked at levels exceeding 30 000 (geometric mean of sample group) 33 days after the second vaccination (Figure 4.7). Agglutinating titres to a heterologous antigen (B was used as an indicator of heterologous protection) rose to nearly 600 following the first, multivalent vaccination but declined below 300 33 days post V2. Titres to homologous antigen (H) were maintained above 8 000 throughout the period that samples were collected - until 22 October, 11 weeks after V2.

Seventeen isolates collected from vaccinated sheep in weeks 16 and 22 were identified to serogroup (Table 4.7). None of these was serogroup H, whereas serogroup H isolates made up 12 of 37 (32%) of the isolates from BATH, AB and CON treatment groups. Amongst the vaccinates, 14 of 17 (82%) were serogroup B, compared with 16/37 (43%) from the BATH, AB and CON groups. The differences in ratios were significantly different (P<0.05). Vaccination caused a shift in the frequency of isolations of the serogroups present, away from the homologous group and toward the most frequently isolated heterologous group.

The protection against homologous challenge afforded by titres over 8 000 is expected. Groups of sheep with mean titres exceeding 5 000 are generally considered strongly resistant to challenge (Thorley and Egerton 1981; Stewart *et al* 1982; Egerton *et al* 1987). Bivalent vaccination of sheep in Nepal, against two previously extant serogroups, led to their apparent disappearance from sheep in the vaccinated flocks (Egerton *et al* 1996; Ghimire 1997). Titres in these sheep remained above 3 000 for at least four months after the second bivalent vaccination but, in the first year of vaccination, titres had declined to 1500 by seven months post vaccination. Schwartzkoff and Handley (1986) found that even relatively low titres against serogroup H provide homologous protection. In general, and not specifically for serogroup H, titres below 3 000 do not provide reliable protection against challenge (Thorley and Egerton 1981; Stewart *et al* 1982; Egerton *et al* 1987).

It might be expected, in the study in flock 1, that the protection against footrot provided by what was effectively monovalent protection would be minimal, because the vaccine caused a shift in challenging strain from an H serogroup strain to a B serogroup strain. The B serogroup strain isolated from this flock had an elastase-clearing capacity similar to the H serogroup strain. Nevertheless, vaccinated sheep had a lower incidence of footrot in weeks 5, 10, 13 and 16 (three cases, compared to a total of 21 in the controls). By week 31 there was no difference in the prevalence of footrot between any group (Figure 4.2). Neither was there any statistically significant difference in the proportion of sheep with score 2, 3 or 4 lesions.

The explanation for the initial high level of protection against footrot following one multivalent vaccination and a monovalent booster is unclear. Titres against B, the serogroup of the other principal *D nodosus* isolate in the outbreak, were recorded at a peak of 581 at week 5, when V2 with monovalent H was given. At subsequent sampling times, titres against B were lower than in week 5 (Figure 4.7). It is not possible to say whether titres against any serogroup were higher at any time before week 5 than at week 5 or if titres continued to rise after week 5 before declining to the level recorded at week 10, nor is there any published information from other studies to indicate the likely trend in titres in the few weeks following one multivalent vaccination. From studies with immune responses of sheep to epsilon toxoid of *Clostridium perfringens* type D, Jensen (1967) found that titres following one vaccination peaked at 3 weeks, then declined. In this study, it seems likely that mean titres against B in the vaccinated group were declining by week 5, and continued to decline to 288 at week 10, 108 at week 13 and 157 at week 16. While such titres are not considered protective, the protection rate apparently afforded by vaccination in this study was 86% (Table 4.6). In light of the observation that transmission was not occurring during this period, it is possible that one vaccination resulted in a high level of cure of sub-clinically affected sheep. A higher proportion of the sheep which were similarly affected in the CON group did develop detectable lesions during the 16 week non-transmission period. If so, the apparent protection afforded by vaccination should more appropriately be considered a cure of sub-clinical infections. This result was achieved with the relatively low antibody titres achieved in the first few weeks following vaccination.

By the time transmission resumed, evident at week 22, the incidence of footrot in the vaccinated sheep was no different from that of any other group. While the vaccinated sheep were protected against infection with *D nodosus* of serogroup H, they developed footrot caused by strains of other serogroups at a similar rate to those sheep susceptible to all serogroups.

It has been proposed by some workers (Lambell 1986; Stewart 1989) that vaccination may mask infection or induce a carrier state, while others (Reed 1986; Egerton 1986) believe that there is no evidence for this. In the study reported here, vaccination was very effective in preventing infection with homologous isolates for five months which included a transmission period, as judged by failure to isolate homologous strains from vaccinates during that period. It is unlikely that a carrier state for H serogroup strains could have existed while clinical footrot lesions associated with strains of other serogroups were expressed.

4.4.3.   Response to treatment

In week 0, the prevalence of footrot in the groups allocated to each treatment group varied, from 11% in the antibiotic group to 20% in the footbath group (Table 4.5). The variation occurred by chance, because the sheep were allocated at random, and the difference was not statistically significant. In week 5, a significant response to treatment in week 0 was apparent in all groups except CON (Table 4.3). In week 5, the prevalence in the BATH group was not statistically significant from the prevalence in the CON group, influenced by the higher number of cases in the BATH group in week 0.

Treatment was repeated in week 5 and the number of cases reviewed in weeks 10 and 13. Any case found at either week 10 or 13 was considered a failure to cure or failure to protect (Table 4.6). The response to treatment with vaccine was poor despite very high titres against serotype H by week 10, which persisted at effective levels in weeks 13 and 16. Sampling of the feet of vaccinated but affected sheep in weeks 13 and 16 demonstrated that serogroup H was absent (to the ability of the detection method employed) from the lesions in these sheep, implying that the treatment with vaccine specifically against one serogroup changed the flora, or the phenotype of the flora in the lesions but, because other serogroups with similar virulence were

also present in the lesions, the lesions persisted apparently unchanged.

Antibiotic treatment cured 63% of cases relative to the CON group. The response was not significantly different from zero. Cure rates with penicillin/streptomycin combinations have given variable results in other studies, with the greatest success (96%) being for treatment of advanced lesions when sheep are returned to dry conditions after treatment (Egerton *et al* 1968). Poorer results frequently occur when sheep are returned to wet or damp pasture conditions, even after 24 hours on dry ventilated floors (Webb Ware *et al* 1993) and when the lesions are principally interdigital lesions (Egerton *et al* 1968). It is likely in this study that the damp conditions in the pasture and the fact that the lesions were principally limited to the IDS contributed to a mediocre response only, despite spending 24 hours on battens in a woolshed after treatment.

Topical zinc sulphate therapy with a footbath (group BATH) gave similar results to antibiotic treatment although it was expected that topical treatment would be more appropriate than systemic therapy for the superficial lesions typical of this flock in winter. The usefulness of 10% zinc sulphate solutions as a footbath has been demonstrated in other studies (Beveridge 1941; Demertzis *et al* 1978; Cross and Parker 1981; Skerman *et al* 1984) and it was considered appropriate to use the solution in this study without the addition of surfactants (Malecki and McCausland 1982; Skerman *et al* 1983, a and b) or prolonged bathing (Malecki and McCausland 1982) because penetration of the horn by the zinc would not be necessary to provide contact with infected tissues. Considering the short exposure time (three minutes), the low frequency of treatment (twice, 33 days apart) and the fact that sheep were immediately returned to damp pastures, the reported success rate in my study is relatively high. The treatment was cheap and simple to administer and resulted in a useful improvement in the health of the treated sheep over winter, when nutritional, climatic and parasitic stresses were substantial.

Treatment (Table 4.6) with antibiotic and vaccine both gave high rates of protection against infection (87% and 86%). As stated above, the steady prevalence in the CON group over winter, with cases waxing and waning between detectable and undetectable states, and the evidence for lack of transmission in early September despite rainfall events, suggest that

transmission of footrot was not occurring at measurable rates in the winter-early spring period. How, then, could antibiotics and vaccines protect against the development of new cases if transmission was not occurring?

Further, antibiotics do not have a prolonged effect; the half-life of procaine penicillin and dihydrostreptomycin is less than 48 hours (Lambert and O'Grady 1992). Two doses of these antibiotics, 35 days apart, do not lead to persisting therapeutic levels for more than 2 or 3 days and yet there appeared to be a protective effect from antibiotic usage when the cumulative incidence of new cases up to and including week 13 was compared (Table 4.2). The most likely explanation is that antibiotic treatment cured, or suppressed the expression of, sub-clinical cases. Thus, while untreated (CON group) sheep with sub-clinical infections were appearing as cases in weeks 5, 10 and 13, antibiotic treated sheep were not. The infections in the feet, presumably in the IDS, of these sheep were either made free of *D nodosus* by the treatment, or other bacteria necessary for the expression of footrot, such as *F necrophorum*, were being removed while *D nodosus* persisted. If this happened, a footrot lesion would not be expected to recur in the foot until inflammatory changes caused by environmental organisms had developed again.

Other evidence of suppression, rather than protection, arises from the incidence of footrot in weeks 16, 22 and 31 (Table 4.2) and the model fitted to the data (Figure 4.3). A total of 61 new cases of footrot were identified in the AB group, whereas only 39 new cases occurred in the CON group at the three spring-summer inspections. Because more cases of footrot had persisted or developed in the CON group over winter than in the AB group the final prevalence in the two groups was similar. This 'bounce-back' phenomenon in the prevalence of the antibiotic-treated sheep could have resulted from the breakdown of suppressed infections. Alternatively, the phenomenon could be explained by the return to infected status of a cohort of susceptible sheep which had truly been cured for several months, then quickly became re-infected when transmission occurred.

The similar proposition that vaccination cured sub-clinical cases and thereby reduced the incidence of new cases in the non-transmission period was discussed in the previous section.

The protection against footrot afforded by monovalent vaccination did not persist and, in week 31, as many VAC group sheep had footrot as sheep from any other group. The footrot lesions were, however, dominated by heterologous strains. Vaccination was effective in protecting against homologous infection but not against footrot caused by a mixed infection. The results obtained here with vaccine were sufficiently encouraging for the owners of the flock to consider a control or eradication program, based on the use of bivalent (H and B) vaccine.

There was a difference in the number of animals of each group present at the end of the trial (week 31) in flock 1. The antibiotic-treated group had the highest survival rate with all 101 AB group animals present in February 1993, seven months after the trial started. By contrast, there were seven CON group, four BATH group and eight VAC group sheep either dead or with missing tags. Seven sheep were present without tags so 12 were dead from these three groups. Although the difference was not statistically significant it was an interesting observation, drawing attention to the low prevalence of footrot lesions over winter in these sheep, when lameness might be a fatal disability in sheep stressed by a cold climate and low nutrition.

4.4.4.   Practical implications

The observations made in these two flocks have confirmed that the disease in the flock was intermediate footrot and have highlighted the need for care when interpreting footscore data to make a diagnosis of footrot in a flock. Both host resistance and the environmental conditions influence the expression of the disease and other observations including the amount of necrotic material present in advanced lesions, evidence of regression in response to a change in the environment and knowledge of the exposure of the flock to particular environmental conditions must be taken into account when making a diagnosis which might be intermediate footrot (Glynn 1993). The study has also shown that, in conditions suitable for the transmission of footrot (flock 2), the proportion of score 4 lesions in intermediate footrot is still restricted compared to virulent footrot and, therefore, it is unlikely that the expression of the disease would be significantly different in other environments. The Monaro is considered only a moderate risk for the expression of footrot, compared to more northerly parts of the southern Tablelands of NSW (Egerton and Raadsma, 1993). The diagnosis of intermediate footrot in the Monaro district has caused problems for regulatory bodies, some of whom believe that

many forms of 'so called' intermediate footrot develop into virulent footrot under suitable environmental conditions (Plant, **pers comm**, 1991). While there is evidence to the contrary in the study reported in this chapter, work reported in Chapter 5 is also relevant to the stated concern.

This study has also suggested that treatments could be used to reduce the prevalence of intermediate footrot in order to assist with eradication of the disease from a flock. While the treatments used here (topical 10% zinc sulphate, systemic antibiotic and vaccination) were moderately effective in reducing disease prevalence over winter they were not used in a transmission period when conditions would have been more testing of their efficacy. Nevertheless, vaccination had a persistent effect on the prevalence of one serogroup and a more specifically designed vaccine (targeting the two or three most virulent strains in an outbreak) may have a role in footrot management. Theoretically, repeated inspections in a non-transmission period could identify all infected sheep, and these could be removed from the flock before a transmission period begins. Clearly, however, a more rigorous identification and removal program than that used in this study would be necessary and concerns about the suppressive, rather than curative effects of antibiotic treatment require further study before their use could be recommended. Glynn (1993) has drawn attention to the possible survival of *D nodosus* of benign or low grade intermediate strains in the interdigital skin, despite the absence of lesions and footbathing with 20% zinc sulphate.

The results of this study suggest that eradication in the winter non-transmission period by identification and removal of affected sheep without whole flock treatments may be difficult because the low levels of moisture from dew and light rainfalls may be sufficient to allow *D nodosus* to persist in mild and sub-clinical lesions which escape detection. Whole flock treatments in combination with inspection and culling are more likely to be effective and, of the treatments investigated here, vaccination is the one which offered the greatest promise.

4.4.5. Flock 2 - vaccine

In flock 2, vaccination with a heterologous vaccine had no apparent effect on the development of footrot in the flock, a finding with implications for the design of a later study (Chapter 5).

4.5.   Summary

An outbreak of footrot was observed in a region of NSW with cold, relatively dry winters and mild, wet summers. The form of the outbreak and laboratory characterisation of the *D nodosus* isolates indicated that the disease was intermediate footrot, rather than benign or virulent footrot. Transmission of footrot did not occur in winter, when temperatures were very low, even after rain events, but lesions of footrot persisted in a mild form. In winter, lesions were generally marked by absence of inflammation of the IDS and a lack of necrotic material in the IDS and hoof, compared to lesions seen when footrot is spreading.

It is hypothesised that sub-clinical cases of footrot existed in the flock during the winter period. Evidence was provided by the fluctuating prevalence and the range of mild interdigital lesions at a time when transmission was unlikely to be occurring. Further evidence was provided by the marked effect of antibiotics and monovalent vaccination on the winter incidence of footrot, but not the spring/summer incidence. These treatments reduced the incidence by curing or inhibiting sub-clinical infections, rather than preventing transmission. Attempts to prevent an outbreak of footrot by identifying and removing affected sheep were not successful because of the impossibility of identifying sub-clinical cases.

Climatic conditions for the extension of lesions are less stringent than those required for transmission. Over the non-transmission period, rain events with slight increases in temperature caused an increase in the severity of lesions of footrot-affected sheep which regressed again when drier, colder conditions returned.

Transmission of footrot began in late October or November in association with 48 mm of rainfall in October and 142 mm in November, and persistent daily mean temperatures exceeding 10°C. Thus the transmission of intermediate footrot in the Monaro was predictable using the conditions described by Graham and Egerton (1968) for an outbreak in parts of NSW where virulent footrot occurs endemically.

Intermediate footrot caused up to 7% of sheep in one flock to develop score 4 lesions, and 22% to develop score 3 lesions. Comparison of footrot scores in two outbreaks in different parts of the same flock, run under different pasture conditions, showed small differences in the characteristics of the footscore profiles. Laboratory characterisation of isolates from both outbreaks showed that there were strains of *D nodosus* common to both outbreaks, although a new serogroup, but not a new RFLP pattern, was detected in the second outbreak. The second outbreak, running in pastures believed to be more suitable for transmission of footrot, involved 88% of the sheep in the flock and persisted at that level for three months. Despite the persistent high prevalence, the proportion of the flock with score 2 lesions was however, higher, and the proportion with score 4 lesions lower, in the flock running in conditions less suitable for footrot transmission. In relatively unfavourable conditions, the ratio of the prevalence of severe lesions to the prevalence of total lesions may be higher than under favourable conditions. This ratio should not, therefore, be used as a diagnostic criterion for intermediate footrot because it will tend to overstate the virulence of a footrot outbreak.

Isolation and characterisation of *D nodosus* from lesions demonstrated that the outbreak was caused by strains of at least five serogroups, and eight different *omp* gene *Hpa*II RFLP patterns, but the serogroup classification did not segregate with the RFLP pattern. The two most frequently isolated serogroups, H and B, were found with five and three patterns respectively, two of which they had in common. Sample isolates of each serogroup were elastase positive with an elastase-clearing time less than virulent type strains. One isolate was adopted as an intermediate reference strain for elastase testing of other isolates found in the course of this series of studies.

Several treatments given in early winter were effective in reducing the prevalence of footrot over winter but only vaccination had a persistent effect once transmission resumed in late spring. Antibiotic treatment gave better cure rates than footbathing in 10% zinc sulphate or monovalent vaccination, and prevented new cases occurring for the winter period by either suppression or curing of inapparent infections. Once transmission resumed in late spring, any effect of earlier treatment with antibiotic, vaccination or footbathing was lost. In the case of vaccination, failure was associated with the mixed serogroup nature of the infection. Vaccination was effective in preventing infection with the homologous strain.

CONTENTS

STUDY OF INTERMEDIATE FOOTROT IN A FOOTROT ENDEMIC ENVIRONMENT

5.1.   Introduction

In Chapter 4, two related outbreaks of intermediate footrot on the properties of origin were described. The characteristic of these outbreaks which distinguished them from outbreaks of virulent footrot was the low prevalence of score 4 lesions, even under conditions which favoured rapid transmission of the disease. The characteristic which distinguished them from benign footrot was the presence of a moderate number (up to 25%) of the exposed sheep with lesions of score 3, plus a small number (up to 7%) of sheep with score 4 lesions.

To enable further study of this form of footrot and to evaluate the effect of a different, warmer environment on the expression of the disease, a portion of the affected flock was moved, with its footrot infection, to property K, a small land holding near Tarcutta, in the south-west slopes region of NSW. This environment is considered suitable for the transmission and expression of virulent footrot and at that time had one of the highest flock prevalences of VFR in NSW. A survey carried out in 1989/90 (Locke and Coombes 1994) found that 31% of sheep flocks with greater than 500 sheep in the Rural Lands Protection Board (RLPB) district of Wagga Wagga had virulent footrot. For the Holbrook district, the figure was 49% and for Gundagai, 48%. Property K lies at the southern extreme of the Wagga Wagga RLPB area, 14 kms north of the boundary with the Holbrook (now Hume) Board area and approximately 20 kms west of the boundary with the Gundagai RLPB.

This further study had the additional objectives of (a) comparing the form of the outbreak to that caused by virulent strains and (b) measuring the differences in productivity between sheep in which footrot was controlled by vaccination and footbathing (group CON), those infected with the intermediate isolates only and protected by vaccination from the virulent strains (group INT), and those infected with virulent footrot (group VIR).

5.2. Materials and methods

5.2.1. The site

Property K is on the northern side of the Hume Highway 23 kilometres south of Tarcutta and was leased for a two year period for the study. The area of the property was 75 ha and included sheepyards and a two-stand woolshed. Permanent water was available from Kyeamba Creek which ran through the property. Apart from a one hectare holding paddock there was only one paddock available for grazing.

5.2.2. The flocks

The experimental sheep consisted of two flocks of wethers. The flocks were purchased from two unrelated sources and brought to the experimental site by truck. Flock 1 comprised 170 four and five year old wethers (born in spring 1988 and 1989) which were purchased from the flock involved in the footrot outbreak described in Chapter 4. The sheep were selected at random from within their age groups in the flock on property B. Eighteen chronically infected seven year old wethers from the same flock were donated by their owner and these sheep are henceforth referred to as the intermediate donors. These chronically infected sheep are not considered part of flock 1 for the purposes of this study because they were older than the other wethers in flock 1 and had been selected on the basis of severe footrot lesions and were not, therefore, a random selection of the flock. Two hundred and thirty wethers, two years of age, were purchased from the owner of a flock on a property in the Tarcutta region. This flock was believed to be free of intermediate or virulent strains of *D nodosus* and no evidence of footrot was seen when the sheep were inspected four days after arrival. This group of sheep formed flock 2.

5.2.3. **The climate**

In reporting climatic conditions in this chapter, daily maximum and minimum temperatures recorded at Wagga Wagga and daily rainfall recorded at Tarcutta, both official Bureau of Meteorology recording stations, are used. Wagga Wagga is 40 kms north-west of property K. Wagga Wagga, elevation 212 m, has hot summers with a mean daily maximum for January of 31.2°C. Winters are mild; July is the coldest month with a mean daily maximum of 12.5°C and minimum of 2.7°C. There are on average 22 days of frost per year. Tarcutta is situated at 35°3' S, 147°7'E at an altitude of 232 m and is slightly more southerly than Wagga Wagga; the temperatures of the two regions are expected to be similar. The rainfall pattern for the region is moderately uniform with a slight winter dominance; May, July and October are the wettest months. The average annual rainfall at Wagga Wagga is 585 mm and the annual precipitation increases to the south-east approaching the western side of the Great Dividing Range. Tarcutta has an average annual rainfall of 670 mm.

Following a very poor season in the second year of the trial and the prospect of a drought, both flocks were moved to property T, 15 kms east of Holbrook and 30 kms south of property K, in September 1994 and meteorological data recorded at official stations at Albury-Wodonga (temperature data) and Holbrook (rainfall) were used to describe the climate experienced by the experimental flock for that period of the study. The annual rainfall at property T is approximately 50 mm higher than at property K.

5.2.4. **Dates of the studies**

The studies were conducted from 3 May 1993 to 20 January 1995. The two flocks were assembled in late autumn and early winter and foot inspections commenced on 21 June 1993. Table 5.1 shows the dates of the main events and observations.

Table 5.1   Main events in the study at properties K and T

Week	Inspection	Date ·	Main events
0		13 May 1993	Flock 1 sheep arrived
5		17 June	Flock 2 sheep arrived
6	1	21 June	V1¶
8		9 July	20 wethers to Camden to become virulent donors
12		5 August	V2
14		19 August	Virulent donors to property K
18	2	20 September	
21	3	6 October	V3
25	4	4 November	
27	5	17 November	
29	6	1 December	
31	7	15 December	
36	8	26 January 1994	
42		8 March	Shearing
46	9	5 April	
50	10	4 May	
65	11	19 August	
69	12	16 September	Moved to property T
79	13	23 November	
86	14	18 January 1995	Shearing
86		20 January	CALM‡ assessed and sold

¶   V1, V2, V3; first, second and third vaccination

‡   Computer Aided Livestock Marketing

5.2.5.   Management procedures

Flock 1 including the intermediate donors arrived at property K on 3 May, 1993. Flock 2 sheep arrived on 17 June, 1993. All sheep were eartagged, drenched with a levamisole-oxfendazole combination anthelmintic (Scanda, Mallinckrodt Veterinary, North Ryde, NSW) and weighed on arrival. Both flocks ran together in one paddock for the duration of the study.   In July 1993 all sheep were treated with triclabendazole (Fasinex 50, Ciba-Geigy Australia Ltd, Pendle Hill, NSW).

Pasture growth in 1994 was poor and insufficient to nourish the sheep adequately. Supplementary feeding with lupin grain was commenced in July 1994 and continued until 15 September. On 16 September 1994, the sheep were transported to property T where they remained until the end of the trial. Pasture availability at property T was relatively high and no supplementation was necessary.

5.2.6.   Experimental procedures

To measure the serological response to vaccination, blood was collected from all sheep in week six and week 46, and from a sample of 30 sheep in each flock in weeks 12, 18, 25 and 34. The sample was composed of 10 sheep in each of the three treatment groups in each flock.

Within each flock, sheep were randomly allocated to one of three treatment groups. One group was exposed to all *D nodosus* strains present in the two flocks, including the virulent strain, without protection from vaccine or topical treatment. This group was expected to develop virulent footrot and was called the Virulent group (group VIR). One group was vaccinated against the serogroup of the introduced virulent strain and, therefore, expected to develop intermediate footrot - the Intermediate (INT) group. The third group was vaccinated against the serogroup of the virulent strain and two strains associated with the intermediate footrot known to exist in flock 1. This group was the Control (CON) group. The first vaccination (V1) for CON and INT groups was given on 21 June. The second (V2) was given on 5 August, the third (V3) on 5 October. The vaccines used are shown in Table 5.2.

Table 5.2   Vaccines used for each treatment group

Group	Serogroups in vaccine	Sheep in each group	
		Flock 1	Flock 2
VIR	no vaccination	70	105
CON	A + H + B	31	29
INT	A	72	96

Ten sheep from the VIR group of each of the two flocks were selected to be virulent donors. On 9 July 1993 these 20 sheep were taken to Camden to be artificially infected with footrot. The sheep were placed on wet mats on 12 July and *D nodosus* VCS1001 was introduced to one hind foot and one front foot of each sheep on 19 July. The method is described in section 2.2.12. A specimen was taken from the foot lesions for bacteriological culture from the sheep on 19 August, the day they were returned to property K.

When it became clear that vaccination alone was not preventing severe lesions in the CON group sheep, footbathing was instituted in an attempt to limit the severity of the footrot lesions in this group. Footbathing was performed with 10% zinc sulphate solution at a depth of 75 to 100 mm in metal troughs and the sheep stood in the bath for approximately three minutes. The first treatment was given in week 27 and repeated in weeks 29 and 31. No further treatment to group CON was given.

All sheep were weighed on 11 occasions and footscored on each of 14 visits between 21 June, 1993 and 18 January, 1995 (Plate I). Fleeces were weighed and side samples were collected for yield and fibre diameter testing at shearing on 8 March, 1994 and 18 January, 1995. After their second shearing, the sheep were inspected by a licensed CALM (Computer Aided Livestock Marketing) assessor who divided the flock into two groups based on their suitability for sale to an abattoir, and they were sold on 21 January, 1995.

Specimens for bacteriology were collected from the feet of selected sheep in July, September, October, November and December 1993 and in January, June and November 1994.

5.2.7. Definitions

Greasy fleece weight (GFW) is the weight of the unskirted fleece of the sheep, measured immediately after removal from the sheep at shearing and includes all the fleece except the belly wool. The March 1994 GFW is referred to as GFW1; the January 1995 GFW as GFW2.

Yield (YLD) is the weight of clean wool obtained per unit of weight of a sample of greasy wool collected from the (greasy) fleece at shearing, after weighing. Yield is measured after scouring - the removal of grease, suint and dirt from the fleece - and drying. Yield is expressed as a percentage of the greasy weight.

Clean fleece weight (CFW) is the product of YLD and GFW.

Fibre diameter (FD) refers to the mean fibre diameter of a sample of wool removed from the mid-side region of the right hand side of each sheep at shearing.

An _affected foot episode_ (AFE) is the occurrence of a footscore of 2, 3 or 4 at one of the 14 inspections between June 1993 and January 1995.

An _affected sheep_ is a sheep with at least one footscore of 2, 3 or 4 and, when classified by footscore, is categorised by the highest footscore.

Year 1 refers to the period from the time of arrival of the sheep at property K in May and June 1993 up to the first shearing, in March 1994. _Year 2_ refers to the period from the first shearing until the second shearing, in January 1995.

5.2.8. Bacterial isolations during the trial periods.

Specimens were collected as described in Chapter 2, _D nodosus_ isolated when possible, and identified by serogroup with slide agglutination. Selected isolates were lyophilised and stored for later examination.

PCR-RFLP of the *omp* gene region was performed on stored isolates. The method described in Chapter 2 was followed.

5.2.9.   Statistical methods

Prevalence data were examined statistically with chi-squared tests of independence. Differences in mean serological titres were analysed using analysis of variance.

A linear model was used to examine the relationship between the presence of footrot lesions and liveweight, wool production and wool characteristics. The liveweight records examined were liveweight in week 29 (WT6), week 36 (WT8) and in week 79 (WT13). Wool production was examined for the first year of the study as greasy fleece weight (GFW1) and the product of greasy fleece weight and yield (CFW) and, for the second year of the study as greasy fleece weight only (GFW2). The wool quality characteristics studied were yield (YLD) and mean fibre diameter (FD) in the first year of the study. The degree to which individual sheep were affected by footrot was calculated as an index called affected feet episodes (AFE), which was the sum of the number of feet which were affected in each year, adjusted for the time between inspection events and the number of inspections in each year. As inspection periods were not at regular intervals during each year but were more frequent during spread periods when the prevalence of infection was changing most rapidly, the lesions detected at any one inspection were deemed to have existed for only 14 days around the time of inspection, which was the minimum time between inspections. Sheep were inspected seven times in the first year, so the maximum AFE index was 28 (up to four feet affected at each of seven inspections), and six times in the second year, so scores in that year were multiplied by 7/6 to give the same maximum score of 28 for both years.

The number of affected feet at the first inspection (AFE1) was not included in calculations of other AFE indices, but was considered a fixed effect for analytical purposes. For examining the relationship between footrot indices and production traits, accumulated footrot episodes up to the time that the production trait was measured were calculated and used. For example, AFE2-6 included the total affected foot episodes in the second to sixth inspections inclusive.

All AFE indices except AFE1 combined the number of feet affected at each inspection and the frequency with which lesions are observed, the frequency being an indicator of duration of lesions.

Linear models for the dependent variables included the effects of flock and treatment group, a flock x group interaction, current and previous footrot indices and the interactions of flock with these indices, except AFE1. If the flock effect on the slope of the linear model was found not be significant at the 0.05 level, a combined slope was assumed.

The associations between AFE1, AFE2-8 and AFE9-14 and any required adjustments for effects of flock or treatment, were also investigated. The distribution of AFE2-8 and AFE9-14 were positively skewed for each treatment group and the frequency of zero values was high, particularly for AFE9-14. Sheep with indices of zero for both years were omitted and both indices were transformed to $\log_e(x+1)$. The linear model for the relations of AFE2-8 and AFE9-14 with AFE1 included the effect of treatment and its interaction with AFE1. The linear model for the relation between AFE2-8 and AFE9-14 included the effects of flock and treatment, a flock by treatment interaction and a treatment by AFE2-8 interaction.

The general form for the linear models is $y = b + mx$ and b is the y intercept and m is the slope of the expression. The proportion of the variability in the independent variable (y) which can be attributed to variation in the dependent variable (x) is given by the coefficient of determination r^2.

In addition to the footrot indices given in Table 5.3, AFE1-14 was used as a measure of total number of footrot episodes. This index was the sum of AFE1, AFE2-8 and AFE9-14 and thus covered the entire length of the study.

A descriptive epidemiological technique was used to examine differences between two groups within each flock. Sheep in each flock were categorised as high or low prevalence sheep, based on their history of footrot lesions during the study period. Differences between the two groups were examined to investigate differences in footrot descriptors (number of feet affected,

duration of infection, incidence, rate of lesion regression and maximum footscore). The same categories were used to examine the relative risk for sheep in each group of being classified as 'light' or 'heavy' when sold at the end of study.

Table 5.3   Dependent variables and relevant footrot index used in statistical analysis

Dependent variables	Indices used
WT6	AFE1
	AFE2-6
WT8, GFW1, Y, FD, CFW	AFE1
	AFE2-8
WT13	AFE1
	AFE2-8
	AFE9-13
GFW2	AFE1
	AFE2-8
	AFE9-14

5.3. Results

5.3.1. Climate

Mean monthly rainfall, mean monthly daily maximum and minimum temperatures for Tarcutta (rainfall) and Wagga Wagga (temperatures) for 1993 and 1994 are illustrated in Figure 5.1. At Wagga Wagga, the lowest temperature recorded in 1993 and 1994 was -3°C, on two occasions (June and August) in 1993 and nine occasions between 16 May and 17 August, in 1994. Mean daily temperatures consistently exceeded 10°C from 24 August 1993 (although there were five days with mean daily temperatures below 10°C in September) and from 26 August in 1994. Although winter rainfall did not consistently exceed 50 mm per month in winter 1993, July and September had rainfalls exceeding 100 mm in each month, and the wet weather continued into spring. The rainfall in September commenced with a heavy fall on the second day of the month (46 mm). In December 1993, 58 mm of rainfall was recorded but it fell on six separate days and did not produce persistently wet pasture conditions. Total rainfall for 1993 was 793 mm.

The pasture conditions present in December 1993 are shown in the photograph in Plate J.

A drought occurred in the region during 1994. Total annual rainfall was 377 mm of which 106 mm was recorded in February. Autumn rainfall (71 mm in March, April and May) was insufficient to produce significant pasture growth, and winter rainfall was 75 mm.

Rainfall and temperature data are shown for property T, the second site, in Figure 5.1b. Although the property was unseasonably dry there had been more rain than at property K and the sheep were paddocked in a low lying area for the remaining four months of the study. Pasture conditions were moist when the sheep arrived in September, and over 50 mm of rain fell in each of October and November.

5.3.2. Establishment of infection

5.3.2.1. Virulent donors ·

Eighteen of the 20 virulent donor sheep developed lesions typical of footrot following artificial infection. Fourteen of these had two feet affected. Twelve sheep had at least one foot with a score 3 lesion and one sheep had a foot with a score 4 lesion. On 19 August, samples were collected from one foot of each of ten sheep and six of the samples were positive for *D nodosus* serogroup A. Despite the introduction of the donors known to be infected with virulent footrot (strain VCS1001), this strain apparently did not transmit to other sheep in the flock and was not isolated from the flock at property K despite repeated attempts.

5.3.2.2. Intermediate donors

The 18 wethers which were used as a source of intermediate footrot had a high proportion of severe lesions when they were selected as donors. The two sheep which were most severely affected were euthanased soon after arrival at property K. Of the 16 remainder, there were 12, 11, 6, 8, 8 and 7 with score 3 or score 4 lesions on each of the six inspections between 20 September and 15 December inclusive. These sheep maintained severe lesions throughout the first transmission period despite topical treatment with 10% zinc sulphate at each of the last three inspections (Table 5.4).

Table 5.4   Footscores of intermediate donors

ID #	20 Sep 1993	6 Oct 1993	4 Nov 1993	18 Nov 1993	1 Dec 1993	15 Dec 1993
109	0044	0012	0022	0022	0022	0022
114	0000	0000	0000	0022	0010	0000
131	3042	0003	2323	4223	----	2322
167	0023	2013	1022	2022	2222	0002
180	0000	0000	----	0202	0202	0000
194	0043	0043	0022	0034	0032	0004
233	0004	0004	0022	2012	2222	0000
285	3033	0223	----	3233	3222	0040
372	0023	2023	2222	2222	2222	0030
374	0044	2003	2023	2023	2224	0022
379	4444	0022	3323	----	2344	3332
380	0000	0000	0000	0022	0022	0000
387	2333	2322	3333	4333	3334	3222
397	0022	0023	2133	2234	2233	0044
398	0022	0023	2133	2234	2233	0044
403	0333	3342	2233	3333	2343	0222

5.3.3.   Efficacy of control measures

The numbers of sheep affected with footrot in at least one foot at each of the 14 inspections are shown in Table 5.5. In the 60 CON group sheep, 76% of the sheep inspection events revealed no footrot lesions. In the INT and VIR groups, 54% of the 2 352 sheep-inspections (group INT) and 52% of the 2 450 sheep-inspections (group VIR) revealed sheep with no footrot lesions.

Chi-squared tests of independence were used to compare the prevalence of affected sheep in groups VIR and INT at each of the 14 inspections. When the virulent donors were excluded from the analysis at the second inspection, none of the differences in prevalence at any inspection was significantly different at the 5% level or lower, with χ^2 values between 0.003 and 3.27. The latter value, for the thirteenth inspection in week 79, was weakly significant (P<0.1). Because 13 of the 14 inspections revealed no significant difference in footrot prevalence between INT and VIR groups, they have been considered one for the purposes of further analysis. It was necessary to exclude the virulent donors from analysis at the second inspection because these sheep had been returned to the field site after artificial infection only 30 days before the second inspection, and seven of them had score 4 lesions at that time.

Table 5.5 **Number of occasions at which affected sheep were identified at all 14 inspections (percentage of group in parentheses)**

	Group		
	CON	**I**	**V**
Score 0/1	642 (76%)	1259 (54%)	1278 (52%)
Score 2	137 (16%)	756 (32%)	809 (33%)
Score 3	26 (3%)	167 (7%)	227 (9%)
Score 4	2 (<1%)	20 (1%)	27 (1%)
Missing	_33 (4%)_	_150 (6%)_	_109 (4%)_
Total	840	2352	2450

5.3.4.   Footrot prevalence

5.3.4.1   Flock 1

In flock 1, the prevalence of affected sheep was low (5.6%) at the first inspection (21 June 1993) one month after the sheep arrived at property K from property B. The prevalence was still low three months later at the second inspection in early spring (Table 5.6, Figure 5.2). Over the course of the next four inspections during spring, the prevalence of affected sheep rose progressively to 81% in 72 days. At the second inspection, seven virulent donors had score 4 lesions and it appeared at this time that virulent footrot had been successfully introduced into the flock from the challenge pens at Camden. These seven sheep have been excluded from prevalence figures for this inspection only; they did not have score 4 lesions at the next inspection and, thereafter, have been considered part of the flock for analytical purposes. Apart from these seven in week 18, the number of sheep with score 4 lesions remained low (fewer than 3% of the flock) until April and most of the increase in prevalence was in the form of score 2 lesions (to 66% of the flock on 1 December, Figure 5.3) and score 3 lesions (a maximum of 22% of the flock in mid-November). The prevalence of affected sheep declined over the following nine months although there was an increase in the number of sheep with score 3 and score 4 lesions in early April which was not matched by an increase in score 2 lesions.

In the second spring, the prevalence of affected sheep in flock 1 rose following the move to property T, with the highest rate of affected sheep detected in late November 1994 (61%, Figure 5.2). No score 4 lesions occurred in the spring - early summer period of 1994-95 and the prevalence of sheep with score 3 lesions remained lower than in the spring of 1993.

The frequency of affected foot episodes was not normally distributed, with 28% of the INT and VIR groups having 50% of the affected foot episodes. Median and mean values for the AFE indices are shown in Table 5.8. CON group sheep were excluded from AFE values used to calculate prevalence data but included when comparing footrot indices to production traits. The median value for AFE1-14 in the 142 sheep in groups INT and VIR was 12.2; 71 sheep had an AFE1-14 score equal to or less than 11.8, 71 had a score equal to or greater than 12.5.

Fourteen sheep (the highest decile) had an AFE1-14 greater than 35 and one sheep had a score of 48.

By classifying sheep into one of two categories based on their total AFEs (AFE1-14), some inferences about differences in susceptibility between individuals were made. Thus, sheep with fewer than the median value formed the *low prevalence sheep*, those with more than the median value formed the *high prevalence sheep*.

A high total AFE (AFE1-14) is likely to be a result of some sheep having either more feet affected per inspection or feet affected for a greater duration of time, or both. The latter was found to be the case; high prevalence sheep were, on average, affected in 2.1 feet per inspection and were found to be affected on 9.1 inspections (out of 14 possible). The values for low prevalence sheep were 1.0 feet, and 3.5 inspections (Figure 5.5).

The extended duration of time for which high prevalence sheep were affected was due to a higher incidence in the early weeks of the outbreak and a more prolonged recovery at the end. This group became affected at the rate of 1.5% of sheep per day between weeks 18 and 25, while the low prevalence group became affected at the rate of 0.5% per day. Between weeks 31 and 36, high prevalence sheep recovered at the rate of 0.1% of affected sheep per day, and low prevalence sheep at 0.5% per day.

High prevalence sheep had more severe footrot lesions than low prevalence sheep. At the peak of the first outbreak, in weeks 27, 29 and 31, 34%, 22% and 24% of high prevalence sheep had score 3 or 4 lesions, while 12%, 10% and 16% of low prevalence sheep had lesions of that severity. The differences were significant for weeks 27 (χ^2=8.84, P<0.005) and 29 (χ^2=3.84, P<0.05), but not for week 31. There was an outbreak of severe lesions in week 46 (5 April 1994) which involved almost exclusively sheep in the high prevalence group.

5.3.4.2 Flock 2

In flock 2, no sheep had footrot·lesions when first inspected in winter 1993 but transmission to this flock from flock 1 had occurred by 20 September. The increase in prevalence of affected sheep in flock 2 was similar to that in flock 1 and the short period of increased prevalence of severe lesions in April 1994 also matched that of flock 1. The seasonal peak prevalences of 72% in December 1993 and 35% in late November 1994 were lower than those of flock 1 (82% and 61%) and both differences were significant (December 1993, 82% > 72%, $P<0.05$; November 1994, 61% > 35%, $P=0.005$). The highest number of sheep with score 4 lesions was eight (4%) in December 1993, the second highest was four (2%) on 5 April 1994.

The distribution of affected foot episodes for flock 2 was similar to that of flock 1, although the median (9.2) and mean (12.7) values for AFE1-14 were less than that for flock 1 (Table 5.7). Classification of flock 2 into low and high prevalence groups based on the median value for AFE1-14 led to results similar to those for flock 1. The incidence between weeks 18 and 25 was 1.1% and 0.3% per day for the high and low prevalence sheep respectively. Recovery rates between weeks 31 and 36, were 0.1% and 0.9% of affected sheep per day for high and low prevalence sheep respectively.

As in flock 1, high prevalence sheep had more severe footrot lesions than low prevalence sheep. At the peak of the first outbreak, in weeks 27, 29 and 31, 30%, 17% and 41% of high prevalence sheep had score 3 or 4 lesions, while 9%, 8% and 15% of low prevalence sheep had lesions of that severity (Figure 5.5). The differences were significant for weeks 27 ($\chi^2=14.04$, $P<0.001$), and 31 ($\chi^2=16.78$, $P<0.001$) but only weakly significant for week 29 ($\chi^2=3.70$, $P<0.1$).

Table 5.6 Prevalence of footrot at property K in groups VIR and INT combined

Date inspected	Week	Flock 1					Flock 2				
		Total inspected	Total affected	Score 2	Score 3	Score 4	Total inspected	Total affected	Score 2	Score 3	Score 4
21 Jun 93	6	142	8 (6%)	3 (2%)	2 (1%)	3 (2%)	201	0 (0%)	0 (0%)	0 (0%)	0 (0%)
20 Sep 93	18	134	21 (10%)	9 (6%)	3 (2%)	2 (1%)**	198	30 (15%)	8 (4%)	22 (11%)	1 (1%)***
06 Oct 93	21	138	36 (26%)	26 (19%)	8 (6%)	2 (1%)	198	41 (21%)	21 (11%)	18 (9%)	2 (1%)
04 Nov 93	25	140	75 (54%)	55 (39%)	20 (14%)	0 (0%)	200	97 (48%)	80 (40%)	16 (8%)	1 (1%)
17 Nov 93	27	139	109 (78%)	77 (55%)	30 (22%)	2(1%)	199	139 (70%)	102 (51%)	35 (18%)	2 (1%)
01 Dec 93	29	140	115 (82%)	92 (66%)	22 (16%)	1 (1%)	197	149 (76%)	128 (65%)	21 (11%)	0 (0%)
15 Dec 93	31	138	113 (82%)	86 (62%)	27 (20%)	0 (0%)	195	154 (79%)	104 (53%)	42 (22%)	8 (4%)
26 Jan 94	36	136	95 (70%)	86 (63%)	9 (7%)	0 (0%)	193	123 (64%)	110 (57%)	13 (7%)	0 (0%)
05 Apr 94	46	112	62 (55%)	34 (30%)	21 (19%)	7 (6%)	194	102 (53%)	54 (28%)	44 (23%)	4 (2%)
04 May 94	50	132	76 (58%)	62 (47%)	13 (10%)	1 (1%)	192	87 (45%)	77 (40%)	10 (5%)	0 (0%)
19 Aug 94	65	125	28 (22%)	27 (22%)	1 (1%)	0 (0%)	189	28 (15%)	27 (14%)	0 (0%)	1 (0%)
19 Sep 94	69	121	21 (17%)	20 (17%)	1 (1%)	0 (0%)	189	29 (15%)	25 (13%)	4 (2%)	0 (0%)
23 Nov 94	79	118	73 (62%)	71 (60%)	2 (2%)	0 (0%)	185	69 (37%)	66 (36%)	3 (2%)	0 (0%)
18 Jan 95	86	111	61 (55%)	56 (50%)	5 (5%)	0 (0%)	177	61 (34%)	59 (33%)	2 (1%)	0 (0%)

** 7 Virulent donors had score 4 lesions and were excluded from the data in week 18

*** 3 Virulent donors had score 4 lesions and were excluded from the data in week 18

Table 5.7   Prevalence of footrot, CON group, flocks 1 & 2 combined

Date inspected	Week	Total inspected	Total affected	Score 2	Score 3	Score 4
21 Jun 93	6	59	1 (2%)	1 (2%)	0 (0%)	0 (0%)
20 Sep 93	18	60	3 (5%)	2 (1%)	1 (2%)	0 (0%)
06 Oct 93	21	60	8 (13%)	5 (8%)	3 (5%)	0 (0%)
04 Nov 93	25	60	21 (35%)	18 (30%)	3 (5%)	0 (0%)
17 Nov 93	27	57	28 (49%)	19 (33%)	9 (16%)	0 (0%)
01 Dec 93	29	59	36 (61%)	30 (51%)	5 (8%)	1 (2%)
15 Dec 93	31	59	18 (31%)	14 (24%)	3 (5%)	1 (2%)
26 Jan 94	36	59	6 (10%)	6 (10%)	0 (0%)	0 (0%)
05 Apr 94	46	57	8 (14%)	8 (14%)	0 (0%)	0 (0%)
04 May 94	50	58	4 (7%)	3 (5%)	1 (2%)	0 (0%)
19 Aug 94	65	58	1 (2%)	1 (2%)	0 (0%)	0 (0%)
19 Sep 94	69	57	0 (0%)	0 (60%)	0 (%)	0 (0%)
23 Nov 94	79	55	21 (38%)	21 (38%)	0 (0%)	0 (0%)
18 Jan 95	86	49	10 (20%)	9 (18%)	1(2%)	0 (0%)

Table 5.8   Values for footrot indices in treatment groups INT and VIR (combined).

Index	Period of index	Max^m possible score	Median value		Mean value	
			n=142	n=201	n=142	n=201
			Flock 1	Flock 2	Flock 1	Flock 2
AFE1	Week 6 only	4	0.0	0.0	0.1	0.0
AFE2-6	Weeks 18 - 29	20	4.0	4.0	5.4	5.0
AFE2-8	Weeks 18 - 36	28	8.0	7.0	9.1	8.1
AFE9-13	Weeks 46 to 79	20	2.5	1.0	4.2	3.3
AFE9-14	Weeks 46 to 86	28	3.5	1.2	6.0	4.6
AFE1-14	Weeks 6 to 86	60	12.2	9.2	15.2	12.7

5.3.5.   Isolation of *D nodosus* and PCR-RFLP typing of isolates

In reporting results of isolations, where the same serogroup was recovered more than once from one sheep it is considered one isolation. If, however, isolates of more than one serogroup are recovered an isolate of each serogroup is reported.

A sample of 50 sheep from flock 1 was examined on 7 June 1993, eight days before flock 2 sheep arrived at property K. Four isolates were made from lesion samples taken from three sheep and freeze dried. Two of these were serogroup H, one was serogroup A and one was serogroup I, which had not been identified previously in the flock (Chapter 4). Tube agglutination tests were used to confirm the serogrouping of the latter two isolates. The serogroup A isolate was serotype A_1.

There were 23 isolates from 22 sheep made from samples collected on 20 September 1993. Sixteen of these were from sheep in group VIR, two of which were virulent donors. None of the isolates was serogroup A (Table 5.9a). Isolates of serogroup B, C and H were identified, in common with isolations made from properties W and B (Chapter 4) but five isolates were identified as serogroup I. Most of the sheep sampled were from flock 2 and all four serogroups were isolated from them, with H predominant. Serogroups H, I and C were also isolated from three flock 1 sheep.

There were 24 isolates made from 24 sheep sampled on 17 November. Serogroups C, H and I were isolated from flock 1 and B, C, H and I from flock 2. Serogroup H predominated (Table 5.9b).

Group VIR sheep from flock 2 were sampled again in January in a last attempt to re-isolate VCS1001. Four serogroups were isolated (B, C, H and I); serogroup H was the most common (Table 5.9c).

Intermediate donors were sampled for culture on several occasions during the study. The isolates typed, and their collection date, were serogroup A (7 June 1993), B and H (20

September 1993), I (6 October 1993) and B (18 November 1993).

RFLP typing was performed on '12 of the isolates which were included in the freeze dry collection. The isolates typed included the range of five serogroups identified in the study and covered the time period from June 1993 to January 1995. The results are shown in Table 5.10. The serogroup A isolate, detected in one sheep in June 1993, before the virulent donors were artificially challenged with VCS1001, was of pattern 1a. An isolate of serogroup A collected at property B in October 1992 was also of pattern 1a (Chapter 4). (The RFLP pattern of VCS1001 is distinct from pattern 1a; see Chapter 7). Two new patterns were found. One isolate of serogroup I tested was of a pattern not seen before at property B or property W, consistent with the fact that the serogroup had not previously been detected. This pattern was called pattern 6. The other serogroup I isolate subjected to RFLP typing was of pattern 4, seen before in isolates of serogroup H and C, collected at property B in 1992. Pattern 6 was seen again in an isolate of serogroup B collected from a flock 1 sheep after the flock had moved to property T. All six patterns other than pattern 6 that were identified at properties K and T had been identified previously at property B. Of the eight patterns identified at property B, only two (1b and 1c) were not seen in isolates collected at properties K and T.

Tube agglutination tests conducted on the first isolate of serogroup I detected and the only serogroup A isolate confirmed their serogroup. The serogroup A isolate was serotype A_1.

Table 5.9   Isolations of *D nodosus* by treatment group and by serogroup

(a)   20 September 1993

Treatment	Serogroup				
group	B	C	H	I	Total
CON	1		1		2
INT		1	2		3
INT D	1		1		2
VIR	3	3	5	5	16
Total	5	4	9	5	23

(b)   16-17 November 1993

Treatment	Serogroup				
group	B	C	H	I	Total
CON		4			4
INT			5	1	6
INT D	1				1
VIR	1	2	5	5	13
Total	2	6	10	6	24

(c)   26-27 January 1995

Treatment	Serogroup				
group	B	C	H	I	Total
VIR	1	3	5	2	11

Table 5.10 PCR-RFLP pattern (*omp* gene) of selected serogroups collected at property K and property T

RFLP pattern	Serogroup					
	A	B	C	H	I	Total
1			1			1
1a	1					1
2			1	1		2
4					1	1
5		1		2		3
6		1			1	2
7				4		4
Total	1	2	2	7	2	14

5.3.6. Relationship between footrot indices

Sheep with AFE2-8 and AFE9-14 values of zero in both years were omitted from the flock 2 data set before log transformation. Data from all sheep in flock 1 were used. The relationship of AFE2-8 and AFE9-14 with AFE1 were significant at the 0.1 level and 0.05 level respectively. There was a significantly lower intercept for CON compared to VIR and INT for AFE2-8 ($P<0.05$) and for AFE9-14 ($P<0.01$). There was no significant interaction of AFE1 with treatment for either index.

The relationship between AFE2-8 and AFE9-14 was highly significant ($P<0.001$) but there was significant difference in intercepts ($P<0.05$) and slopes ($P<0.01$) between CON and the other two groups. There was no effect of flock or interaction of flock with treatment. The regression coefficients for VIR and INT were close to 1.0, which suggests that AFE9-14 values for both groups were roughly proportional (72% and 73% respectively) to the corresponding AFE2-8 values.

5.3.7. Effects on production

The CON group were excluded from analysis of the results of footrot prevalence because treatment with footbathing and vaccine had contributed to a lower prevalence and a lesser severity of lesions in that group compared to the INT and VIR groups. The CON group have, however, been included in the examination of the relationship between foot lesions and production traits on the basis that the treatment received by the CON group had an effect on foot lesions but no direct effect on production.

5.3.7.1. Bodyweight

Bodyweights were not recorded at inspections 3, 9 and 14. The mean bodyweight of flock 1 at the beginning of the study (week 6) was 47.8 kg and of flock 2 was 41.5 kg (P<.005). The bodyweight of both flocks increased through the winter and spring period of 1993 and reached a peak of 61.0 kg (flock 1) at the eighth inspection (WT8), in late January 1994 and 55.5 kg (flock 2) at the seventh inspection, in mid-December (Figure 5.6). Mean bodyweights declined over the subsequent seven months to a low of 46.5 kg (flock 1) and 43.0 kg (flock 2), increased by around one kg in early September before the sheep were transported to property T, then gained approximately 10 kg in the following two months, which was the final weight recording (WT13), performed in week 79.

In year 1, bodyweight in week 29 (WT6), when the prevalence of footrot was highest, was examined in relation to the footrot index for the period from week 18 to week 29 (AFE2-6) and bodyweight in week 36 (WT8), the highest bodyweight for the season, was examined with respect to AFE2-8, the footrot index for the period from week 18 to 36. In the second year, WT13 was examined with respect to AFE9-13, the footrot index for the period from week 46 to week 79.

There were strong negative relationships (P<0.001) between bodyweight and footrot index in both years (Table 5.11). The slope was shallower for flock 2 for WT8 (P<0.05) but for WT6 and WT13 there was no significant difference in the slope between flocks, and a common slope

was derived. The intercept was lower for flock 2 for WT6, WT8 and WT13 (P<0.001) consistent with the lower initial weight of the flock 2 sheep.

WT6 and WT13 represented the bodyweights of the sheep at the peaks of the outbreaks in years 1 and 2 respectively. For both flocks in both years, the slope of the relationship between footrot index and bodyweight at that time was -0.46, implying a loss of bodyweight of 0.46 kg for every affected foot episode detected in the preceding transmission period. The mean values for AFE2-6 and AFE9-13 were 5.4 and 5.0 respectively, suggesting an average depression of bodyweight across the flocks of 2.5 kg (year 1) and 2.3 kg (year 2) as a consequence of footrot.

Table 5.11   Relationship between footrot index and bodyweight

Bodyweight measure	Footrot index	Slope (±se)	Intercept	100 x r^2
WT6	AFE2-6	-0.46±0.06 ***	Flock 1 62.6 ***	25.7
			Flock 2 56.5	
WT8	AFE2-8	Flock 1 -0.51±0.07 ***	Flock 1 65.4 ***	27.9
		Flock 2 -0.28±0.06 ***	Flock 2 56.2	
WT13	AFE9-13	-0.46±0.07 ***	Flock 1 59.1 ***	19.1
			Flock 2 55.3	

*** P<0.001

For WT8, the bodyweight in late January and the highest mean bodyweight recorded in the study for flock 1, there were significantly different relationships (P<.05) between flock and footrot index for the two flocks. The predicted effect of one AFE was to reduce bodyweight by 0.51 kg (flock 1) or 0.28 kg (flock 2).

The r^2 values (Table 5.11) are the squared correlation coefficients of each of the relationships and indicate the percentage of the variation in each measure of bodyweight which is explained by variation in footrot index. Considering the large number of factors which can variably

influence the bodyweight of sheep at pasture, the values of 19.1% to 27.9% are relatively high, which suggests that footrot severity, as measured by footrot index was an important contributor to the variation of bodyweight within flocks.

5.3.7.2.   Wool production at first shearing (GFW1, CFW, FD, YLD)

For the relationship between footrot index AFE2-8 and greasy fleece weight at first shearing (GFW1), the slopes for both flocks were negative but significantly different from zero for flock 1 only (P<0.05). As there was no significant interaction between flock and AFE2-8 affecting the slope of the relationship with GFW1, a common slope was fitted for both flocks which was statistically significant (P<0.01) (Table 5.12). The flock differences in intercept were highly significant (P<0.001).

Table 5.12 Relationship between footrot index and wool production

Wool production measure	Footrot index	Slope (±se)	Intercept	$100 \times r^2$
GFW1	AFE2-8	-0.013±0.005 **	Flock 1 5.80 *** Flock 2 5.40	9.6
Y	AFE2-8	-0.051±0.031 (P<0.1)	78.0	0.8
FD	AFE2-8	-0.029±0.014 *	22.11	1.2
CFW	AFE2-8	-0.009±0.004 *	Flock 1 4.55 *** Flock 2 4.18	11.6
GFW2	AFE9-14	-0.015±0.004 ***	Flock 1 4.30 *** Flock 2 3.87	19.1

*** P<0.001 ** P<0.01 * P<0.05

There were no significant flock effects on the intercepts or the slopes of the relationship between AFE2-8 and YLD or FD, so the data from the two flocks were combined. For each trait, the slope was negative. The slope for FD was significant at the 0.05 level, but for YLD was only significant at the 0.1 level.

There was no significant difference in the interaction of flock and AFE2-8 on CFW. The flock difference in intercept for CFW was highly significant (P<0.001) and the combined slope was significantly different from zero (P<0.05) (Table 5.12).

The mean value for AFE2-8 was 9.1 (flock 1) and 8.1 (flock 2). The predicted effect of the footrot outbreak in year 1 is a depression of greasy fleece weight of 118 g and 105 g, 82 g and 73 g of clean fleece weight, and of 0.26 and 0.23 micrometres of fibre diameter, for flocks 1 and 2 respectively.

5.3.7.3.   Greasy fleece weight at second shearing (GFW2)

For greasy fleece weight in year 2, there was no significant interaction between flock and AFE9-14. The slope of the relationship for the combined flocks was highly significant (P<0.001) and similar to the slope for GFW1 (0.015, cf 0.013 for GFW1)

Flock had a highly significant effect on intercept for GFW2 (P<0.001).

Plate I

On property K, sheep were weighed on 11 occasions and footscored on 14 occasions. Inspections were carried out on a sheep handler (illustrated in Plate C) or by dragging sheep 'over the board' in the woolshed.

Plate J

TOP   Property K on 15 December 1993, looking south towards the woolshed and sheepyards. The inspection at this time found the prevalence of footrot was at a seasonal peak for flock 2, and 2 weeks after the seasonal peak in flock 1. This inspection also coincided with the highest bodyweight recorded for flock 2.

BOTTOM   Property K on 26 January 1994, looking west from the woolshed. Following a period of hot dry weather pastures had dried out substantially since December.
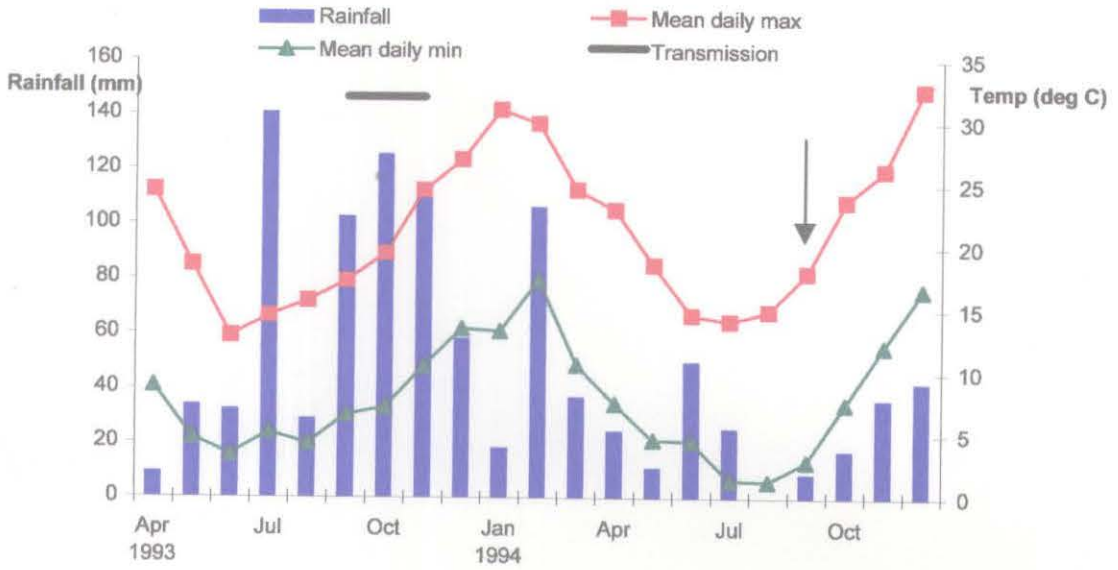
Figure 5.1(a) Mean monthly rainfall, Tarcutta, and mean monthly maximum and minimum temperatures at Wagga Wagga. The expected footrot transmission period, predicted from the report of Graham and Egerton (1968), is shown as a solid black line. 1994 was a drought year and the sheep were transferred to property T, near Holbrook, in September 1994 (arrowed).
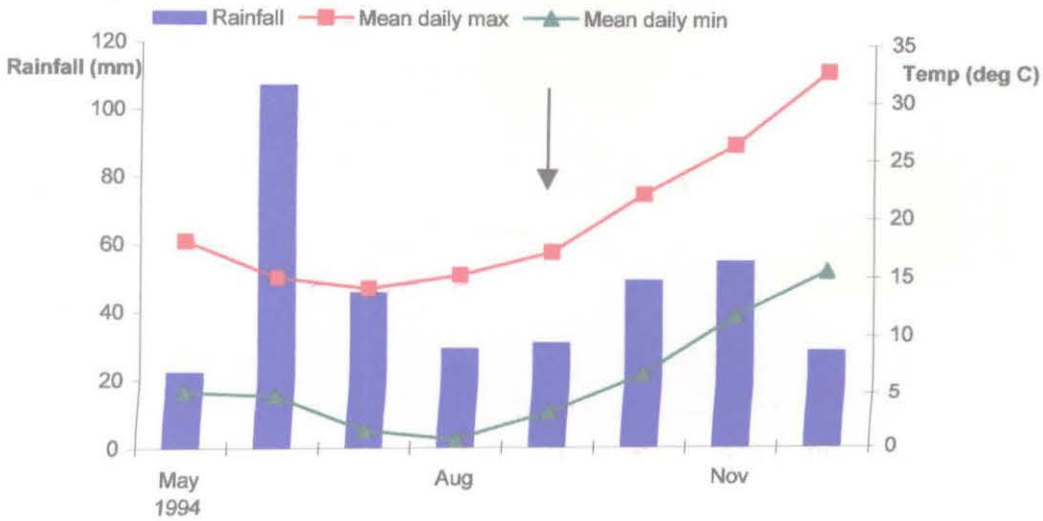
Figure 5.1(b) Mean monthly rainfall, Holbrook, and mean monthly maximum and minimum temperatures at Albury. Property T is near Holbrook, in south-eastern New South Wales. The sheep were transferred in September 1994 (arrowed).
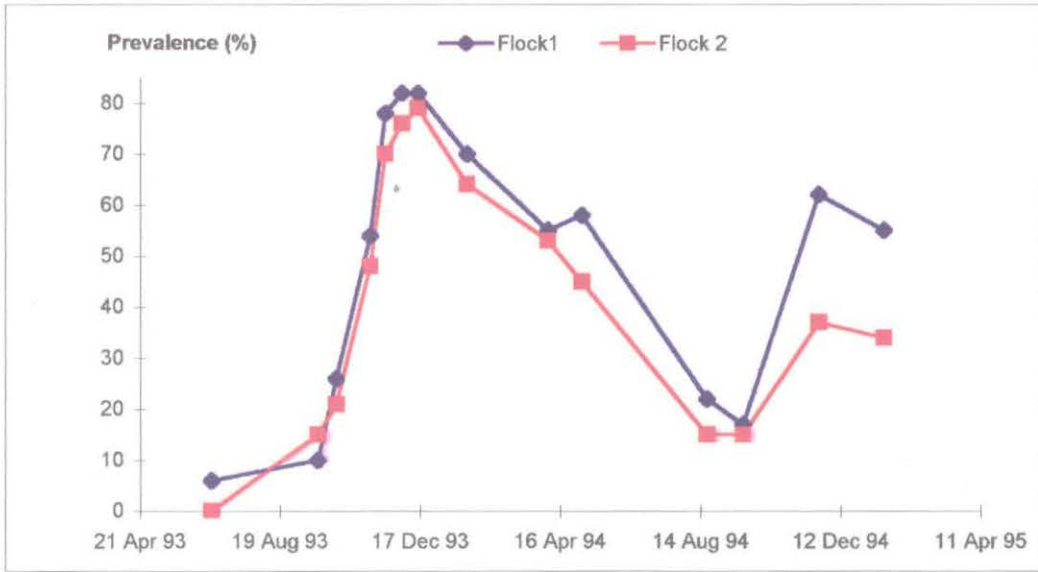
Figure 5.2 Prevalence of footrot in both flocks at property K, Tarcutta. Flock 1 was moved from property B and included infected donor sheep from the flock described in Chapter 4. Footrot was endemic in the flock although prevalence was low and lesions were mild, except in the donors, when the sheep arrived at property K. Flock 2 was free of footrot when it arrived at property K but, by the time of the second inspection on 20 September 1993, footrot was well established. The prevalence in the two flocks remained similar throughout the study period despite different exposure histories, different ages and different genetic backgrounds.
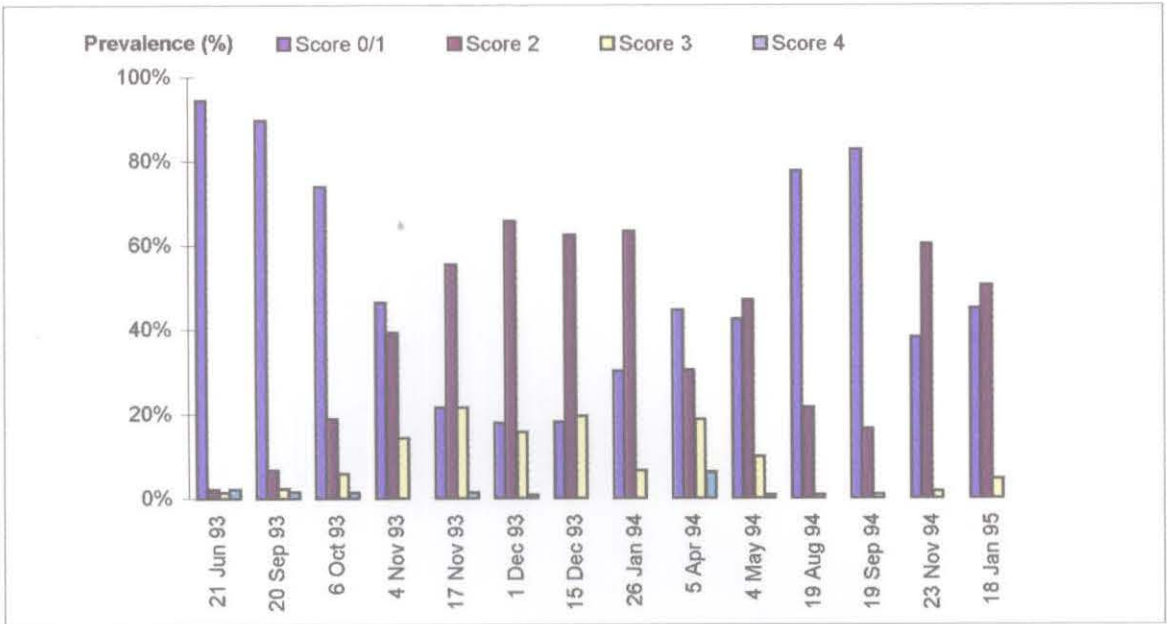
Figure 5.3 Flock 1; prevalence of lesions of each score category. The prevalence of footrot was 5.6% at the first inspection in June 1993. The highest prevalence of sheep with score 3 lesions was 22%, in mid-November 1993; the highest prevalence of sheep with score 4 lesions was 6% in April 1994.
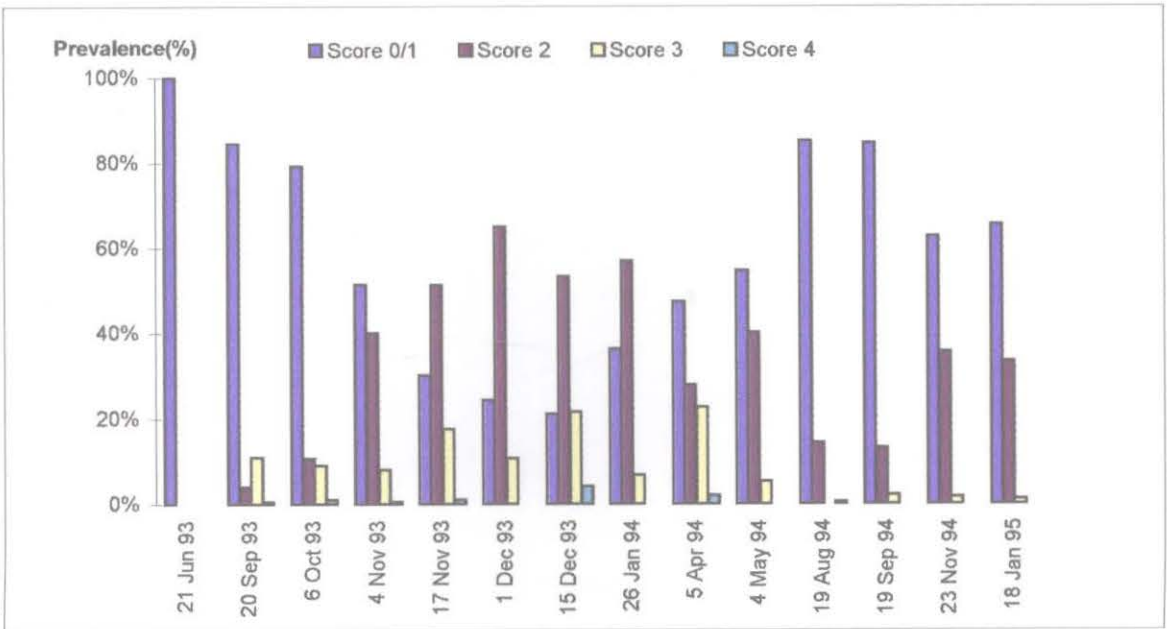
Figure 5.4 Flock 2; prevalence of lesions of each score category. No sheep had footrot lesions when first inspected but transmission from flock 1 and the inter-mediate donors had occurred by 20 September 1993. The highest number of sheep with score 4 lesions was eight (4%) in December 1993.

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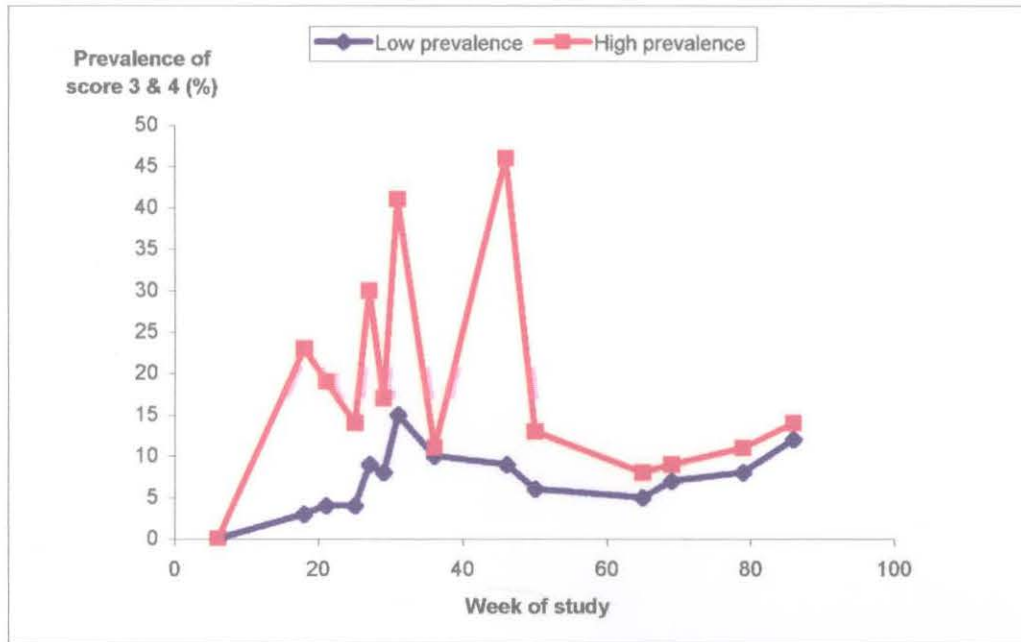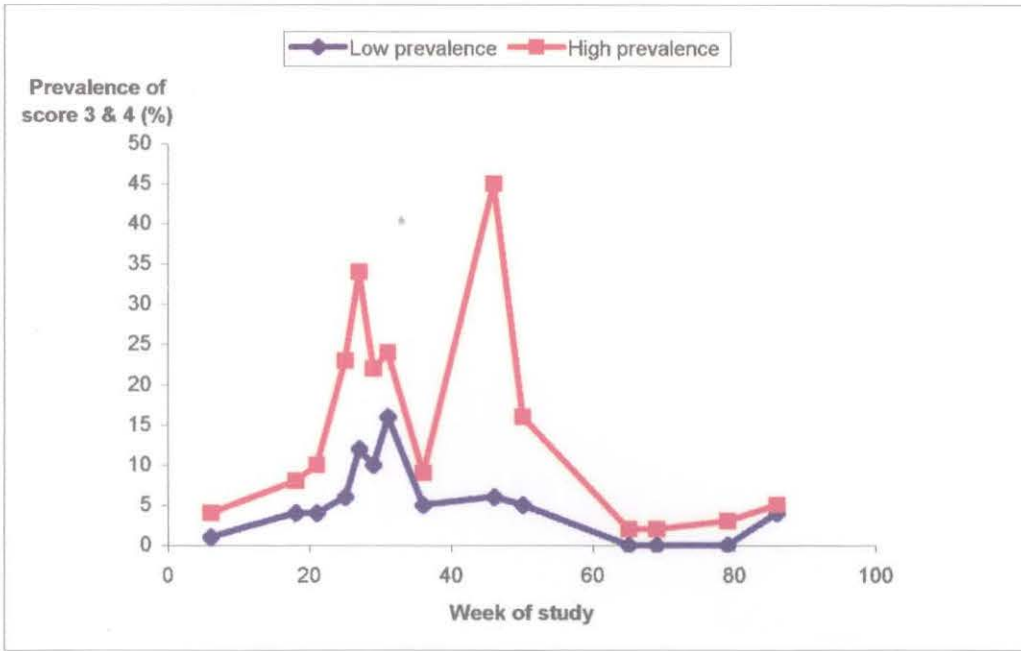
Figure 5.5 Prevalence of sheep with score 3 and 4 lesions after categorisation into low and high prevalence groups; flock 1 top, flock 2 bottom. Sheep in the high prevalence group of each flock had more feet affected and more persistent lesions and also had more severe lesions than sheep in the low prevalence group. The outbreak of severe lesions in week 46 involved almost exclusively the sheep in the high prevalence group.
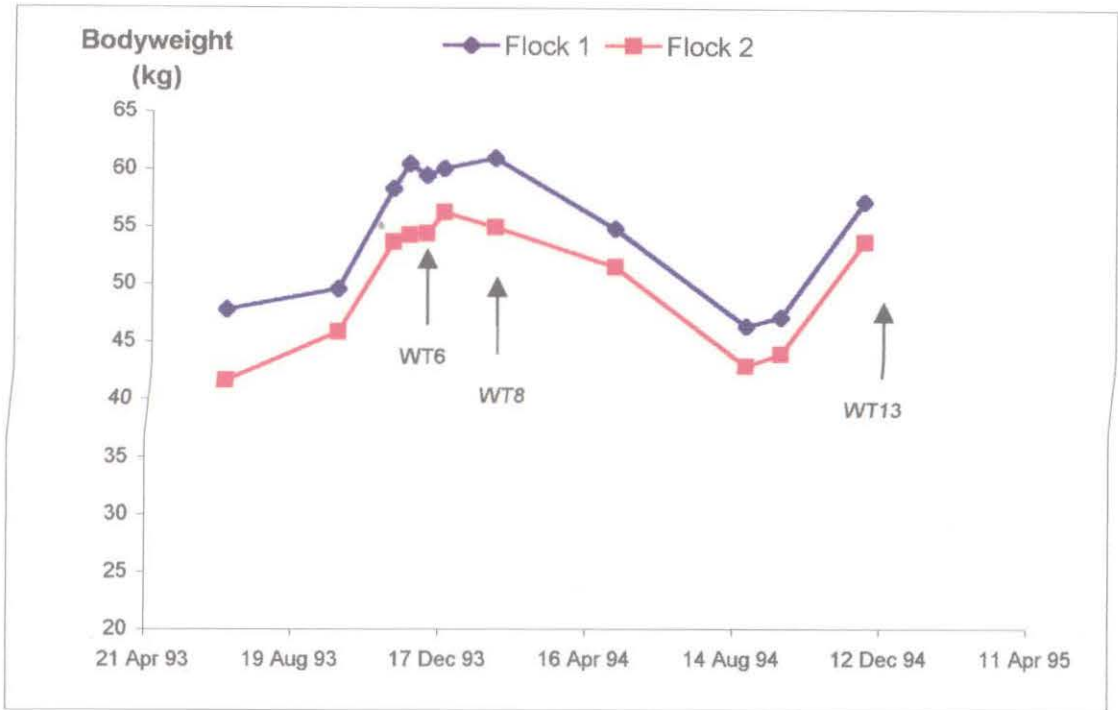
Figure 5.6 Bodyweights of flock 1 and flock 2 sheep, all treatment groups. The three bodyweight variables examined in the analysis of the relationship between bodyweight and the presence of footrot were WT6, WT8 and WT13. WT6 was the bodyweight of the sheep at the peak of the outbreak, WT8 was the maximum weight achieved by flock 1 sheep and WT13 was the maximum weight in year 2.

(a)

(b)

(c)
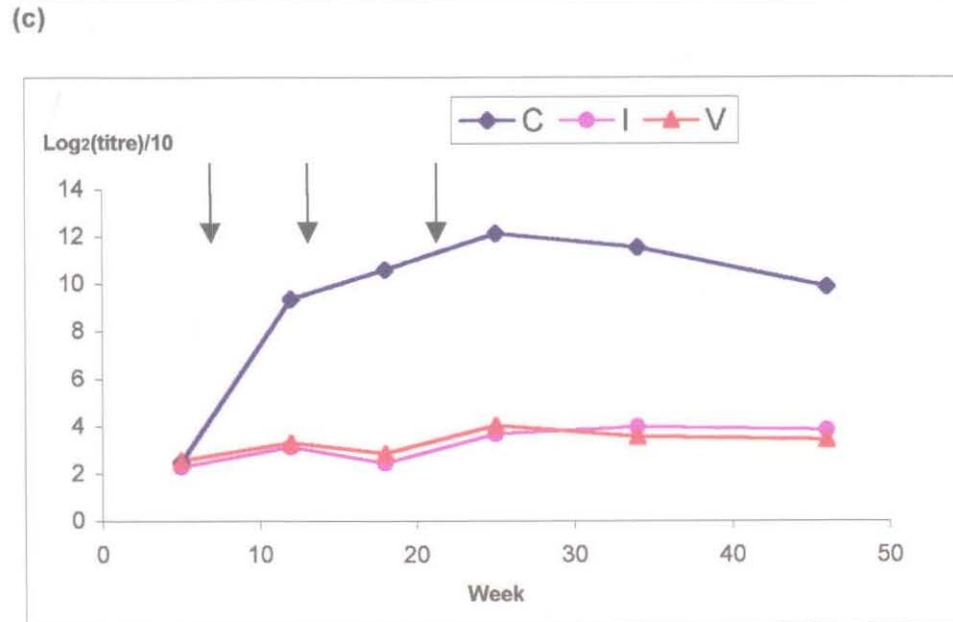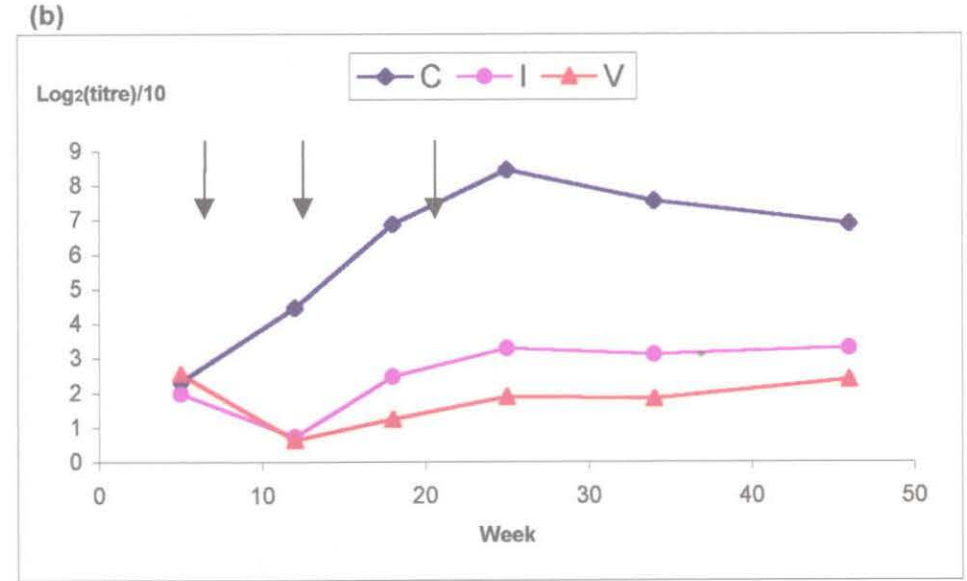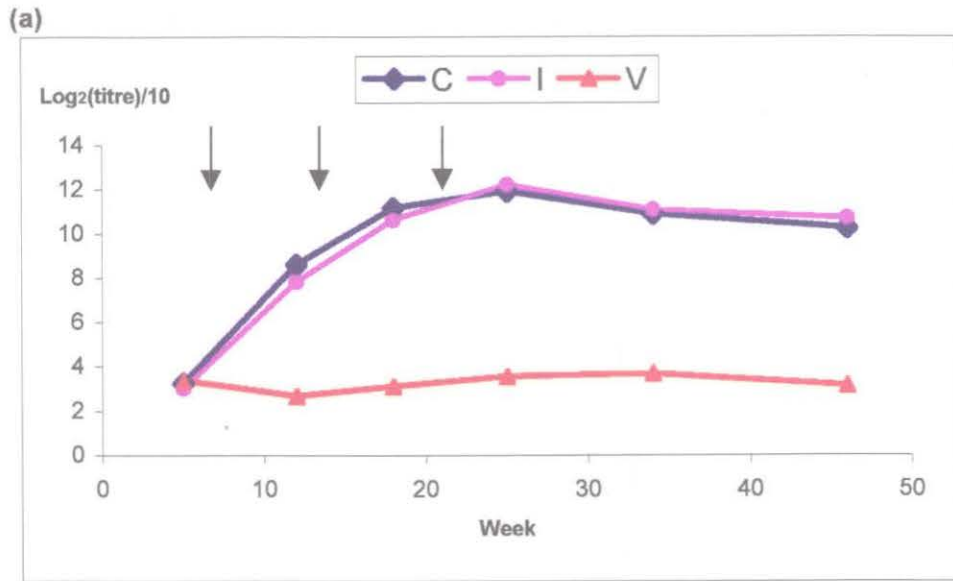
Figure 5.7 Sheep in groups CON (C) and INT (I) were vaccinated in weeks 6, 12 and 21. Group CON were vaccinated against serogroups A, B and H. Group INT were vaccinated against serogroup A. Group VIR (V) were not vaccinated. Figures illustrate agglutinating titres to (a) serogroup A, (b) serogroup B, (c) serogroup H. Arrows indicate vaccination times.

5.3.7.4. Condition and bodyweight at sale off-shears

Three days after shearing, on 20 January 1995, the flock was inspected by a licensed CALM assessor in preparation for sale. He divided the flock into 2 groups based on condition and bodyweight. The group of 107 sheep classified as 'light' was described as "80% fair average quality, 20% poor low quality". A randomly selected sample of 25 of this group had a mean liveweight of 44.5 kg.

A group of 216 'heavy' sheep was described as "80% good medium quality, 20% fair average quality". A random sample of 40 had a mean liveweight of 52.5 kg.

The classifications of sheep of each flock by sale category and footrot infection frequency are shown in Tables 5.13 and 5.14. There was a significant association between the classification into high or low prevalence group and the CALM classification into heavy or light sale category. The association was significant for both flocks; $\chi^2 = 7.6$, P<0.01 for flock 1, $\chi^2 = 11.5$, P<0.005 for flock 2.

The association between the footrot prevalence history and sale classification was also used to determine the relative risk (RR) of being in the 'light' category. For flock 1, the RR was 2.0, for flock 2 the RR was 2.06. Sheep with a history of a high prevalence of footrot during the previous season were twice as likely to be categorised as 'light' at sale as sheep with a history of low prevalence.

Table 5.13 **Number of sheep in flock 1 categorised by footrot lesions and sale description**

	'Heavy' sale category	'Light' sale category
> 12.2 affected-feet episodes	36 (27%)	30 (23%)
< 12.2 affected-feet episodes	51 (39%)	15 (11%)

Table 5.14 **Number of sheep in flock 2 categorised by footrot lesions and sale description**

	'Heavy' sale category	'Light' sale category
> 9.2 affected-feet episodes	56 (28%)	45 (22%)
< 9.2 affected-feet episodes	80 (39%)	22 (44%)

5.3.8. **Serology**

Blood samples were collected from the entire flock at the beginning of the study (week 6) and in April 1994 (week 46 of the study). The first vaccination (V1) was given to the sheep in treatment groups CON and INT when the first blood sample was taken.

On four other occasions blood samples were collected from a sample of each flock. Ten sheep were identified in each of the three treatment groups in each of the two flocks and samples were collected from these 60 sheep in weeks 12, 18, 25 and 34. Results are presented both for the 60 sheep which were sampled on all six occasions and for the entire flock, which was sampled twice (Appendix 4)

Agglutinating titres were measured against three serogroups, A, B and H. Titres to serogroup A rose markedly after V1 in the CON group and INT group sheep, which had been vaccinated with trivalent (A, B, H) and monovalent (A) respectively. Titres continued to rise after V2 and again, but less markedly, after V3 (Figure 5.7a). The pattern was similar for titres to B (Figure 5.7b) and to H (Figure 5.7c) in the CON group sheep.

Titres to A and H in sheep which were not vaccinated with those serogroups remained at low levels over the period of measurement. Titres to B in group INT sheep (vaccinated with serogroup A) rose after V2 and V3 and were significantly different from group VIR sheep in week 25 (P<0.01) but not significantly different in weeks 18 or 34.

The use of the trivalent vaccination (group CON), rather than monovalent vaccination (group INT) had no significant effect on the titre to serogroup A (Figure 5.7a)

The whole flock was sampled in weeks 5 and 46. There was no significant difference in the mean titres of the entire group or the selected sample of 20 sheep in each group at either sampling occasion. This applied to all three antigens.

5.4.   Discussion

5.4.1.   Climatic conditions

The climate at property K in 1993 was substantially warmer than the climate at property W in 1992 (see Chapter 4, Figure 4.1). Monthly minimum temperatures, recorded at Wagga Wagga in 1993, were between 4°C and 5°C higher than those recorded at Cooma in the previous year. At Cooma in 1992, the monthly average mean daily temperatures exceeded 10°C from October to April inclusive; at Wagga Wagga in 1993 the period was extended to include September and May.

The conditions of warmth and moisture likely to permit an outbreak of footrot occurred earlier in 1993 at property K than at property B or property W in 1992. Winter rainfall (June, July, August) was 100 mm at property W in 1992 and 202 mm at property K in 1993. Spring rainfall was 211 mm at property W in 1992, 340 mm at property K. The summer months, however, were drier at property K (173 mm rainfall) than property W (246 mm) and the highest rainfall occurred in February at property K after a relatively dry December and January period, in contrast to the high rainfall in December at property W. To a large extent, these differences are characteristic of the differences in the two environments; the south-western slopes with a winter-dominant rainfall pattern and dry summer, the Monaro with a summer dominant rainfall pattern. The expected transmission period for footrot, based on the environmental conditions proposed by Graham and Egerton (1968), was September to November in 1993 at property K (Figure 5.1) compared to November 1992 to March 1993 at properties B and W (Figure 4.1).

Footrot transmission started in September 1993 at property K and the prevalence peaked in early December. By contrast, the peak prevalence at property W occurred in January and, at property B, there was no evidence of transmission in September or October. The difference in the timing of the outbreaks at property K and in the Monaro is consistent with the differences in climatic conditions between the two environments.

5.4.2.   Footscore profiles within flocks

At or near the peak of the outbreak, the profile of footscores in each flock at property K and in the Monaro was very similar. Figure 5.8 compares footscore prevalence data selected from Figures 4.5, 4.6, 5.3 and 5.4. There was some difference in the prevalence of score 2 lesions, associated with less suitable conditions for transmission at property B, as discussed in Chapter 4, but the prevalence of score 3 lesions was between 15% and 21% in all flocks and of score 4 lesions was between 1% and 7%. These data support the view that the expression of intermediate footrot in the Monaro, in terms of the proportion of the flock with score 3 and score 4 lesions, was not limited by any environmental factors unique to the Monaro sites but was a consequence of the inherent limitations in virulence of the infecting strains of *D nodosus*.

In the discussion of the footscore profiles in the Monaro flocks (section 4.4.1.), it was suggested that characterising the virulence of intermediate strains of *D nodosus* by dividing the proportion of a flock with score 4 lesions by the proportion of the flock which is affected, as suggested by Allworth and Egerton (1999), may overestimate the severity of the outbreak when conditions do not encourage high levels of transmission. Data from this study at property K also support this view. First, the differences between flocks 1 and 2 at property K at the time of peak prevalence (Figures 5.3 and 5.4) show that flock 2 had a higher proportion of unaffected sheep than flock 1, yet a higher proportion had score 3 and score 4 lesions. Considering their different age and genetic background but the fact that both flocks were run together, the differences are likely to be due to differences in the interaction of sheep of each flock to the environment. Second, at times when environmental conditions led to regression of footrot prevalence, such as in April 1994, the relative proportions of score 3 and score 4 lesions increased (Figures 5.3 and 5.4). This latter phenomenon was also observed at property B, and is discussed in section 4.4.1.

In view of this observation, it is interesting to note the small increase in score 4 lesions in flock 2 at the 15 December inspection (Figure 5.4) which did not occur in flock 1. Environmental conditions were becoming unsuitable for footrot transmission during the early part of December; flock 1 had a peak prevalence on 1 December and flock 2 on 15 December.
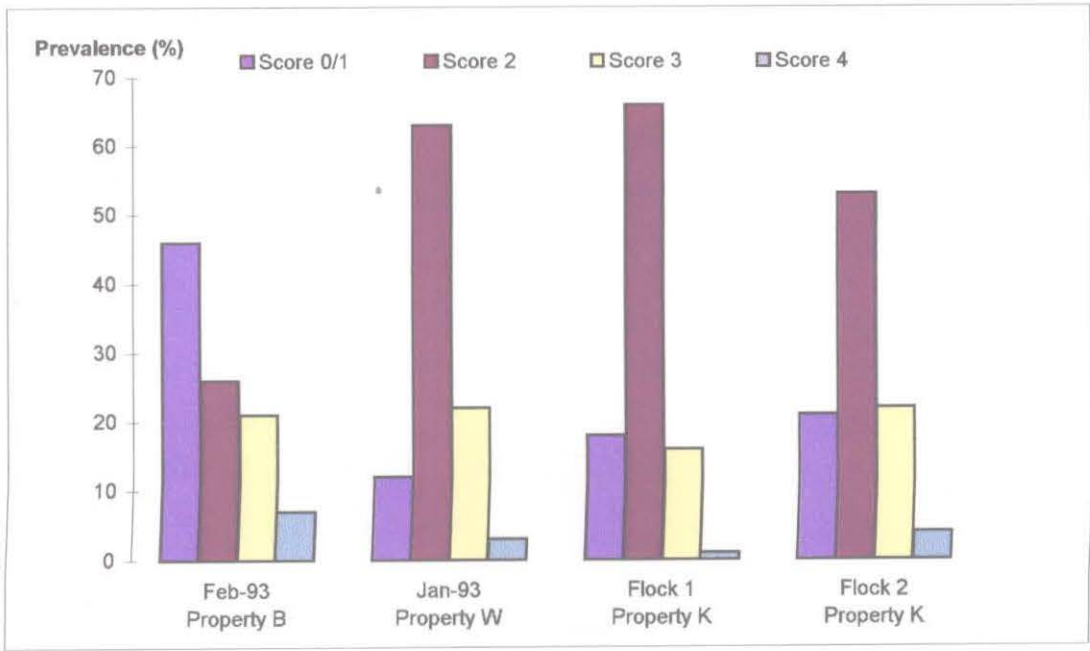
Figure 5.8 The footscore profile of intermediate footrot outbreaks in four flocks, at different times and at three sites. From left, February 1993 at property B, January 1993 at property W, 1 December 1993 for flock 1 at property K and 15 December for flock 2 at the same site. Sheep are classified by maximum footscore.

Although no definite conclusions can be drawn from this observation, it suggests that the two flocks were reacting in slightly different ways to the changing conditions and, once again, the prevalence of score 4 lesions changed in an unexpected way. These observations infer that the environmental conditions which favour transmission of footrot may, at least partly, prevent the extension of score 2 and score 3 lesions into more severely underrun lesions. As environmental conditions become drier, transmission ceases or slows, but some lesions extend, at least temporarily, before most of them regress in response to continuing dry conditions. A hypothesis that the free moisture restricts proteolytic activity was advanced in section 4.4.1, following a similar observation in the study reported in that Chapter. As environmental conditions change towards warmer, drier weather, in the short period when the feet are exposed to less free water but are still moist, bacterial activity in the feet lesions may increase. Then, as conditions continue to become drier, bacterial activity declines and healing of lesions commences in most sheep. If this proposition were true, it may also be more characteristic of intermediate footrot than virulent footrot because of the lower protease activity of intermediate strains.

In summary, it appears that, in a flock infected with intermediate footrot, the number of sheep with score 4 lesions increases and the number of sheep with score 2 lesions declines when conditions are less than fully favourable for footrot transmission compared to the numbers in each category under more favourable conditions. This observation has implications for diagnostic classification of outbreaks based on the relative proportion of sheep with score 4 lesions to affected sheep in a flock (Egerton 1989, Allworth and Egerton 1999). It also has implications for pen studies of footrot used to assess strain virulence (Stewart *et al* 1995). If, for example, conditions in the pens mimic the field conditions which suit lesion progression, rather than transmission, the proportion of artificially infected sheep which develop severe lesions may be overestimated in comparison to the proportion which develop severe lesions for more than one or two weeks in the field.

The recommendations arising from these studies concerning the most reliable approach to classification of natural footrot outbreaks are that

- the ratio of sheep with score 4 lesions to *total sheep exposed*, rather than *total sheep affected*, should be used to categorise outbreaks

- where the proportion of the flock which is affected is sufficiently low to suggest that environmental conditions have limited transmission, say less than 60%, field workers should consider the possibility that a high number of score 4 lesions may be only a short-term characteristic of the outbreak

5.4.3.   Footrot spread between flocks

The proportions of each flock which were affected by footrot were very similar throughout the period of the study. Once transmission to flock 1 had occurred, the only two occasions on which significant differences in prevalence occurred were at, or close to, the inspections at which seasonal peaks in prevalence were observed, December 1993 and December 1994 (Figure 5.2). Nine sheep in flock 1 were affected with footrot at the first inspection (week 6) after arrival from the Monaro, eight of them in groups INT and VIR. Arriving with these sheep were 18 chronically infected intermediate donor sheep and transmission from the donors and infected flock 1 sheep to unaffected sheep in both flocks had occurred by week 18. The incidence of footrot in each of the two flocks during weeks 18 to 27 was almost identical; 7.9% per week in flock 1 and 7.2% per week in flock 2.

The very rapid increase in footrot prevalence during a period when environmental conditions are apparently suitable for transmission has been observed in other studies (Marshall *et al* 1991, Allworth 1995). In discussing an outbreak of mid to low range intermediate footrot in which 98% of sheep were affected in July, Glynn (1993) suggested that the organism was already present in the foot of many of the sheep in the flock and lesions developed very quickly following the advent of favourable conditions, developing faster than might occur if transmission of organisms between sheep was necessary before lesions could develop. The sheep in his study had not, however, been examined since the previous November, so accurate estimates of incidence could not be made. The rapid rise in prevalence in my study, occurring in both flocks simultaneously, showed that exposure to footrot organisms in previous outbreaks

(flock 1) made no difference to the rate at which new footrot cases developed once conditions were suitable for transmission. It is clear that the rapid spread of footrot cases within a flock is not dependent on the activation of already present bacteria but can occur as a result of bacteria spreading from infected sheep to previously unaffected sheep.

To confirm that the outbreak of footrot in flock 2 was caused by bacteria derived from flock 1, or its associated intermediate donors, there was evidence that the bacterial flora in the footrot cases in both flocks was the same. Lesion samples collected from sheep in both flocks revealed a similar mixture of *D nodosus* serogroups, the most common isolations, in order, being H, C, I and B. An isolate of serogroup A was identified once, in a small survey of flock 1 wethers soon after they arrived at property K. The most commonly isolated serogroups in the source flock (at property B, Chapter 4) were, in order of frequency, B, H, C, A and E. (At property B, the relative frequency of isolations, particularly of H and B, was disturbed by the use of a monovalent vaccine against serogroup H. The serogroup E isolate was identified only once.) The appearance, at significant frequency on property K, of the serogroup I isolate was surprising. The isolate was found in samples collected one week before flock 2 sheep arrived and 24 days after flock 1 sheep arrived at property K. Property K had been destocked of sheep and cattle for more than one month before flock 1 sheep arrived, so it is more likely that the strain arrived with the flock 1 sheep than from any other source. Further support for that view was provided by the RFLP pattern of the first I serogroup isolate found, which was pattern 2, the same as two isolates found at property B in 1992, in a serogroup H isolate and a serogroup C isolate.

5.4.4.   Failure of VCS1001 to establish

Strain VCS1001 failed to transmit to the flocks despite apparently successful establishment in the artificially infected 'virulent donors'. This was an unexpected occurrence because previous experience had suggested the technique employed would lead to establishment of the strain. In retrospect, it is likely that the relatively non-advanced lesions present in the donors did not provide sufficient protection from drying during the five hour journey from Camden to property K. At the time of their arrival at property K (19 August 1993), pastures were still short and

relatively dry, although green, and conditions suitable for transmission were not present until September. It would appear that the VCS1001 strain became extinct on the property before conditions were suitable for its transmission and establishment.

5.4.5.   Differences in prevalence between flocks

Differences in prevalence did occur between the two flocks from time to time and minor differences also occurred in footscore profiles of each flock, as discussed above. The reasons for the differences cannot be determined because the flocks arose from different farms, with different histories of footrot exposure, were of different bloodlines although both were of medium-woolled Merino type and of different ages. Little is known of differences in resistance to footrot due to any of these factors. Skerman (1986) found that the mean lesion severity score and prevalence of footrot was higher in Romney sheep four years of age or greater, compared to younger sheep, an observation which, if true for Merinos, could at least partly explain the differences between flock 1 and flock 2 in the current study.

5.4.6.   Differences in susceptibility to footrot within the flocks

The footscores of the intermediate donors were indicative of much more severe footrot lesions than was suffered by the rest of the flocks. Some of these sheep (Table 5.4) had very persistent score 3 and score 4 lesions and, at no inspection between September and mid-December 1993, did fewer than 37% of them have score 4 lesions, despite topical treatment. These wethers had been selected from the flock of origin on the basis of their severe footrot lesions and they remained a highly susceptible group throughout the time they were present in the study.

Although of the same genotype, these 'intermediate donors' were two to three years older than the flock 1 study sheep and their greater age could have contributed to their increased susceptibility (Skerman 1986). It is also likely that these sheep were particularly susceptible to footrot, and there was evidence in the study that some sheep were markedly more susceptible to footrot than others.

The half of each flock with the highest AFE1-14 value formed the high prevalence group. As would be expected from the way that AFE1-14 was calculated, the high prevalence sheep had more feet affected with footrot and for longer, than the low prevalence group. They also were slower to recover after the peak of the outbreak, and had more severe lesions than sheep in the low prevalence group. All of these observations were confounded by the way in which the footrot indices were calculated, but it is of interest to note that the sheep which had the highest AFE scores were, in general, the first ones affected, the most severely affected and the last ones to recover. They were also twice as likely to be classified as 'light', rather than 'heavy', at sale and to receive a financial penalty accordingly.

5.4.7.   Effect on productivity

5.4.7.1.   Bodyweight

The study demonstrated a strong association between the bodyweight of sheep and an index of footrot severity which combined the number of times they were found to have footrot, and the number of feet that were infected. When bodyweight at inspection six was examined in relation to the number of footrot episodes detected in the five previous inspections conducted over the previous 11 weeks (AFE2-6), the following relationships were found

(1) $WT6$ = 62.6 - 0.46 AFE (Flock 1)
(2) $WT6$ = 56.5 - 0.46 AFE (Flock 2)

While the intercepts of the expressions differ between flocks, in accordance with the different initial weight, age and genetic and environmental backgrounds of the two flocks, the effect of footrot lesions was similar in both flocks and equivalent to a reduction of 0.46 kg in bodyweight at the peak of the outbreak (early December) for every foot found to be affected with footrot at inspections conducted at approximately two week intervals.

No significant relationship was detected between the severity of the foot lesions and the variation in bodyweight of the sheep but the inability to demonstrate a relationship could be due to the relatively small proportion of the flock which had severe lesions.

These models predict that a sheep with one foot found to be affected with footrot at each of the five inspections over spring would be 2.3 kg (5 x 0.46) lighter at the peak of the outbreak than it would otherwise be if unaffected.

Marshall *et al* (1991) used a regression model to predict the effect on bodyweight of severe footrot lesions. Their model predicted that one severely affected foot over two transmission periods would lead to a 12.3 kg reduction in bodyweight compared to an unaffected sheep. Interpolation from their model suggests that over a period comparable to the 11 weeks preceding WT6, one severely affected foot would reduce bodyweight by approximately 5 kg, about double that predicted from this study for the effect of any footrot lesion, score 2, 3 or 4.

The sheep reached their highest bodyweight for the season seven weeks after the sixth inspection, at inspection 8, in week 36. By this time, footrot prevalence had declined, from a peak of 82% to 70% in flock 1 and from 76% to 64% in flock 2. The prevalence of score 3 and score 4 lesions had declined relatively more (Table 5.6). The relationship between bodyweight at inspection 8 and the number of footrot episodes detected in the seven previous inspections conducted over the previous 18 weeks (AFE2-8), the following relationships were found

(3) $WT8 = 65.4 - 0.51\ AFE$ (Flock 1)

(4) $WT8 = 56.2 - 0.28\ AFE$ (Flock 2)

For this weight period, the slope of the relationship between footrot lesions and bodyweight was different between the two flocks. It appeared that sheep in flock 2 were, by January, less affected by their footrot history over the preceding spring than sheep in flock 1, while in flock 1 the depression of bodyweight of approximately 0.5 kg per AFE persisted. There are a number of possible explanations for the difference in the effect of footrot on the sheep in each flock, including the difference in age of the sheep. Possibly the younger sheep (flock 2) were better able to regain weight as footrot lesions became less severe in the dry climate of summer. Alternatively, the genetic or environmental differences in the sheep of the two flocks could have been associated with the differences in resilience to footrot infection.

The interpretation of the relationship between footrot lesions and bodyweight in year 2 of the study is different from that applied to year 1. The variable WT13 was related to AFE9-13, the footrot index for the period from week 46 (April 1994) to week 79 (23 November). This period was much longer than the period examined in year 1 (33 weeks, compared to 11 weeks) and the footrot lesions present in the first 23 weeks were lesions persisting from the outbreak in the previous spring. From April 1994 until September 1994, the sheep were living in drought conditions. Footrot transmission was unlikely and lesion prevalence was gradually declining, from 54% in April to 16% in September. Following the move to property T and a wetter environment, footrot spread and, by 23 November, 62% of flock 1 and 37% of flock 2 were affected.

Equations 5 and 6 represent the observed relationship between WT13 and AFE9-13. Again, the slope of the relationship was -0.46, with no significant difference between flocks, the same result as the relationship in year 1 for WT6. In the case of year 2, the presence of lesions was associated with the previous season's outbreak, rather than an outbreak in the current year. It is significant that the effect of the lesions on bodyweight was similar, whether the disease was spreading through the flock at the time or was at least partly the result of persisting lesions from the year before. The footscores in year 2 were generally less active during winter and early spring, which may have reduced the effect of foot lesions on bodyweight, but the greater duration of the period of observation in year 2 implied that affected sheep were affected longer before WT13 was recorded than they were before WT6.

(5) $WT13 = 59.1 - 0.46\,AFE$ (Flock 1)
(6) $WT13 = 55.3 - 0.46\,AFE$ (Flock 2)

To assess the impact of intermediate footrot on bodyweight across the entire flock in year 1, the mean footrot index for that year can be substituted into equations (1) to (4) above. The mean values for AFE2-6 were 5.4 and 5.0 in flock 1 and 2 respectively, not including the CON group, in which the prevalence, severity and duration of footrot lesions were reduced by treatment. The model predicts that the effect of uncontrolled intermediate footrot in the two flocks was a 2.5 kg (flock 1) and 2.3 kg (flock 2) depression of WT6. For WT8, the predicted depression is 4.6 kg (flock 1) and 2.3 kg (flock 2).

This result is similar to the result reported by Glynn (1993 and personal communication), which showed that an outbreak of benign footrot associated with grass seed infestation of the interdigital skin led to a difference in bodyweight of 2.6 kg between affected and treated, unaffected sheep. The form of footrot in the outbreak of Glynn (1993) was described as benign, although a large proportion of the affected sheep had lesions more severe than score 2 and some laboratory characteristics of the isolates were typical of *D nodosus* of intermediate virulence.

The virulent footrot outbreak described by Marshall *et al* (1991) caused a greater effect on liveweight than was measured in my study or that of Glynn (1993). In their experiment, sheep in which control measures were used to limit the effect of footrot were 4.1 kg heavier by summer following the first outbreak than unprotected sheep, and 6.4 kg heavier after a second outbreak.

5.4.7.2.   Wool production

There was a significant relationship between greasy fleece weight, clean fleece weight, fibre diameter and AFE2-8 at the first shearing, and between greasy fleece weight and AFE9-14 at the second shearing (yield and fibre diameter were not measured at the second shearing). The relationship between footrot index and yield of clean wool at the first shearing was close to zero and only weakly significant (P<0.1).

The significant relationships are given as equations 7 to 10.

(7) $GFW1 = Y - 0.013\ AFE$ where Y = 5.80 (flock 1) or 5.40 (flock 2)

(8) $CFW = Y - 0.009\ AFE$ where Y = 4.55 (flock 1) or 4.18 (flock 2)

(9) $GFW2 = Y - 0.015\ AFE$ where Y = 4.30 (flock 1) or 3.87 (flock 2)

(10) $FD = 22.1 - 0.029\ AFE$

To generalise across both years, the results show that, for each unit increase in AFE, fleece weights are expected to decline by 13 g to 15 g in greasy weight or 9 g in clean weight.

The mean values for AFE2-8 were 9.1 (flock 1) and 8.1 (flock 2). The models predict that the effect of the outbreak of intermediate footrot on wool production was a depression of 118g (2.0%) and 105 g (1.9%) in greasy weight for each flock respectively, 82 g (1.8%) and 73 g (1.7%) in clean weight. The predicted effect of the outbreak in year 2, despite a less severe outbreak, was similar; a 2.1% (flock 1) and 1.8% (flock 2) depression in greasy fleece weight.

Footrot lesions also had a depressing effect on fibre diameter, with a slope for both flocks of -0.029. Given the flock mean values for AFE2-8, intermediate footrot is predicted to reduce flock mean fibre diameter by 0.26 (flock 1) and 0.23 (flock 2) micrometres.

The effect of footrot on mean fibre diameter is greater than predicted from the effect on fleece weight alone, assuming that both are depressed by the reduced food-energy intake of sheep which is a result of footrot infection. White and McConchie (1976) found that a reduction in clean fleece weight of one kilogram brought about by increased stocking rate would reduce mean fibre diameter by 1.8 micrometres. This relationship suggests that the 82 g depression in CFW caused by footrot would be associated with a 0.15 micrometre reduction in mean FD. The unexpectedly large impact on FD may be a chance effect, or there may have been a specific effect of footrot on wool fibre diameter in addition to an effect mediated by energy intake.

The depression of greasy fleece weight by intermediate footrot of approximately 2% found in this study is much less than the 7.8% and 10.0% reduction found by Marshall *et al* (1991) with virulent footrot and the 10% reduction found by Symons (1978) in pens following artificial infection of an isolate of *D nodosus*, the virulence of which is not described but which caused severe footrot lesions in affected sheep. Glynn (1993), in a comparison of treated sheep and untreated sheep suffering an outbreak of intermediate footrot, found a 5% difference in greasy fleece weight, despite a significant outbreak of footrot in the treated group. In Glynn's study, grass seeds appeared to add to the impact of footrot lesions on productivity. He noted that grass seed penetration of the interdigital skin was a significant factor contributing to the

variation in bodyweights between untreated and treated sheep, so it is likely that grass seeds also contributed significantly to differences in wool weights.

5.4.8. Vaccination responses

Vaccination was used in an attempt to protect CON group sheep against intermediate footrot (caused by strains of serogroup B and H) and to protect INT and CON group sheep against virulent footrot (caused by strain VCS1001). Strain VCS1001 did not establish in the flocks and virulent footrot did not occur. Vaccination against serogroup A is likely to have protected the INT and CON group sheep against the benign strain of serogroup A endemic in the flocks from properties B and W, but this strain did not appear to be an important component of the flora of the footrot lesions in these outbreaks, based on frequency of isolation, and the small preliminary study on the effect of vaccination with serogroup A reported in Chapter 4 indicated that it would make no diference to the clinical features of the outbreak of intermediate footrot. For these reasons and because no differences were detected between groups, group INT and VIR were considered together for analysis of clinical and production aspects in this study.

Vaccination against B and H serogroups did have an effect in group CON. Serogroup B and H isolates were isolated only once for each serogroup from group CON sheep, while serogroup C was isolated from four group CON sheep. Although the total number of strains isolated was small, vaccination did appear to reduce the frequency of isolation of serogroup B and H and increase the frequency of isolation of serogroup C. The isolation of a serogroup B and H soon after two vaccinations was unexpected. In one sheep, tag number 409, the serogroup H isolate was cultured in week 18 from a foot with a footscore of zero, selected only for sampling on the basis of a small degree of inflammation of the IDS. This sheep was not one of the sheep blood sampled regularly, but its titre to H was only 640 in week 46, when the mean titre of CON group to H was 9 519. It is possible that these sheep responded poorly to vaccination or, given that no lesion was present, the isolate cultured was a surface contaminant on the IDS. Sheep 421 yielded a serogroup B isolate from a sample from a score 3 lesion in week 18. This sheep had a titre to B in week 46 of 320, compared to a mean titre of its group of 1 190 so, like sheep 409, this animal may have responded poorly to vaccination.

Vaccination of group CON against serogroups A, B and H reduced the proportion of the flock affected with footrot and the severity of lesions. Before footbathing was introduced in week 27, fewer CON group sheep were affected than VIR and INT group sheep and, in week 46, 15 weeks after the last footbath, CON group sheep did not show the outbreak of severe lesions shown by VIR and INT group sheep and only 14% of the group were affected with footrot, all with score 2 lesions, whereas 54% of groups VIR and INT were footrot affected, 25% with score 3 or score 4 lesions. It was evident that vaccination made a significant contribution to reducing the prevalence and severity of the outbreak by reducing the frequency of occurrence of the more virulent serogroups.

5.5.   Summary

A flock of sheep in which intermediate footrot was associated with a mixture of *D nodosus* strains showing different phenotypic and genetic characteristics, was moved from the Monaro, a region of NSW considered by some to be only moderately suitable for footrot expression, to a farm on the south-western slopes of NSW where footrot was expected to be well expressed. The flock was mixed with another flock, of different origin and apparently free of footrot, and footrot spread from the first flock to the second.

Conditions were suitable for transmission of footrot in spring after the flocks were mixed, and the transmission period occurred approximately two months earlier in the year than had occurred the previous year in the Monaro. At the peak of the outbreak, in early December, 82% of one flock was affected with footrot and 79% of the other. There were two peaks of severe lesions, one during the latter part of the main transmission period when up to 23% of flock 1 and 30% of flock 2 were affected, and one in autumn when 25% of both flocks had score 3 or score 4 lesions. The proportion of the flocks which had score 4 lesions did not exceed 6% of the flock.

The profile of maximum footscores across the flocks at the peak of the outbreak was similar to the profiles seen in related flocks in the Monaro in the previous year, in which the same bacterial strains were involved. The key similarities were that, at a maximum, between 15%

and 25% of sheep had score 3 lesions and fewer than 8% of sheep had score 4 lesions. This study demonstrated that these characteristics of intermediate footrot were constant across two sites, one in an area considered only moderately suitable for footrot expression and one considered highly suitable.

Examination of the footscore profiles also demonstrated that a relatively high number of score 4 lesions can occur when conditions are becoming, or have become, less than optimal for transmission of footrot and, therefore, can occur at times in the season when the prevalence of affected sheep is past its peak. This observation led to the recommendations that the most reliable approach to classification of natural footrot outbreaks should include the ratio of sheep with score 4 lesions to *total sheep exposed*, rather than *total sheep affected*. Additionally, where the proportion of the flock which is affected is relatively low, say less than 60%, field workers should consider the possibility that a high number of score 4 lesions will not persist.

It is hypothesised that the reason for relatively high numbers of score 4 lesions when conditions are less than optimal for transmission is the effect that water on pastures has on the footrot lesions, an effect which removes footrot bacteria and their extracellular products which are necessary for advancing underrunning lesions. Further, it is suggested that this characteristic may be more a feature of intermediate footrot rather than virulent footrot because of the less effective or lower concentrations of proteases produced by strains of lesser virulence.

Comparison of the rise in prevalence of footrot at the beginning of a spread period in the flock endemically infected and the flock with no history of footrot demonstrated that it is not necessary for sheep to be harbouring inactive or sub-clinical infections with footrot organisms for a rapid spread of the disease to occur within a flock. Characterisation of the *D nodosus* isolates by serogroup and with RFLP patterns showed that the same bacteria were represented in the outbreaks in both flocks, and with the exception of an isolate of serogroup I, the same as the *D nodosus* isolates found most frequently in the outbreaks studied in the Monaro flocks.

Relationships between various indices of footrot and effects on bodyweight, wool weight and other fleece characteristics were determined. Uncontrolled footrot led to a mean depression

in bodyweight of approximately 2.5 kg at the peak of the outbreak and 4.6 kg later in the season in the flock which showed less resilience to the effects of footrot. This effect on bodyweight was approximately half that reported for virulent footrot (Marshall *et al* 1991) and similar to that reported for benign or low grade intermediate footrot complicated by grass seed infestation (Glynn 1993).

Similarly, the outbreak of intermediate footrot depressed the weight of annual production of greasy wool by approximately 2%, of clean wool by 1.7% and reduced mean fibre diameter by 0.25 micrometres. The reduction in annual greasy wool production was much less than that recorded for virulent footrot of 7.8% to 10.0%.

CHAPTER 6

CONTENTS

A COMPARATIVE STUDY OF INTERMEDIATE AND VIRULENT FOOTROT UNDER CONTROLLED ENVIRONMENTAL CONDITIONS

6.1. Introduction

Earlier chapters described outbreaks of intermediate footrot occurring naturally in flocks in a region considered marginal for the expression of footrot (Chapter 4) and another considered favourable for footrot expression (Chapter 5). While these outbreaks allowed a characterisation of intermediate footrot, comparisons with other forms of footrot, particularly virulent footrot, were not possible in the field studies. The possibility remained that the limited expression observed in the outbreaks was a consequence of environmental effects, rather than bacterial virulence factors.

There is only one other report of experiments which have compared virulent footrot and intermediate footrot simultaneously in the same environment. Stewart *et al* (1984) compared four strains of *D nodosus* in an experiment with two replicates and eight sheep per treatment. The virulent strain (A198) caused the most numerous severe footrot lesions; the two intermediate strains caused fewer and the benign strain the least. In association with the differences in footrot severity, the virulent strain caused a marked effect on bodyweight and an effect on wool growth rate which was significantly greater than one of the intermediate strains and the benign strain. The experiment clearly demonstrated the difference between the strains.

The experiment reported in this chapter was designed to compare the intermediate strain of footrot characterised in the studies reported in Chapter 4 and 5 with virulent footrot, under controlled conditions and to compare both to uninfected sheep. The aim of the experiment was to create an artificial outbreak of footrot equivalent in virulence to the natural outbreaks described earlier and to demonstrate how that disease differed from virulent footrot. Differences to be measured were the severity and number of lesions and the effects of the disease on wool growth and bodyweight. In this respect, the planned experiment was similar to that of Stewart *et al* (1984), but with a strain of intermediate footrot which had been very extensively described in field outbreaks. The demonstration that the form of footrot in the field

outbreaks (described in Chapters 4 and 5) was clearly different from virulent footrot was considered necessary in order to draw more confident conclusions about the behaviour of intermediate footrot. In other words, it could not be deemed to be intermediate with certainty until it was demonstrated that it was different from virulent footrot in the same environment.

A second aim was to estimate the extent of the depression of wool growth and bodyweight change caused by intermediate footrot. To allow this estimation, a control group, unaffected by footrot, was required. A subordinate goal of this aim was to determine if the effect on productivity could be directly related to the severity of footrot, regardless of the infecting strain. If this could be demonstrated, it would be possible in future to predict the cost of any form of footrot from information about the footscores, without measurements of production directly, for every form of footrot.

A third aim was to examine the difference between a mixed infection of two strains of footrot and the two strains separately. While it is recognised that most outbreaks of footrot are associated with more than one strain of *D nodosus*, there are no published reports of the difference between single strain infections, as most experimental outbreaks have been, and multi-strain infections.

In the experiment described here, only two strains were used, one virulent and one intermediate. Although it was recognised that the single strain infection would probably not be the same as the highly mixed infection identified in the field, it was necessary to use just one strain in order to make a valid comparison between the two strains acting separately and the mixed infection of both strains together.

6.2. **Materials and methods**

6.2.1. **The experimental site**

The experiment was conducted at the Colonel JB Pye Farm, Bringelly, NSW. The experimental site was divided into six blocks and each block was further subdivided into four plots. Two blocks were split such that three plots were contiguous and one plot was separated from the other three by about 100 m (Plate K). Each of the 24 plots was assessed for gradient, pasture cover and position on the slope and ranked on expected proclivity for remaining wet after rainfall or irrigation with a wetness ranking of one to four. The plots were either 3 600 m^2 or 2 500 m^2. Within blocks, all plots were the same size. Four blocks had plots of 2 500 m^2 and two had plots of 3 600 m^2.

6.2.2. **The animals**

The experimental flock consisted of 162 Merino wethers, aged two years, purchased from a property near Condobolin, NSW. The sheep had been shorn in July and arrived at the experimental site on 22 September 1995. There was no history of footrot in the flock of origin. From the 162 sheep which arrived, 156 were selected for the experiment on the basis that sheep of similar weight could be matched across all treatment groups.

6.2.3. **The bacteria**

Two strains of bacteria were used in the experiment. The virulent strain was VCS1001, a prototype serogroup A, sub-type 1, also known as A198 and of the same strain used by other workers who published reports relevant to the aims of this study (Stewart *et al* 1984; Marshall *et al* 1991). The intermediate strain was VCS1728, a serogroup H isolate from property B (Chapter 4) which has been widely studied in the work reported in this thesis, and used also by Allworth (1995).

6.2.4.   Records of climate

Climatic data were recorded by the Bureau of Meteorology at Penrith Lakes, approximately 15 kms north of the JB Pye Farm. The site is an automated weather station situated at latitude 33°43'S longitude 150°41'E at an elevation of 25 m above sea level.

Irrigation water was applied by a travelling high pressure sprinkler irrigator to four of the blocks and by portable aluminium spray lines fitted with knocker-type spray heads in the other two blocks. Water was pumped from a dam and irrigation of each block was done in an eight to ten hour period on one day per week.

6.2.4   Dye banding

Dye banding of wool (Chapman and Wheeler 1963) was performed by a method similar to that of Wheeler *et al* (1977). A 1% w/v stock solution of the oxidising agent Nako H (Hoechst Roussel Vet Pty Ltd) was made. Immediately before applying to the fleece, hydrogen peroxide was added to a small volume (usually 100 ml) of stock solution at the rate of 1 ml per 100 ml Nako H solution to prepare the dye banding solution (DBS).

The fleece of each sheep was parted in the upper part of the left side, immediately behind the shoulder, while the sheep was restrained on a V-belt sheep handler. Approximately 1 ml of DBS was applied in a thin line, about 100 mm long, at the base of the parted fleece, so as to lie on the skin and move by capillary action up the adjacent wool staples to a height of 1 to 2 mm (Plate L). A Gilson pipette was used to apply the DBS. The fleece was then closed and the sheep returned to the flock.

The result of the application of DBS was to produce a dark black stain of the wool which persisted as the fleece grew. The band in the wool staples could be up to 10 mm wide depending on how far the DBS diffused up the staple after application but the bottom of the band corresponded to the skin-fleece junction at the time of application.

For repeated applications, the first band was located and the fleece parted in the same position as had been used for the previous application, so that banded staples were visible on both sides of the parting. The result of three banding procedures, followed by a period of three months wool growth, is illustrated in Plate L.

In April, two months after the third dye band was made, four staples of wool clearly bearing three bands were removed from all sheep with scissors, cutting the staple between the most recent band and the skin. The staples were then placed in a paper envelope, labelled with the number of the sheep, and stored in a dry environment.

For this experiment, it was necessary to compare the rate of wool growth of each sheep in the second dye-banding period to that in the first. This was performed by cutting each staple of wool at the base of each band after ensuring that all wool fibres were smoothed longitudinally along the staple. Each of the eight fragments of staples from each sheep was then washed as an individual sample. Washing was performed in plastic beakers containing 100 ml of a solution of 0.3% sodium hydroxide (w/v) in a 0.26% (v/v) solution of Teepol at 55°C for 10 minutes, repeated twice. During washing, the beakers were gently agitated on a stage. After washing, the solution was poured off and the wool rinsed twice in cold water. The wool was recovered from a sieve and allowed to partially dry on blotting paper at room temperature until approximately 100 samples were available and these were dried at 100°C for one to two hours, cooled to room temperature and weighed. All segments of staples from any one sheep were weighed consecutively to avoid errors due to variable moisture regain over time.

To calculate the relative rate of wool growth in the second period, the weight of each of the bottom segments of each staple was divided by the weight of the top segment. Four ratios were calculated for each sheep and the mean of the four ratios was used in analysis of wool growth rates.
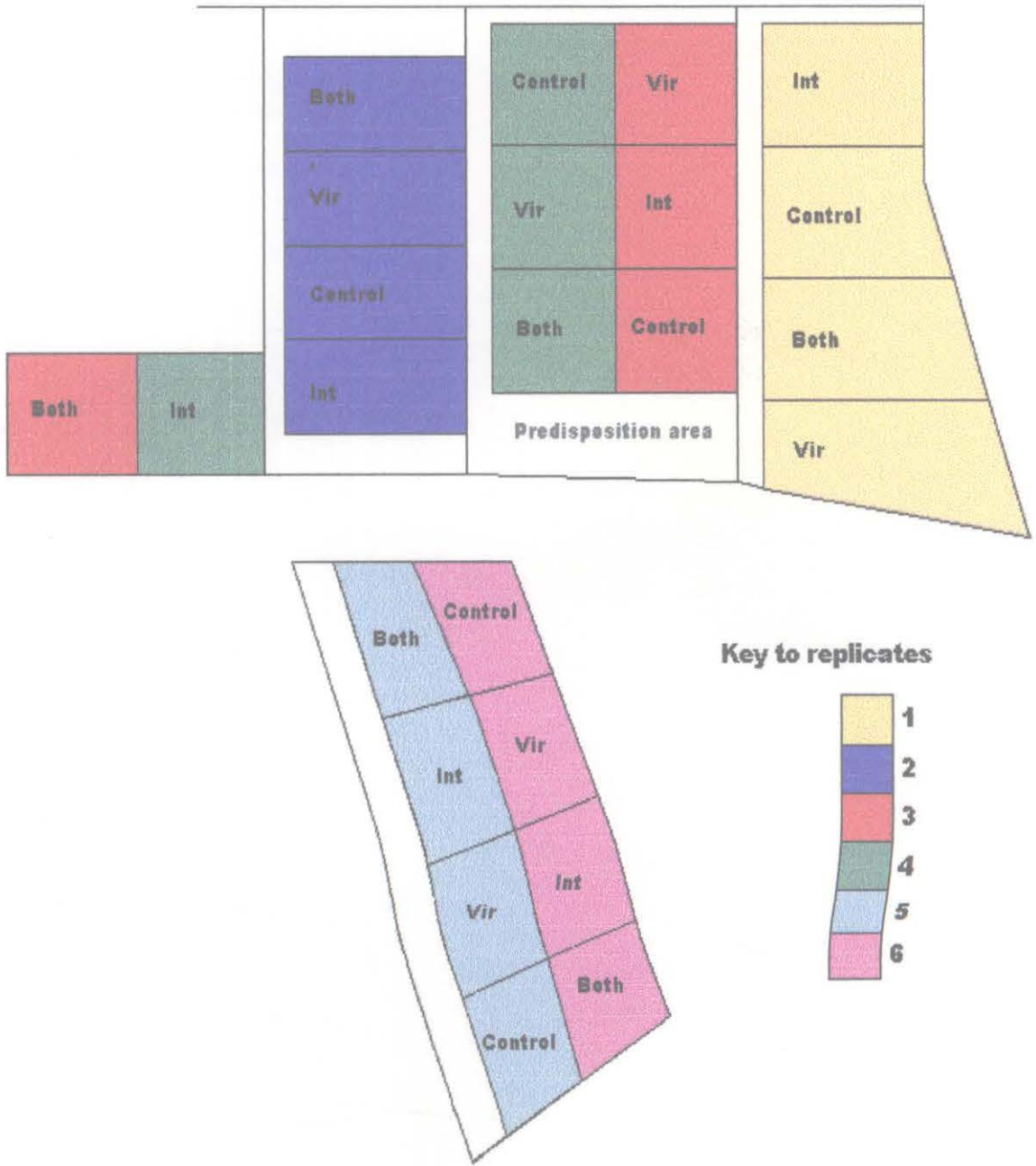
Plate K

Diagrammatic representation of the experimental site at the Colonel JB Pye Farm, Bringelly. The site was divided into six blocks or replicates, each with four plots. Each plot was either 2 500 m² or 3 600 m². Within blocks, all plots were the same size. Treatment groups were allocated at random to plots within blocks after stratification of plots for wetness. The four treatments were VIR (virulent strain infection), INT (intermediate strain infection), BOTH (virulent and intermediate strains combined) and CON (control group, not infected).

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Plate L

Dye banding of the fleece was performed three times. A 1% w/v stock solution of the oxidising agent Nako H (Hoechst Roussel Vet Pty Ltd) was made. Immediately before use, hydrogen peroxide was added at the rate of 1 ml per 100 ml (Wheeler *et al* 1977; Chapman and Wheeler 1963). The Nako H solution was applied to the fleece with a Gilson pipette, along the bottom of the staple for a length of about 10 cm. Banding was applied on day 0, day 35 and day 118 (February). The bottom photograph was taken in April and shows the three bands separated from the skin. At this time, four staples were removed from each sheep, cutting between the lowest staple and the skin. The wool growth between days 35 and 118 was compared to the wool growth between days 0 and 35 by comparing the weights of staple sections cut at the lower edge of the three bands.

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Plate M

TOP   Infected donor sheep were placed onto plots by carrying from a trailer and lifting over the fence from the access laneways into the plots, to avoid contamination of laneways and cross-infection of donors. Placement of donors on plots on day 35 marked the start of transmission of footrot. Rain was falling during the procedure and pasture was green and abundant.

BOTTOM   Experimental sheep were predisposed to infection by confinement in a heavily irrigated part of the site. For one week prior to challenge on day 35, all experimental sheep were confined in a predisposition area. Pasture and soil was kept wet by almost continuous irrigation. Sheep were fed hay and grain from troughs.

6.2.5. Time course of the study

The first dye band was applied to the fleece of all experimental sheep on 12 October 1995, and this day became day 0 of the experiment (Table 6.1). Sheep were then run in one of six replicate groups, moving between plots within blocks every few days. The second dye band was applied five weeks later (day 35) on which day the experimental sheep were moved into their plots and exposed to the donor sheep. The donor sheep were removed three weeks later and all experimental sheep were footscored. The second post-challenge inspection occurred four weeks later and the third five weeks after the second (day 118). A third dye band was applied and the sheep removed from their experimental plots to a dry, short-pastured environment. Dye-banded wool staples were harvested in April 1996.

Table 6.1   Main events in the study at JB Pye Farm

Day	Date	Inspection	Main events
0	12 October 95	1	First dye band, sheep in one flock
11	23 October		Donor sheep onto mats; balance in replicate groups grazing across all plots within each block
18	30 October		Donor sheep infected
28	9 November		All experimental sheep onto predisposition area
35	16 November	2	Second dye band
			Donors onto plots
56	7 December	3	Donors removed from plots
85	5 January 96	4	
118	7 February	5	Third dye band. Sheep removed from experimental site.

6.2.6. Management procedures

On 12 October 1995 (day 0), the experimental sheep were drenched with closantel (Seponver, SmithKline Beecham) and tagged in both ears. On day 28 all sheep were vaccinated with a combined clostridial-CLA vaccine (Cheesyvax 6, SmithKline Beecham) and were jetted with cyromazine (Vetrazin, Ciba-Geigy) and drenched with a levamisole-oxfendazole combination (Scanda, Mallinckrodt Veterinary). Closantel treatment was repeated on day 85. During the week that the sheep were confined in the predisposition area they were fed meadow hay and lupin grain at the rate of 1 kg and 0.3 kg respectively, per head per day.

6.2.7. Experimental design

The experiment was a completely randomized two by two factorial design with six replicates (plots) per treatment. The two by two factorial consisted of a control (CON), an intermediate strain (INT), a virulent strain (VIR) and both intermediate and virulent strains together (BOTH).

Sheep in group VIR were exposed to infection with *D nodosus* VCS1001; sheep in group INT were exposed to infection with strain VCS1728; those in group BOTH were exposed to infection with both strains and sheep in group CON were not deliberately infected. Each treatment group was allocated to one plot in each of six blocks on the experimental site. The groups were allocated to plots within blocks such that each treatment was equally represented in plots of each wetness ranking.

Sheep were stocked at five per plot on the 16 smaller plots (20 sheep ha^{-1}) and seven per plot on the eight larger plots (19.4 sheep ha^{-1}), so that stocking rates in all blocks were similar and were identical within blocks.

Before entering the experimental plots, the sheep destined to be the experimental sheep (not the donors) were predisposed to infection in a confined area adjacent to one of the experimental plots which was maintained in a wet condition by frequent repeated watering with a travelling irrigator (Plate M). Approximately 90 000 litres were applied to the 0.4 hectare area every

second day, equivalent to 23 mm of rainfall. The sheep remained on this site for seven days before being individually inspected, footscored, dosed with anthelmintic, jetted with insecticide, dye banded and moved to their plots. While awaiting inspection in the sheepyards, the sheep were held on rubber mats which were frequently wetted to maintain the interdigital skin in a moist condition.

Sheep in groups VIR, BOTH and INT were exposed to infection by the introduction of artificially infected donor sheep (see section 2.2.12). Each plot received two sheep; one infected donor and one uninfected sheep for groups VIR and INT, two infected donors in group BOTH and, in the case of the controls, two uninfected sheep. The six BOTH groups each received one donor infected with each strain of *D nodosus*. After artificial infection, all candidate donors were examined and their footrot lesions scored. Donors were classified as mildly, moderately severely or very severely affected based on their lesion scores. Donors were allocated to treatment groups after stratification on severity classification such that plots within blocks received donors of similar severity of footrot.

Donors were carried to each plot by trailer and lifted over the fence into each plot to prevent contamination of laneways (Plate M). Three weeks after introduction all donors were removed and treated by foot paring and antibiotic injection to aid recovery from footrot.

On days 56, 85 and 118, sheep were inspected in yards placed strategically in laneways. Treatment groups were brought to the yards in strict order; CON, VIR or INT then BOTH so that sheep were not exposed in laneways to strains with which they were not already infected. VIR group and INT group sheep did not travel to the same yards at any one inspection.

Wool growth was measured over two periods. The first, over five weeks, was the period immediately before sheep came in contact with donor sheep. The second, over twelve weeks, was the period commencing immediately after the donors were introduced and ending when healing was induced by footbathing and exposure to dry environmental conditions.

6.2.8. **Measurements**

All sheep were weighed and all feet inspected and scored for footrot on five occasions; on days 0, 35, 56, 85 and 118. Wool was dye-banded on three occasions; days 0, 35 and 118, producing two intervals of measurable wool growth, one of five weeks and one of twelve weeks.

Feet were sampled for bacterial culture at each inspection after the donors were removed. Lesion material was collected from one foot of each of two sheep in each of the 24 plots at each inspection on days 56, 85 and 118. From successful cultures, up to three isolates were sub-cultured and grown in pure cultures for subsequent serogroup identification.

Sheep were exposed to infection and to conditions suitable for transmission of footrot over a defined 12 week period and two production traits, liveweight change (WTCHANGE) and rate of wool growth (WOOL) over that 12 week period were the dependent variables examined.

Rate of wool growth in the 12 week challenge period was expressed as a multiple of the rate of wool growth in the five week period before the sheep were exposed to infection.

6.2.9. **Statistical analysis**

6.2.9.1. **Footscoring system**

Foot lesions were scored in the same manner as described previously (Chapter 2), with a classification system similar to that described by Egerton and Roberts (1971). Whereas in previous chapters a sheep was considered to be either affected or not affected with footrot with a binary classification based on the presence of at least one score 2 lesion, it was appropriate in this controlled study to grade the degree of severity further and, if possible, develop a classification system which would lead to a wider distribution of grades of disease. Consequently, two related systems were developed; one, a points system allocating increasingly higher numbers of points to increasingly severe lesions and, two, a six grade classification system based on the allocation of points for each footrot lesion recorded for each sheep (Table

6.2). The value of the points was selected to separate sheep with two or more score 4 lesions from sheep with mostly score 3 lesions and sheep with score 3 lesions from sheep with mainly score 2 lesions. The points score of each sheep then classified it to an overall grade, from 0 to 5, and both the points and the grade were used in statistical analysis.

An overall footrot severity score, calculated for each sheep for the whole challenge period, was calculated from the severity scores of each sheep on days 35, 56, 85 and 118, after weighting each day's score according to the time interval between measurements. This variable was called OVERALLSEV for analytical purposes.

It was felt that the grading system would adequately separate sheep with lesions of differing severity without overstating the understanding of the effects on production of lesions of different severity. The system classified sheep in a way which was intuitively sound and, in this study, led to a distribution of sheep in each category which was largely expected. The system was also similar to the six level, overall animal grade used by Raadsma _et al_ (1993).

Throughout the studies reported in this thesis sheep with footscores of two or greater have been considered to be affected with footrot. This association is based on the assumption that _D nodosus_ is present and has a causative role in the foot lesion. While this is probably a valid assumption in most cases, it is recognised that scores of 2 and even 3 can occur with OID and in the absence of _D nodosus_. In the study reported in this chapter, the definition of an affected sheep remains the same, even when evidence is presented that _D nodosus_ was absent from the foot. This approach is considered valid, however, because the study examined the relationship between footscore and productivity and the effect of a footscore of 2 on the productivity of a sheep is likely to be similar, whether _D nodosus_ is present or not.

6.2.9.2.   Statistical methods

Results were analysed as a completely randomised factorial design, repeated measures for variables that were measured more than once. Means were compared using a Tukey's test. Analysis of the number of feet with score 4 lesions was performed after exclusion of the controls, which had no score 4 lesions, and log transformation of the data from the infected

groups, and comparison by least significant difference of the transformed means at the 5% level. The relationship between wool growth, weight change and footrot severity were examined using an analysis of covariance and regression. All analyses were carried out using SAS version 6.12 (SAS Institute Incorporated, Cary NC, USA).

Table 6.2   Description of scoring system used to classify sheep on footrot severity

Severity grade	Points range¶	Scores	Description
0	0	All feet 0 or 1	No footrot
1	1 - 2	1 or 2 score 2 lesions	Very mild
2	2.5 - 4	3 or 4 score 2 lesions, or 1 score 3 and up to 1 score 2 lesion	Mild
3	4.5 - 7.5	2 score 3 lesions, or 3 score 3 lesions and no score 2	Moderate
4	8 - 15.5	4 score 3 lesions, or 1 score 4 lesions plus up to 3 score 3	Severe
5	16 - 32	2, 3 or 4 score 4 lesions	Very severe

¶   Points are allocated to footrot lesion scores; 0 points for score 0 or 1, 1 point for score 2, 2.5 points for score 3 and 8 points for score 4.

Plate N

Pasture in December 1995 was abundant in plots, following a period of high natural rainfall and irrigation, with moderately high temperatures. This photograph was taken on day 56, when donors were removed from the plots.

6.3.   Results

6.3.1.   Sheep health

One sheep died on the 26 October 1995 with post–mortem signs characteristic of enterotoxaemia. This sheep had been allocated to an experimental plot and was replaced by a sheep from those which had not been allocated to a treatment after matching, as closely as possible, on liveweight. No further mortalities occurred.

6.3.2.   Climate

Mean monthly rainfall and mean monthly daily mean temperatures recorded at Penrith Lakes for the period September 1995 to May 1996 are illustrated in Figure 6.1. In the two months before the experimental sheep were placed on pastures rainfall was low (40 mm in September and 17 mm in October). In November, when sheep were placed in their experimental groups at pasture and then exposed to footrot, rainfall was high (141 mm). On the day infected donors were added to the plots (20 November) 42 mm of rain fell and rain fell on 12 of the subsequent 21 days. Rainfall in the latter part of December was low but 113 mm was recorded in January, occurring over a series of seven days in early January and six days in late January.

From 25 October 1995 until 3 January 1996, irrigation water was applied to all of the experimental sites at the rate of approximately 176 000 litres per hectare per week, equivalent to 17.6 mm of rainfall. Irrigation was continued throughout rain periods but was discontinued for one week in early December, due to mechanical breakdown. Irrigation added significantly to the total water falling on plots during November and December. Total precipitation (rainfall plus irrigation) in November and December was 212 mm and 104 mm respectively. Care was taken to ensure that the same amount of irrigation water was applied to all replicates within a one week cycle but, during December, the cycles were extended to 10 days.

Mean daily temperatures consistently exceeded 15°C throughout the period of the study until

17 April and the mean monthly mean temperature for the study period peaked at 23°C in January 1996.

6.3.3.　　　Pasture growth

Following irrigation and natural rainfall in late October and early November, pasture in all plots grew abundantly and was 30 to 45 cm high in all replicates (Plate N). Pastures consisted of grass legume mixtures including cocksfoot (*Dactylis glomerata*) and white clover (*Trifolium repens*).

6.3.4.　　　Establishment of infection in donors

The footscores in the donor sheep on day 35, 17 days after infection, are shown in Table 6.3. Sheep were categorised into four groups, based on the extent of their lesions (footscore) and the apparent activity, as judged by the degree of inflammation and amount of necrotic material in the lesions. Twelve donors from each serogroup were selected as donors for specific plots such that footscores were similar within replicates. Where a donor from each serogroup was required (group BOTH), two donors were selected with similar foot lesions in an attempt to cause similar levels of challenge with each serogroup (Table 6.3).

Table 6.3   **Footscores of artificially infected donor sheep**

| Serogroup | Activity and extent of lesions | | | |
	Mild	Moderate	Severe	Very severe
A	1101	1022	1131*6	1244*2
		2201	0032*5	2133*1
		1121	0044	
		1122	1034	
		1212*4	1133*3	
H	0000	0030*6	2022	2123
		1042	0122	3122
			2022	3322*2
			0122*4	
			0123*5	
			3123*1	
			1132*3	

*1 to * 6 indicates sheep which were paired for challenging group BOTH in each of the six replicates

6.3.5.   Infection in experimental sheep

6.3.5.1.   Establishment of infection

Donors were removed from the experimental flocks on day 56, 21 days after introduction. All sheep were footscored at the time. Footrot was well established in all plots containing groups VIR, BOTH and INT. In one plot of control (CON) sheep four out of five sheep had at least three feet affected with score 2 lesions and it was suspected at the time that footrot had been transmitted to this plot. Subsequent events showed that this was not the case and that the sheep in this plot had been affected with OID. In the infected groups, lesions were generally score 2 and score 3 lesions and the only score 4 lesion was in one foot of a sheep in group INT.

6.3.5.2.   Number of affected feet

At the first post–challenge infection INT and BOTH had significantly greater numbers (P<0.05) of affected feet than the controls but there was no significant difference between VIR, BOTH and INT in the number of affected feet (Table 6.4).

On day 85, 50 days after the donors had been introduced, lesions had progressed in severity in the groups VIR and BOTH. There were fewer affected feet in groups CON and VIR than there were on day 56. All infected groups had significantly (P<0.05) more affected feet than did the CON group (Table 6.4). On day 118 the numbers of affected feet in all infected groups were similar and were higher than at either of the two earlier inspections, except for INT, which was the same as at day 85. (Table 6.4). There was no significant difference in mean number of affected feet between any of the infected groups but all were significantly greater than in the controls (P<0.01).

There was no significant difference between treatment groups in the mean number of affected feet averaged over the three post–challenge inspections, but all three treatments produced significantly more affected feet (P<0.01) than the controls (data not shown).

6.3.5.3.   Number of score 2 affected feet

At all inspections (days 56, 85 and 118) the INT group sheep had significantly more feet affected with score 2 lesions than the controls (P<0.05), and significantly more than the VIR group at days 85 and 118. Groups VIR, BOTH and CON were not significantly different in numbers of score 2 feet at any inspection; groups INT and BOTH were not significantly different at any inspection (Table 6.4).

The mean number of feet with score 2 lesions, averaged over the inspections on days 56, 85 and 118, was significantly greater (P<0.05) in the group infected only with the intermediate strain (INT) than in the controls and in the group infected with the virulent strain only (VIR) but not significantly different from BOTH. The numbers in the VIR and BOTH groups were not significantly different from the controls (Table 6.5).

6.3.5.4.   Number of score 3 affected feet

Group BOTH sheep had a significantly higher (P<0.05) number of feet with score 3 lesions at day 56 than INT and CON despite having the same mean number of affected feet as the INT group. The INT group feet were predominantly score 2 lesions, the BOTH group sheep were a mixture of score 2 and score 3 lesions.

At days 85 and 118 there were no significant differences in numbers of feet with score 3 lesions between any infected treatment groups. Only BOTH had significantly more score 3 lesions than the controls at day 85; both VIR and BOTH had significantly more feet with score 3 lesions at day 118 than the controls.

The factorial nature of the experiment provided additional insights into the behaviour of the two strains in separate and combined infections. Factorial analysis of variance indicated that there was no significant interaction between the intermediate strain and the virulent strain in number of feet with score 3 lesions but that the combined infection (BOTH) produced significantly more score 3 lesions than either strain alone on day 56 (P<0.01 for VIR and

P<0.05 for INT) and day 85 (P<0.05 for VIR and INT). On day 118, there were more score 3 lesions in group BOTH than in VIR (P<0.01). The lack of interaction implies that the two strains acted in an additive way and that together the two strains produced more score 3 lesions than either alone (see Appendix for analysis).

The mean number of score 3 lesions over the three inspection days (Table 6.5) was not significantly different between infected treatment groups but the groups VIR and BOTH had significantly more score 3 lesions than the controls (P<0.05).

6.3.5.5.   Number of score 4 affected feet

The numbers of sheep with score 4 infections and the numbers of affected feet are shown in Table 6.6. The mean values are shown in Table 6.4. The analysis of variance and comparisons between the mean number of score 4 affected feet used log transformed data from the treatment groups, after excluding the controls for which all values were zero. After transformation, all variances on the replicate level were homogenous. Comparisons of the means, after the analysis of variance, using *least significant difference* (lsd), indicated a significant difference (P<0.05) between the INT group and the VIR group. The group BOTH was not significantly different from either INT or VIR.

Table 6.4   Mean number of affected feet per sheep (34 sheep in each group)

Footscore	Day 56				Day 85				Day 118				sem	msd
	CON	INT	VIR	BOTH	CON	INT	VIR	BOTH	CON	INT	VIR	BOTH		
2	0.68^a	1.82^b	1.15^{ab}	1.21^{ab}	0.09^a	2.44^b	0.47^a	1.32^{ab}	0.09^a	2.21^b	0.94^a	1.24^{ab}	0.305	1.19
3	0.09^a	0.45^a	0.56^{ab}	1.09^b	0^a	0.44^{ab}	0.44^{ab}	0.74^b	0^a	0.50^{ab}	0.79^b	0.74^b	0.159	0.61
4	0	0.03	0.03	0	0	0	0.65	0.53	0	0.18	0.94	0.85		
Total	0.76^a	2.29^b	1.74^{ab}	2.29^b	0.09^a	2.88^b	1.56^b	2.59^b	0.09^a	2.88^b	2.68^b	2.82^b	0.370	1.47

a, b, c Values in same rows within days with different superscripts are significantly different at P<0.05

sem, standard error of the mean

msd, minimum significant difference for P<0.05, calculated with weighted Tukey's *w* procedure

NOTE Data on numbers of feet with score 4 lesions were not subjected to the same statistical analysis as data on scores 2 and 3. (See Table 6.6)

Table 6.5   Mean number of feet affected with score 2 and score 3 lesions, calculated over all three post–infection inspections.

Treatment	Mean number of feet with score 2 lesions sem = 0.49	Mean number of feet with score 3 lesions sem = 0.22	Mean number of feet with score 4 lesions
CON	0.28^a	0.03^a	0.00
INT	2.16^b	0.46^{ab}	0.07
VIR	0.85^a	0.60^b	0.54
BOTH	1.25^{ab}	0.85^b	0.46

a, b Values with different superscripts within the same columns are significantly different (P<0.05)

sem, standard error of the mean

NOTE Data on numbers of feet with score 4 lesions were not subjected to the same statistical analysis as data on scores 2 and 3, see Table 6.6.

Table 6.6   Number of score 4 affected sheep (and feet in parentheses) on day 118 (34 sheep per treatment group)

	Treatment			
	CON	INT	VIR	BOTH
Number of score 4	0 (0)	$4 (6^a)$	$18 (32^b)$	$15 (29^{ab})$

a,b Values with different superscripts were significantly different (P<0.05). Controls were excluded from the comparison; see section 6.3.5.5 for explanation

6.3.5.6.   Footrot severity grades

Mean footrot severity grades for each inspection day are shown in Table 6.7 and illustrated in Figure 6.2. This method of grading footrot severity did not differentiate any of the infected groups from each other but the infected groups were all significantly different from controls on day 85 and 118, and overall.

The summary variable (OVERALLSEV) was also calculated for each treatment, which was a mean overall severity grade, weighted for time interval and including the data from day 35 when all feet had severity grades of zero. This overall grade discounted the effect of day 56 and day 118 values compared to the simple means used in the repeated measures analysis. Consequently, the differences between all treatments were reduced and there was no significant differences detected between infected groups, although all were significantly different from the controls (data not shown).

Table 6.7   Mean footrot severity grades (OVERALLSEV)

Treatment	Day 56	Day 85	Day 118	Mean
	-----------	sem = 0.348	-----------	sem = 0.569
CON	0.56^a	0.09^a	0.09^a	0.25^a
INT	1.74^{ab}	1.91^b	2.15^b	1.93^b
VIR	1.47^{ab}	2.32^b	3.18^b	2.32^b
BOTH	2.15^b	2.56^b	2.91^b	2.54^b

a, b Values with different superscripts within the same columns are significantly different (P<0.05)

6.3.5.7.   Footrot lesion points

When analysed using points, rather than severity grades, no insights about differences between treatments were gained beyond that provided by severity grades (previous section). The variable OVERALLPTS, calculated in a way analogous to OVERALLSEV, required log transformation to homogenise variances between treatments, probably reflecting the higher value given to the more severe lesions. After transformation, OVERALLPTS was no better in differentiating treatments on the basis of foot lesion severity than OVERALLSEV. The data are not shown.

6.3.6.   Wool growth rates

The two time periods between the three dye bands were five weeks (period 1) and twelve weeks (period 2). During period 1, the experimental sheep were maintained as single flocks within blocks so that all sheep within replicates were exposed to equivalent feed conditions. During period 2, the experimental sheep were in experimental plots and, in the case of groups VIR, BOTH and INT, exposed to footrot infection.

The weight of wool grown in period 2 was two to three times the weight grown in period 1, with a range between sheep of 1.74 to 3.61. For purposes of analysis this variable was named WOOL. Factorial analysis of variance indicated that there was a significant (P<0.05) interaction between the two infecting strains. There were significant differences (P<0.05) in WOOL between the control group (CON) and the two groups infected with the virulent strain (VIR and BOTH) (Table 6.8). The difference in WOOL between the control group and the group infected with the intermediate strain approached significance at the 0.05 level with a difference in mean values of 0.25, compared to the value of 0.29 calculated by the Tukey's test for $\alpha = 0.05$. Groups VIR and BOTH grew wool at a rate which was 15.2% and 12.3% less than the controls. Group INT grew 9.3% less wool than the controls.

Table 6.8   The mean ratio of wool grown in period 2 to period 1 (WOOL)

Treatment	Mean ratio of wool growth sem = 0.072
CON	2.70[b]
INT	2.45[ab]
VIR	2.29[a]
BOTH	2.37[a]

a, b Values with different subscripts are significantly different (P<0.05)

sem, standard error of mean

Table 6.9   The weight change (kg) during 12 weeks of footrot infection

Treatment	Mean weight change (kg) sem = 0.49
CON	5.97[b]
INT	2.78[a]
VIR	2.25[a]
BOTH	2.04[a]

a, b Values with different subscripts are significantly different (P<0.05)

sem, standard error of the mean

6.3.7.   Weight change in treatment groups

Between days 35, when donor sheep were introduced onto the plots, and day 118, when the challenge period ended, sheep in the control group (CON) gained an average of 5.97 kg liveweight. The infected groups gained between 2.04 kg (BOTH) and 2.78 kg (INT) (Table 6.9). Factorial analysis of variance indicated that there was a significant (P<0.01) interaction between the two infecting strains. There was a significant difference between the weight change of the control group and all infected groups (P<0.05) but no significant difference

between the other three groups.

6.3.8.   Severity of footrot (OVERALLSEV) related to productivity traits

6.3.8.1.   Wool growth

When the measure of footrot severity OVERALLSEV was included in a one way analysis of variance (Table 6.10) the effect of treatment on WOOL was no longer significant, whereas it was highly significant (P<0.01) in the unadjusted analysis (Figure 6.3).

Table 6.10   One way analysis of variance for WOOL including OVERALLSEV

Source	DF	Type 1 SS	Mean square	F value	P value
OVERALLSEV	1	3.9706	3.9706	33.53	0.0001
Treatment	3	0.7252	0.2417	1.76	0.1864
Replicate (within treatment)	20	2.7780	0.1389	1.69	0.1445
Error	111	9.0963	0.0819		

The relationship between wool and OVERALLSEV was quantified using linear regression. The resulting model was

WOOL = 2.66 – 0.14 x OVERALLSEV

The slope was highly significant (P<0.001). The relationship indicates a 5.3% (0.14/2.66) decrease in wool production for every one point increase in severity grade. For example, a sheep with an average of grade 3 severity infection of footrot (two or three score 3 lesions), will be expected to produce 15% less wool during the period of infection than it would if it were uninfected.

6.3.8.2. Liveweight change

When OVERALLSEV and OVERALLPTS were included in a model to examine liveweight change during the period of infection (WTCHANGE), the better predictor was OVERALLSEV but the effect of treatment group was still significant (P<0.01). OVERALLSEV had a highly significant relationship (P=0.0001) with WTCHANGE (Table 6.12, Figure 6.4).

Table 6.11   One way analysis of variance for WTCHANGE including OVERALLSEV

Source	DF	Type 1 SS	Mean square	F value	P value
OVERALLSEV	1	178.26	178.26	25.93	0.0001
Treatment	3	171.74	57.25	7.33	0.0016
Replicate (within treatment)	20	157.75	7.89	1.56	0.0766
Error	111	562.22	5.07		

The linear model relating WTCHANGE to OVERALLSEV was

WTCHANGE = 4.63 – 0.91 x OVERALLSEV

The slope was highly significant (P<0.0001).

Using the same example as for the effect on wool growth, the model predicts a sheep with an average severity score of 3 will gain 2.73 kg less during a period when it would gain 4.63 kg if it were uninfected.

6.3.9.   Isolations of *D nodosus*

Samples for culture were taken from a total of 144 sheep at the three post–challenge inspections (days 56, 85 and 118). Of these, 51 were successfully grown and the serogroup of the isolate determined (Table 6.12). The success rate of isolation was generally low on the first two post–challenge inspections, probably as a result of the muddy conditions in which the sheep were examined and subsequent overgrowth of culture plates with environmental contaminants.

D nodosus was not cultured from specimens collected from any sheep from group CON, despite the presence of score 2 lesions in eight sheep and score 3 lesions in two sheep on day 56. These lesions resembled those of footrot although the extent of underrun in the score 3 lesions was less than 10 mm.

The only serogroups recovered were the introduced serogroups (A and H) and they were only recovered from the groups into which they had been introduced. From group BOTH, into which both serogroups were introduced, both serogroups were recovered.

Isolates were recovered from all replicates of groups VIR, BOTH and INT at some stage of the experiment although isolates were not recovered from every replicate on each of the three post–challenge inspections. Mixed infections were not demonstrated in any one foot of BOTH group sheep.

On most occasions, at least three isolates were grown in pure culture and tested for serogroup.

Table 6.12   Number of sheep providing isolates which were identified to serogroup.
Number of isolates shown in parentheses.

| Day | Group | | | |
---	VIR	BOTH	CON	INT
56	4 A (11)	4 H (13)	0	4 H (9)
		1 A (3)		
85	5 A (11)	2 H (6)	0	3 H (9)
		2 A (7)		
118	9 A (27)	5 H (15)	0	8 H (22)
		6 A (15)		
Total	18 A (49)	11 H (34)	0	13 (40)
		9 A (25)		
Success rate	50%	42%	–	36%
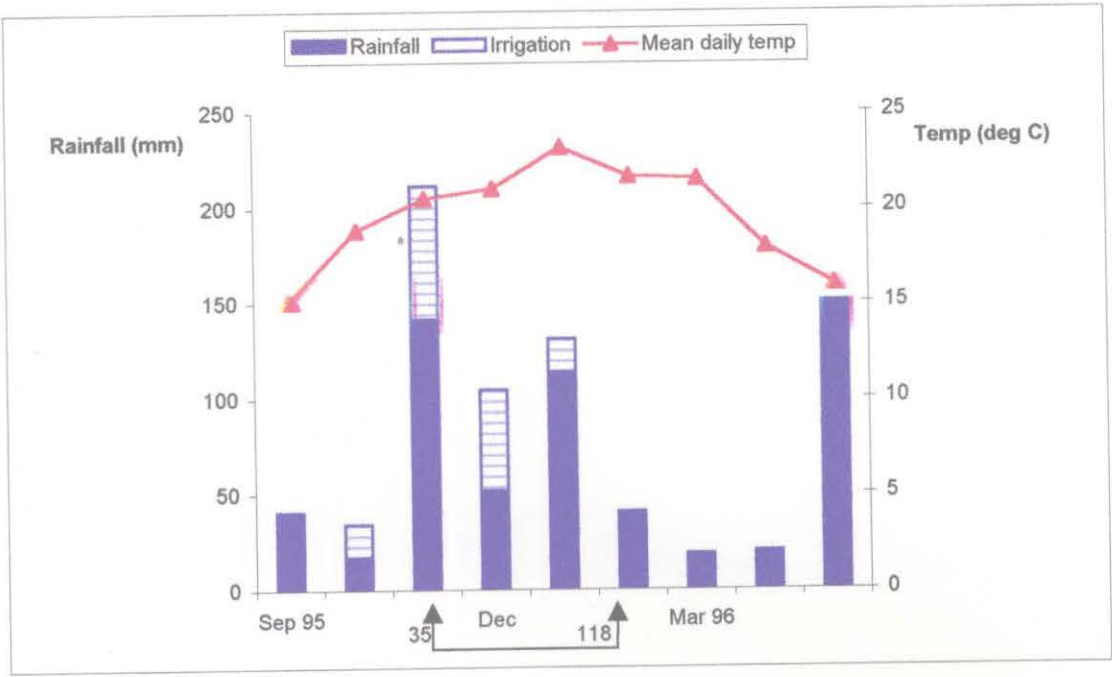
Figure 6.1 Rainfall, irrigation water and mean monthly temperatures at JB Pye Farm. Donors were placed on plots on day 35 (arrowed) in mid November 1995 and were removed 21 days later. The experiment ended on day 118 (arrowed).
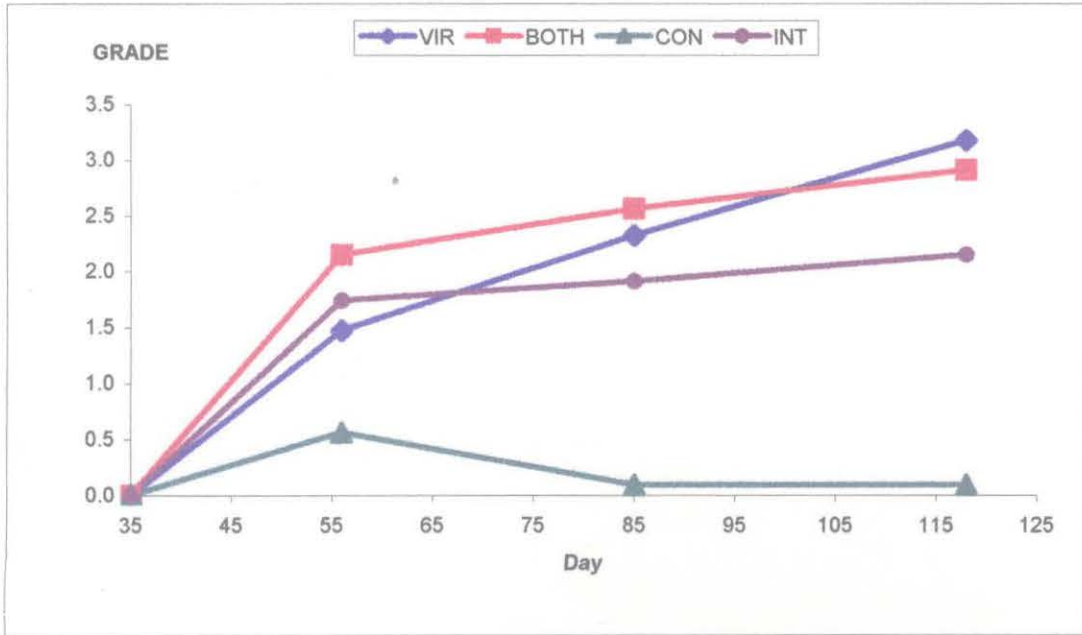
Figure 6.2 Mean footrot severity grade of each treatment group at four inspections over 83 days. Infected donors were introduced into groups VIR, BOTH and INT at day 35, matched by the same number of uninfected sheep in the CON group. Within each group there were six replicates and each replicate contained 5 or 7 sheep. Sheep in group VIR were infected with the virulent strain VCS1001, sheep in group INT were infected with intermediate strain VCS1728 and sheep in group BOTH were infected with both strains. Sheep in group CON were uninfected controls. All three infected groups had significantly different mean severity grades from the control group on days 85 and 118.
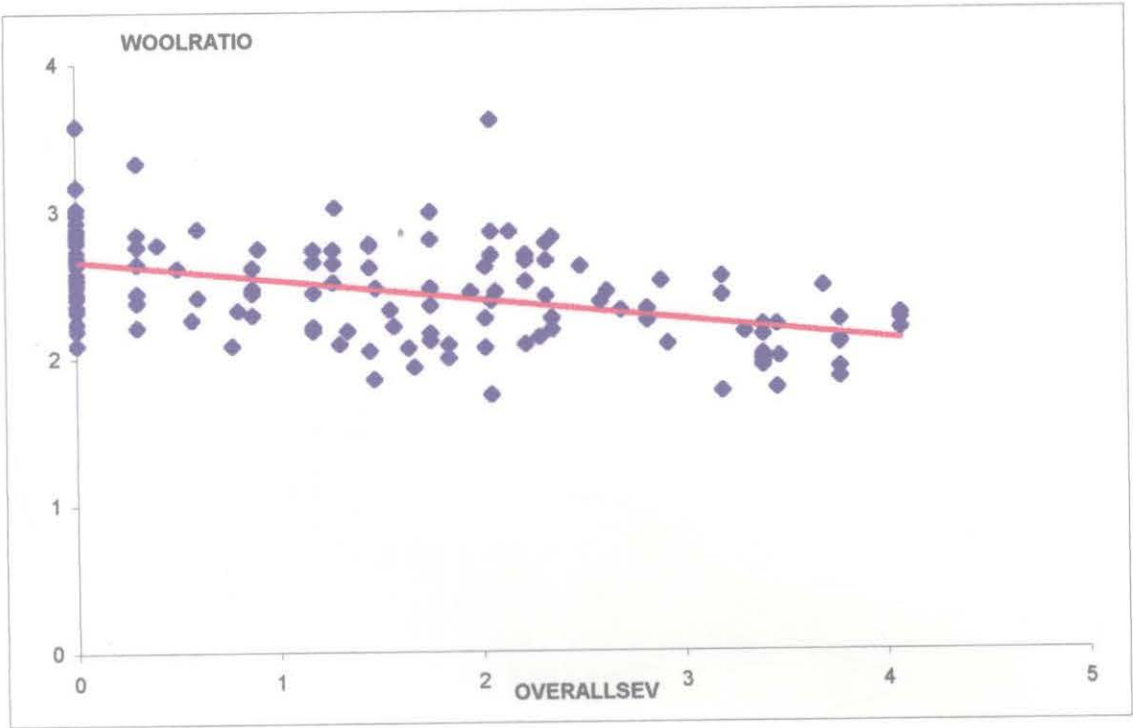
Figure 6.3 Regression plot for the wool growth measure WOOL and the footrot severity grade variable OVERALLSEV. The relationship was calculated for sheep in all treatment groups. The model was :

WOOL = 2.66 - 0.14 OVERALLSEV. WOOL was the ratio of wool grown during the 83 day period of active footrot infection to the amount grown in the 35 day period immediately preceding footrot infection.
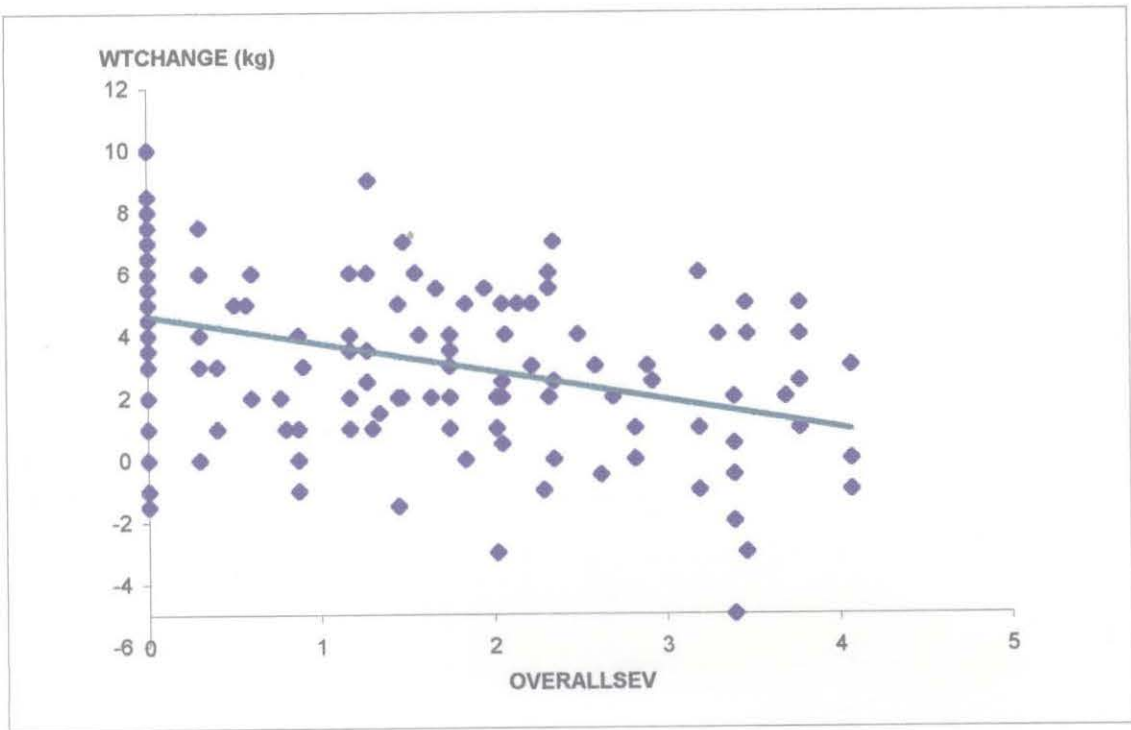
Figure 6.4 Regression plot for the weight change measure WTCHANGE and the footrot severity grade variable OVERALLSEV. The relationship was calculated for sheep in all treatment groups. The model was :

WTCHANGE = 4.63 - 0.91 OVERALLSEV. The variable WTCHANGE was the change in bodyweight of sheep in the 83 day period of active footrot infection.

6.4.   Discussion

6.4.1.   Prevalence of footrot and footscores

In this experiment, sheep were exposed to infection with either virulent footrot, intermediate footrot, a combination of both or were uninfected controls. Their bodyweight change, wool production and footrot lesion scores were recorded over the twelve week period that they were maintained in an environment suitable for footrot transmission and development. There were significant differences between the infecting strains of footrot in the rate at which footrot lesions developed and in the severity of lesions. At the first inspection, 21 days after infected donors were introduced, treatment groups infected with the intermediate strain had significantly more feet affected with footrot than the controls. The group infected only with the virulent strain was not significantly different from the controls. Of the two groups infected with intermediate footrot, the single strain–infected group (INT) was the only group with significantly more score 2 lesions than the control group, while the intermediate plus virulent infection led to a significant number of score 3 lesions in the BOTH group. These data suggest that the intermediate strain was relatively precocious in causing footrot lesions and the presence of the intermediate strain facilitated the development of infection with the virulent strain, allowing it to cause score 3 lesions faster than when it was present alone.

At subsequent inspections at four week and five week intervals, there were no differences between any infected group in the mean number of affected feet, but the distribution of lesion scores differed between groups. The presence of the intermediate strain led to high numbers of score 2 lesions, rather than score 3 and 4 lesions, while the presence of the virulent strain led to a relatively high proportion of score 3 and score 4 lesions. The intermediate strain rapidly developed a characteristic profile of maximum footscore distribution (the relative numbers of score 2: score 3: score 4) which changed little between days 56 and day 118, while the virulent strain profile developed steadily over the 83 day period of observation. This difference was highlighted by the BOTH group which, after 21 days, had a pattern similar to the INT group but with significantly more score 3 lesions, and after 83 days had a pattern similar to the VIR group but a mean number of score 2 lesions between the high number of

the INT group and the low number of the VIR group.

In natural outbreaks of virulent footrot, progression of lesions is often much faster than was recorded in this experiment after day 56 and the number of affected feet and, particularly, the number of severely affected feet, are often much higher than recorded here. The relatively slow development of virulent footrot in this experiment, and the relatively low number of affected feet in the VIR and BOTH groups, suggest that environmental conditions were not highly favourable for disease transmission or development at least not for the whole period. In the first 21 days after donors were introduced, conditions were apparently highly suitable. The experimental site received over 130 mm of rainfall plus irrigation in the three week period, and rain fell on 15 of the 21 days. Over the same period mean daily temperatures averaged 20°C. The control group developed such severe OID that some sheep had mild score 3 lesions and it was feared that *D nodosus* had inadvertently spread to at least two control plots. Over the following four weeks, only 68 mm of rain fell, most of it in one day (41 mm) late in the period (day 54). Although irrigation continued, evaporation rates were higher than had been the case in late November. Lesions in the control group reduced dramatically in both number and severity, and the number of affected feet in the VIR group also declined, although statistically it was not different from the other two infected groups. In the next five week period, 5 January to 7 February 1996, 70 mm of rain fell, the irrigation system failed for a few days and temperatures were several degrees higher. Although footrot progressed in all groups, conditions were less favourable for transmission and lesion progression than they had been from day 35 – 40.

The prevalence data (Table 6.4) suggest that the limiting conditions had at least as much effect on the virulent strain, if not more, than they had on the intermediate strain. The data show that the intermediate strain was equally or better able to spread and persist compared to the virulent strain in relatively unfavourable conditions. The number of score 4 lesions produced by the virulent strain is less than it is known to produce under favourable conditions. The intermediate strain, however, caused four of 34 sheep (12%) in the INT group to develop score 4 lesions, a higher proportion than occurred under natural conditions. Once again, as in Chapter 4 and 5, there is the suggestion that, for intermediate footrot, the occurrence of

slightly unfavourable conditions after an outbreak has commenced actually increase the proportion of sheep with severe lesions, compared to persisting favourable conditions. Intermediate footrot, rather than being limited in its expression by the environment as measured by the proportion of score 4 lesions, in fact appears more severe than when conditions remain wet.

6.4.2.   Effect of footrot on wool growth

Sheep affected with virulent footrot grew 15% (VIR) and 12% (BOTH) less wool during a twelve week period of challenge with footrot than did unaffected control sheep. During a part of this period, footrot infection was spreading from donors to experimental animals and the effect of the disease on production for the first one to two weeks would have been minor. Given that fact and the relatively slow spread and low proportion of severe lesions, 12% to 15% depression of wool growth rates by active infection with virulent footrot is almost certainly an underestimate of its effect in most natural outbreaks.

Comparison of this result to other reports is complicated by the differences in the way wool production has been measured in this and other experiments. Usually, effects of footrot on fleece weight have been assessed by measuring annual production (Marshall *et al* 1991; Glynn 1993), which will normally include periods of wool growth when most sheep are unaffected by footrot. The estimate of Marshall *et al* (1991) of 7.8% and 10% reduction in annual fleece production suggests that the effect of the disease on wool production must be significantly more than 10% during periods of the year when footrot lesions are severe. The relationship is unlikely to be simple, however, given that footrot is most severe in spring, when wool growth rates are highest (Robards 1979).

To measure the effect of VFR on wool production, Symons (1978) used tattooed skin patches, a technique which, like dye banding, measures wool growth over a defined period. In his experiment, wool production for the infected period was not measured until footrot lesions had developed in the infected period, an approach which would be expected to catch the depression of wool production at its peak. The six infected sheep in his experiment had

moderate to severe lesions of footrot and yet the depression in wool production was only 10%, less than measured in the experiment described here, and similar to the effect recorded by Marshall *et al* (1991) on annual production. A likely explanation is the fact that, in the experiment of Symons (1978), sheep were fed in pens and not required to graze, an activity likely to have been limited by lameness.

Stewart *et al* (1984) used a technique very similar to the one used in this experiment and found a significant depressing effect on wool production of infection with VFR relative to both an intermediate strain and a benign strain but, from the data given, it is not possible to estimate the proportional depression in wool growth rates.

Intermediate footrot was found to depress wool production in this experiment, and the estimate of 9.3% reduction in fleece weight compared to the controls approached statistical significance at the 5% level.

A clearer understanding of the relationship between wool production and footrot was presented by the linear regression model, relating a footrot severity grade score (OVERALLSEV, range 0 to 5) to wool production. The analysis found a highly significant relationship between the severity grade and the depression of wool growth rate. The model predicts that wool growth rate will be depressed by 5.3% for every one grade increase in severity and the model is such that predictions could be made for a variety of hypothetical outbreaks. For example, in an outbreak of VFR, one might expect 60% of a flock to have a severity grade of 5, 15% of grade 4, 15% of grade 3 and 10% might average grade 1 (Table 6.2). The flock average grade score is 4.15 and the predicted depression of wool growth rate during the period of infection is 22%. In an outbreak of intermediate footrot, with 2% of the flock with grade 5 severity, 5% of the flock with grade 4, 15% with grade 3, 60% with grade 2 and 18% grade 1, the flock average grade score is 2.13 and the predicted depression of wool growth rate during the period of infection is 11.2%. It will be possible to develop more complex models to compare the economic impact of footrot of differing severity characteristics with relationships such as this, although data on the relative persistence of lesions in the different forms of footrot are still anecdotal.

6.4.3.   Effect of footrot on bodyweight change

All infected groups gained significantly less weight during the 12 week challenge period than the controls. The differences ranged from 3.2 kg less weight gain in the INT group to 3.9 kg in the BOTH group, but differences between the infected groups were not statistically significant. The average bodyweight of the experimental sheep on day 35 was 41 kg, so the relative differences in weight were around 7.5% to 9%. This estimate of the effect of footrot is less than that of Stewart *et al* (1984), from an experiment in which infection was generally more severe, but greater than that found by Symons (1978) in a pen experiment. The result is not readily comparable to the longer–term experiment of Cummins *et al* (1991) and Marshall *et al* (1991), nor with the longer duration of infection with intermediate footrot described in Chapter 5 but, broadly, the results are consistent.

The linear regression model relating overall severity grade to weight change was, like the model for wool growth, highly significant and provided useful insights into the relationship. The model predicts a 0.9 kg difference in weight change over a 12 week period of infection for every one grade increase in severity. The long term effect of the depression of weight change is not easily predicted from the model. Ultimately, a sheep with a feed intake depressed by a stable footrot lesion will cease to change weight relative to uninfected sheep and will maintain a lower bodyweight as a consequence of its lower feed intake. Estimation of the depression in feed energy intake which would lead to a loss of weight or lowered gain in weight can be made using predictive equations (Anon 1990). A difference in gain of 0.9 kg is predicted to arise from a difference in energy intake of 45 megajoules (MJ) of metabolizable energy (ME) which, over 83 days, is approximately 0.5 MJ ME per day. A difference in feed energy intake of 0.5 MJ ME extended during a period of weight maintenance would lead to a difference in liveweight of approximately 5 kg (Anon 1990).

Although this conclusion requires that a number of assumptions, particularly that energy intake differences due to footrot would be similar for the same severity grade regardless of pasture availability or quality, the prediction allows a comparison to the results of Marshall *et al* (1991). My results suggest that a sheep with a grade 4 severity score would maintain a

bodyweight 20 kg less than it would if uninfected. Marshall *et al* (1991) found that a sheep with one foot severely affected with footrot over two years would be 12.3 kg lighter, due to footrot. There is also the suggestion in their data that the bodyweight effect was still increasing at the end of the two year study period, and may well have increased beyond 12.3 kg had the experiment run longer.

In field outbreaks, the average severity score is rarely maintained all year at such high values. With VFR, the flock average severity score may exceed four at the peak of the outbreak, but decline markedly as the environment becomes less suitable for footrot. During this period, it would be expected that sheep which had been affected and then recovered, would regain at least some of the lost weight. Thus, the effects of VFR on bodyweight are not as dramatic as a 20 kg depression across the flock. The approach suggested here, however, is to relate the severity of the footrot lesions and the duration of the lesion to the amount of feed energy ingested, and predict the effect on bodyweight from the estimate of feed energy intake. For example, a flock infected with intermediate footrot (average severity grade of 2 during a transmission period), is predicted to be 1.8 kg lighter after 12 weeks as a result of footrot, but a flock infected with virulent footrot and an average severity score of 4 for the same period will be 3.6 kg lighter.

6.4.4.   Footscoring systems

The scoring system for footrot lesions used in this study was that proposed by Egerton and Roberts (1971), which has been used consistently throughout the studies reported in this thesis. In this system, a footrot lesion is either score 2, score 3 or score 4. Score 1 lesions are non–specific and can be caused by inflammatory processes not associated with *D nodosus*. For some analyses in this thesis, a sheep has been considered to be affected with footrot if it has at least one foot with a score 2, score 3 or score 4 lesion, as did Stewart *et al* (1982b). Different approaches were taken by Marshall *et al* (1991), who considered a sheep to have footrot with a footscore of 1 or greater, and others (Egerton *et al* 1979; Egerton and Thorley 1981) who considered a sheep affected with footrot only if it had at least two feet with score 2 lesions or at least one foot with a score 3 lesion or greater. For some purposes it is

convenient to allocate a single score to affected sheep to reflect the overall severity of the disease in each animal. Such a score can reflect either the extent of the lesion or lesions, or the number of feet affected, or both. Egerton and Roberts (1971) summed the scores from each foot to give a total footscore (TFS) and allocated group mean footscores (GMFS) to affected treatment groups, as did Lee _et al_ (1983), Schwartzkoff and Handley (1986) and Woolaston (1993). Sheep have also been classified by their maximum footscore, as they have in studies reported in Chapters 3 to 5, and this method seems appropriate for describing the advance of footrot through a flock during an outbreak and comparing virulent footrot to intermediate footrot (Allworth and Egerton 1999).

Sheep have been considered to have severe lesions if they have at least one foot with a score 3a lesion or greater (Marshall _et al_ 1991; Depiazzi _et al_ 1998) or at least one foot with a score 3c lesion or greater (Stewart _et al_ 1982b; 1984; 1986b).

Some systems attempt to classify sheep with an overall score reflecting the severity of lesions as a distinct component of the score from the number of affected feet. Raadsma _et al_ (1993) has used an overall animal grade, with a range from 0 to 5. Whittington and Nichols (1995) compared a number of scoring systems and fund that the ones which gave the best indication of immune system responses to infection were those which considered both the severity of the lesions and the number of lesions. In addition, weighting the underrunning lesions and considering all eight claws, rather than just the worst-affected claw on each foot, improved the correlation between footscore and immune response, measured by ELISA.

In the study reported in this chapter, two systems of allocating scores to sheep were compared. The method which was most useful in explaining the effect of footrot on the production traits bodyweight and wool growth rate was the one which allocated a severity grade on a linear scale from 0 (unaffected) to 5 (severely affected). Sheep were allocated to a grade category based on the number of feet affected and the severity of the foot lesion, measured by the 0 to 4 foot score system of Egerton and Roberts (1971). Thus, the system used was an extension of the Egerton and Roberts (1971) method which allowed for variations in the number of feet affected.

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This method was a better predictor of the effect on productivity than one which allocated points to footscores on a sliding scale, with severe lesions awarded more points than mild lesions.

6.4.5. Predictions of production effects from foot score alone

An important outcome of the linear regression analysis of the relationship between OVERALLSEV and WTCHANGE and WOOL was the fact that treatment (virulence of the infecting strain) was not a significant contributor to the model. In other words, the effects on the two production traits could be explained by severity score alone, regardless of the virulence of the strain. The inference from this is that the effect of footrot on productivity can be estimated from a knowledge of footscores and persistence; there is nothing inherent in virulent footrot that causes it to depress productivity more than intermediate footrot beyond the differences in footscore and chronicity of infection.

6.4.6. Synergistic activity in multistrain infections

In this experiment, single strain infections were compared to a bi–strain infection and there was evidence of synergistic activity between the two strains. The evidence was not strong statistically, although the number of score 3 lesions at day 56 in the BOTH group was higher than INT and VIR, similar to the sum of the two, and approaching significance with respect to group VIR. The suggestion lingered at day 85, where the BOTH group was the only group with significantly more score 3 lesions than the controls (Table 6.4).

The implication from these data, and from the way that the dual infection behaved like the fast–establishing intermediate strain early then like the underrunning virulent strain later, that the combined infection had the capacity to adapt to the micro–climatic (hoof level) and macro–climatic (pasture level) environments better than either strain alone. Interestingly, the isolates from the BOTH group were predominantly serogroup H (intermediate) at day 56, and predominantly serogroup A (virulent) at day 118, although these differences were not statistically significant.

6.5.   Summary

One strain of footrot, isolated from an outbreak of footrot which showed characteristics of the intermediate virulence form of the disease, was compared to a virulent strain in an outbreak of footrot under controlled experimental conditions. The single strain infection with each strain showed significant differences in footscores early and late in the 12 week infection period, and in the proportion of severe lesions to mild lesions. This difference in footscore proportions was expected and supported the view that the form of the disease which had been studied and reported in earlier chapters was of intermediate virulence. It was demonstrated that virulent footrot was slower to establish than intermediate footrot but that it caused more score 3 lesions and, particularly, more score 4 lesions. Intermediate footrot was characterised by a high proportion of score 2 lesions, few score 3 lesions and very few score 4 lesions.

The same experiment examined the effects of each strain of footrot, and a combination of the two strains of footrot, on wool growth rate and bodyweight change during a period of active infection. While the experiment detected minor differences between the three treatments in their effects on the two productivity traits, it allowed two models to be developed which related the effect on production to an overall footrot severity grade. These models showed that, regardless of the strain causing the infection, wool growth and weight gain are depressed by footrot lesions and are depressed more by more severe lesions. On a scale ranking footrot severity from 0 (unaffected) to 5 (severely affected) wool production was reduced by 5.3% and weight gain over 12 weeks was reduced by 0.9 kg for every one grade change in footrot severity. These models can be used to estimate the production effects and, therefore, the economic costs of footrot of any virulence type given a knowledge of the severity of footscores and the duration of infection. This knowledge will be useful to producers, advisers and regulators who are evaluating the cost–benefit of eradication of footrot.

CONTENTS

CHAPTER 7

THE USE OF MOLECULAR EPIDEMIOLOGY IN STUDIES OF OVINE FOOTROT

7.1. Introduction

Molecular genetic techniques offer the opportunity to identify bacterial genera, species and strains within species cheaply and quickly. The sensitivity of many of these techniques relies on the polymerase chain reaction (PCR) (Mullis and Faloona 1987) which can reproduce specific parts of the bacterial genome to levels at which visualisation techniques can detect the millions of copies of DNA product.

Identification of bacteria by examination of their genetic characteristics can offer some advantages over phenotypic characterisations, such as measurement of bacterial secretory products, and surface antigens or colony morphology, beyond the advantages of cost and speed. Genetic characteristics may be more constant features of a bacterial species than phenotypic characters, which vary with the environment in which they are grown (Liu and Yong 1997).

To be useful, therefore, in taxonomy and epidemiological studies, areas of the bacterial genome which are to be used in this way must be stable within the classification level under study, yet show differences from closely related genetic lines of the superior taxonomic level.

Generally, for these techniques to be applicable in epidemiological studies of disease caused by bacteria, the classification level of interest is *strains* within *species*. Hence, in footrot epidemiology, while *D nodosus* is the bacterial species of interest, epidemiological studies of footrot outbreaks require the identification of strains of the species. Strain identification techniques are useful in studies such as those described in this thesis, in which the relationship of the environment to the bacterial flora associated with the footrot outbreak has been examined. In order to examine the effect of two or more different environments on the form of the outbreaks, it is necessary to demonstrate that the bacteria responsible are of the same genotype in each environment.

In the absence of genetic techniques, a range of phenotypic characterisations has been used for this purpose. The most commonly used has been based on the antigenic characteristic of the bacteria, either serogroup or serotype. At present, in Australia, there are 19 serotypes recognised within 10 major serogroups (Claxton 1986, Ghimire *et al* 1999). The immunogen used to produce antisera is the K antigen (Egerton 1973) associated with the fimbriae on the surface of *D nodosus* cells (Stewart 1973).

There are a number of problems associated with using serotyping for epidemiological studies. First, there is no relationship between virulence and serotype (Claxton 1989). Second, outbreaks of footrot frequently involve more than one serogroup of *D nodosus*, and may involve six or more, as found in this study and others (Claxton *et al* 1983). If the occurrence of serogroups in an outbreak is considered a random event, the probability of finding one serogroup represented in two distinct strains when six strains are present is $1 - .9^5$, or 47%. Similarly, the probability of two serotypes occurring twice is 24%. Third, there is some evidence that strains of *D nodosus* may change antigenicity. Raadsma *et al* (1994) described the isolation of a virulent isolate of serogroup G from lesions in an outbreak where previously a benign isolate of that serogroup had existed in a mixed infection with virulent strains of serogroups A and H. They suggested that there may have been a change in fimbrial expression of one of the virulent strains, or that genetic recombination involving virulence genes had occurred between strains of the different serogroups. Allworth (1995) observed a similar occurrence in isolates collected during a field study and concluded that recombination between strains was a more likely explanation than rearrangement of genes within a genome.

What is required, therefore, to identify strains of *D nodosus* for epidemiological studies is a characteristic which is stable within strains, *ie*, is unlikely to change in the course of a study, but which shows much greater variation between strains than the 19 variations in serotype that have so far been described. The discovery (Linn and Arber 1968) and application (Smith and Wilcox 1970) of restriction endonucleases has offered some promise as a molecular epidemiological tool.

Restriction endonucleases (RE) are enzymes which cut DNA molecules by breaking the phosphodiester bonds between nucleic acids. Some of these REs cut DNA at specific positions (restriction sites) which are determined by the sequence of bases at the restriction site and it is these site-specific endonucleases which are most useful in molecular biology. When genomic DNA is digested with REs the DNA is cut into fragments of varying length which can be separated by using gel electrophoresis. When gels are stained with dyes that bind to DNA, a pattern of bands is produced which can represent a 'genetic fingerprint' of a particular bacterial strain (Dale 1998).

Various genetic events can change the size of the fragments produced by digestion with a particular RE. An insertion or deletion event between restriction sites may make fragments larger or smaller, or a mutation at the restriction site may prevent the DNA being cut at that position, thus two fragments become one larger one. The variation in size of the fragments produced by RE digestion is called *restriction fragment length polymorphism* (RFLP). Genetic polymorphism of bacterial strains within species that can be exploited through *restriction endonuclease analysis* (REA) has the potential to be used in molecular epidemiology.

McGillivery *et al* (1989) used an RE (*Bam*HI) to digest the whole genome of *D nodosus* isolates from a number of sources. REA showed that isolates from unrelated sources produced different patterns, regardless of serogroup or virulence. Further, isolates from outbreaks which were believed to be related were shown to have the same patterns, confirming the relatedness of the outbreaks.

While the technique was successful, REA of the whole genome with a 'frequent cutter' like *Bam*HI produces many hundreds of bands which can make differentiation between genetic fingerprints difficult. Before REA could be applied more widely it was necessary to identify a smaller portion of the *D nodosus* chromosome which was unique to the species, present in some form in all strains of the species but showing variability between isolated strains.

Moses (1993) and Moses *et al* (1995) described aspects of the genetic expression of the major outer membrane protein (Omp1) of *D nodosus*. Using VCS1001, they identified four *omp1* genes in the *D nodosus* chromosome and described a gene inversion system which led to a

structural arrangement within the genome such that only one gene would be expressed in any one *D nodosus* cell. Limited variation in the structure of these genes led to differences in the structure of the Omp1 proteins produced by each of these genes.

Parts of each of these genes, *omp1A*, *omp1B*, *omp1D* and *omp1E*, were sequenced and some regions were found to be highly conserved across all four genes. The sequences of two conserved regions, about 490 bases apart in each gene and separated by several variable regions, were selected as primers (primer A and primer C) for PCR amplification of these four regions of the chromosome. Moses predicted that four fragments, between 465 and 489 base pairs (bp) in length, would be produced but the close similarity in size of the four fragments prevented easy identification of four different bands after gel electrophoresis. The areas of different DNA sequence within each fragment did, however, present restriction sites at different locations within the fragments, so digestion with a restriction endonuclease could produce a number of fragments with more variation in fragment length than the four undigested PCR products. If so, these fragments would form a pattern of bands after electrophoresis which would allow the construction of four restriction maps as predicted by the sequence analysis.

Using this technique with the RE *Sau*3A, Moses (1993) was able to demonstrate that his predictions were correct and the band pattern on agarose gel was consistent with the production of 10 fragments from 5 bp to 488 bp long. Further, the presence of only the predicted fragments demonstrated that there were no more than four variants of the *omp1* gene present in the genome of VCS1001.

To determine the degree of conservation of these genes in *D nodosus* of serotypes other than A_1 (VCS1001), representatives of 15 further serotypes were used in PCR reactions (Moses 1993). PCR products of approximately 500 bp were produced from each isolate. With one possible exception, two or more polymorphic fragments were produced from each strain. It was not possible to determine how many fragments were produced in all cases because, if multiple fragments were produced, they were likely to be of similar size.

The variation in size of PCR products between isolates, which was evident from the slightly different positions of bands produced by undigested PCR products, led Moses and coworkers

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(RT Good, EK Moses and CJ Langford, cited by Moses 1993) to exploit polymorphism within the fragments to produce unique genetic fingerprints for a range of *D nodosus* isolates by the action of an RE on PCR products of primers A and C. Further studies of two additional *D nodosus* strains suggested that these two strains contained only *omp1A* and *omp1D* genes, introducing a further level of variation between strains in addition to polymorphism in structure of the regions amplified by primers A and C.

The first reported application of this technique to molecular epidemiology was by Allworth (1995) who determined the PCR-RFLP fingerprints of a range of isolates using primers A and C (Moses 1993) and the RE *Hpa*II. In a survey of 12 flocks, 37 isolates were characterised by slide agglutination to serogroup, and *Hpa*II fingerprints. Twenty four different patterns were found. In general, the more isolations made, the more serogroups and the more patterns were found. In the flock where the most isolates were made (eight), seven different PCR-RFLP fingerprints were found amongst a total of four serogroups.

To that author's knowledge, all 12 flocks shared no footrot epidemiological history and no pattern was found in more than one flock. Ghimire and Egerton (1999), who also used PCR-RFLP of the *omp* gene with *Hpa*II, identified 11 distinct patterns, but only four serogroups, from 66 isolates collected from migratory flocks in Nepal.

In an experimental flock, Allworth (1995) was able to use the PCR-RFLP technique to provide more convincing evidence that two benign isolates persisted through an eradication program which successfully eradicated virulent strains. The evidence he presented was the presence, post-'eradication', of an isolate of serogroup B with the same pattern as the serogroup B isolate he introduced pre-'eradication', and similarly for a serogroup C isolate. The fact the same *omp* gene fingerprint was present with the same serogroup was much more convincing evidence that eradication had failed with these two isolates than would have been the case had he based his claim on serogroup alone.

The same experiment also showed that, for some strains, the relationship between serogroup and genetic fingerprint remained constant in isolates collected over a period of several weeks, even in mixed infections. Other isolates were collected, however, which indicated that

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previously undetected patterns were occurring. Six patterns were known to be introduced into his experimental flock but eight were recovered. Another isolate, of serogroup H, was found to have two distinct fingerprints and the author was not able to determine if the second fingerprint was the result of a change which had occurred after the introduction of the strain or if the serogroup had been inadvertently introduced as a mixed (two patterns, two strains) infection. Interestingly, the pattern which was detected after the introduction also occurred in association with a different serogroup, indicating mobility between strains of either *omp* genes, serogroup or both.

When *Hpa*II fingerprinting was used to investigate the epidemiology of footrot in flocks between which transmission of *D nodosus* may have occurred, flocks were found to have a different 'set' of patterns with none in common between flocks except in cases where there was also a known movement of footrot-affected sheep from one flock to another.

Within flocks, different serogroups could have the same or different patterns. In his studies, Allworth (1995) found that two isolates of different serogroup had the same pattern within flocks in five flocks, and in one case, four serogroups shared one pattern. Ghimire and Egerton (1999) found two (on two occasions) and three serogroups (once) with the same genetic fingerprint in Nepalese flocks. Because both serogroups and *omp* gene fingerprints remain constant within flocks but change association with each other, it appears that exchange of genetic material between strains occurs. Evidence of frequent recombination events in the region of the fimbrial subunit gene *fimA* suggest that exchange of genes determining serogroup between strains is likely in these cases (Ghimire 1997).

The implication of these findings, and the fact that the same fingerprint was not found in unrelated flocks, is that PCR-RFLP may be useful to show that isolates from two different sources are related, if their fingerprints are the same, even if their serogroup is different. The technique may have limitations, however, in determining that two isolates are not of the same origin if their patterns are found to be different. If the *D nodosus* flora of two outbreaks are compared and, for example, a fingerprint is found in the second which was not detected in the first, the 'new' fingerprint may have come from a genetic mutation, or it may have been already present in the sheep of the second outbreak, or it may have been present, but undetected, in the

sheep of the first outbreak.

In the studies described in Chapters 3, 4 and 5 of this thesis, there was a need to show that the *D nodosus* strains present at the start of an investigation were the same as those present at some later date. In the eradication study at property F (Chapter 3) it was felt that evidence that the same strains persisted in an untreated control flock, while they were eradicated from the main flock, would confirm that the procedures used were responsible for the disappearance of the strain, rather that the environment. Further, if benign footrot recurs in this flock it will be useful to compare the *D nodosus* strains with those identified in 1993.

In the studies described in Chapters 4 and 5, evidence was sought that the same mixture of strains of *D nodosus* were present in flocks of sheep run in difference environments. It was recognised that serogroup and virulence tests were inadequate epidemiological markers to achieve this because of their limited variability and, in the case of virulence tests, lack of repeatability.

It was decided to evaluate PCR-RFLP of the *omp* gene as an epidemiological tool in both of these separate studies. The results of that evaluation are reported in this chapter in addition to the brief results which have been presented in Chapters 3, 4 and 5.

It was also felt that further evidence of the genetic stability of PCR-RFLP *omp* gene fingerprints over time in the one strain should be investigated and the the archival material of the Department of Clinical Sciences presented this opportunity. These results are also presented here.

7.2.   Materials and methods

7.2.1.   Bacteria

7.2.1.1.   VCS1001

Strain VCS1001 is the prototype of serogroup A, serotype 1 and is held in the collection of the Department of Veterinary Clinical Sciences, University of Sydney. The strain originated from strain A198 (ATCC 25549), held by the Commonwealth Scientific and Industrial Research Organisation in Sydney (Claxton *et al* 1983) and isolated originally from a flock at Goulburn, NSW.

Twenty nine isolates believed to be clones of VCS1001 from the freeze dry collection of the Department of Veterinary Clinical Sciences were selected for PCR-RFLP. The samples represented the type strain of virulent *D nodosus* held in the Department which had been used in field experiments and re-isolated from sheep, purified, and returned to the collection, over a period of 23 years. The histories of the 29 isolates are tabulated in Table 7.1. All isolates were expected to be clones of the original accession of VCS1001 which entered the Department collection in 1972. Often, when *D nodosus* was re-isolated from sheep after field challenges, an isolate which was believed to be of the same strain as the original VCS1001 was lodged in the collection. The field re-isolations were not named in the same series as 1001, but given a new accession number.

Sixteen of the isolates tested in this study were field isolates, the other 13 were selected directly from the collection of lyophilised VCS1001. Isolates were selected for testing with the objective of covering a wide range of histories, including isolates which were closely related, some which were descended from different sub-culture pathways of the primary accession, and some which had been frequently sub-cultured, stored for many years, and used in field experiments (Table 7.1).

7.2.1.2. Other *D nodosus* isolates tested with PCR-RFLP

Other *D nodosus* strains tested and reported in this chapter were collected at property F (see Chapter 3), property B, property W, property K and property T (see Chapters 4 and 5). The latter four properties ran sheep which were originally from property B and property W, two properties in the Monaro district of NSW, so the flocks at these four study sites are referred to as the Monaro-origin flocks.

7.2.2. PCR-RFLP procedures

The procedures used to produce genetic fingerprints through PCR-RFLP procedures involving the *omp* gene region of isolates of *D nodosus* have been described in Chapter 2.

Table 7.1 History of isolates of VCS1001 used in examination of the stability of PCR-RFLP genetic fingerprint (*omp* gene)

Sample number	Freeze dry num	Comment
1	1001/12	Fourth level sub-culture from original accession, through 'E'
2	1001/12	As above
3	1001/12	As above
4	1001/12	As above
5	1001/aa	Sixth level sub-culture from original accession, through 'B'
6	1733	Re-isolated from a sheep challenged with 1001/7
7	1734	Re-isolated from a sheep challenged with 1001/7
8	1732	Re-isolated from a sheep challenged with 1001/7
9	1001/12	As above
10	1001/12	As above
11	1001/G	Second level sub-culture from original accession, through 'B'
12	2201/B	Re-isolated from a sheep challenged with 1001/11
13	2272/A	Re-isolated from a sheep challenged with 1001/11
14	1640B	Re-isolated from a sheep challenged with 1001/3
15	1664	Isolate involved in 1987 attempts to develop streptomycin resistance.
16	1585	Re-isolated from a sheep challenged with 1001/ab
17	1581	Re-isolated from a sheep challenged with 1001/1
18	1586	Re-isolated from a sheep challenged with 1001/ab
19	1587	Re-isolated from a sheep challenged with 1001/ab
20	1588	Re-isolated from a sheep challenged with 1001/ab
21	1659	Re-isolated from a sheep challenged with 1001 suffix unknown 1985)
22	2202	Re-isolated from a sheep challenged with 1001/11
23	2213A	Re-isolated from a sheep challenged with 1001/11
24	1223	Re-isolated from a sheep challenged with 1001 suffix unknown 1978)
25	1604	Re-isolated from a sheep challenged with 1001/G
26	1625B	Re-isolated from a sheep challenged with 1001/2
27	1626B	Re-isolated from a sheep challenged with 1001/2
28	1730	Re-isolated from a sheep challenged with 1001/10
29	2244A	Re-isolated from a sheep challenged with 1001/11

7.3.   Results

7.3.1.   Stability of genetic fingerprints within a strain

7.3.1.1.   VCS1001

PCR amplification of genomic DNA from all 29 isolates of VCS1001 with primers A and C (Moses 1993) produced DNA fragments which, after electrophoresis in an agarose gel, were estimated to be 520 to 540 bp long by comparison with a molecular weight marker . There was no evidence of any fragments less than 520 bp, contrary to the findings of Moses (1993). Digestion with *Sau*3AI was predicted to produce one fragment of 488 bp from the *omp1E* gene which does not have a *Sau*3AI restriction site. In the studies reported in this thesis there was evidence of a fragment of the same approximate length as undigested product, but it was clearly and consistently approximately 520 bp in length (Figure 7.1b), 32 bp longer than expected.

The second and third longest fragments found by *Sau*3AI digestion of VCS 1001 by Moses (1993) were 305 and 275 bp. In this study, however, the band produced by the second largest fragment(s) was only slightly smaller than the 331 bp band of the marker and, therefore, approximately 20 bp longer than predicted by Moses (1993) (Figure 7.1b).

Using the sequence information published by Moses (1993) it is possible to predict the length of segments produced by digestion of PCR product from VCS1001 with *Hpa*II. The four amplified *omp* gene segments would be expected to be cut into the fragment lengths shown in Table 7.2.

*Hpa*II digestion of VCS 1001 in this study produced a series of bands all of which corresponded to bands less than 500 bp (Figure 7.1a), as expected, but there appeared to be a large fragment of approximately 480 bp, rather than the one expected of 446 bp, and a second band produced by a fragment of approximately 270 bp, which was also larger than expected. The pattern was similar to that reported by Allworth (1995) with the same strain.

Table 7.2 **DNA fragment lengths produced by *Hpa*II digestion of *omp1* gene PCR products, based on sequence information published by Moses (1993)**

Omp gene	Fragment lengths (bp)				
ompA	253	211			
ompB	214	196	79		
ompD	123	123	105	61	42
ompE	446	42			

To examine the genetic fingerprints of all 29 isolates of VCS1001, digested samples were subjected to electrophoresis in either 2.5% agarose gels or 4-20% gradient polyacrylamide gels (Novex, San Diego, CA). The gradient gels gave a better resolution of bands than the homogenous agarose gels so, thereafter, gradient polyacrylamide gels were preferred for visualisation of RFLPs, rather than agarose gels. The patterns of sample numbers 2, 3, 4 and 5 (see Table 7.1) produced on agarose gels are shown in Figure 7.1a and b. The *Hpa*II pattern produced by one isolate in a gradient gel is reproduced in Figure 7.3c. All 29 isolates of VCS1001 gave identical genetic fingerprints with both *Hpa*II and *Sau*3AI.

7.3.1.2. Samples from property F flock (Chapter 3)

Eleven isolates collected from property F were subjected to *omp* gene PCR-RFLP with the restriction enzymes, *Hpa*II and *Sau*3A. The isolates, whose origins were described in Chapter 3, represented two serogroups although serogroup I was isolated only once.

PCR amplification of genomic DNA of all 11 isolates produced PCR products which appeared, following agarose gel electrophoresis, to be identical in size at approximately 520 to 540 bp (Figure 7.2). A faint band evident at approximately 330 bp was believed to be a result of non-specific primer annealing and not PCR product. To test this hypothesis, DNA from the larger fragment was extracted from the gel, purified and subject to RFLP with *Hpa*II. Patterns obtained from electrophoresis of these digests were identical to those obtained when all DNA

produced from the PCR reaction was subject to digestion (data not shown), indicating that the smaller fragment was not responsible for any visible bands on the *Hpa*II fingerprints. The band corresponding to a fragment length of 520 to 540 bp was likely to consist of at least two fragments of similar size, according to the prediction of Moses (1993).

The procedure with *Hpa*II was duplicated, the second set of procedures was performed on isolates which had been lyophilised from cultures which were grown and sub-cultured for the first PCR-RFLP. The *Hpa*II genetic fingerprints of both sets of accessions of the same isolate were identical. The results are summarised in Table 7.3 and illustrated in Figure 7.3.

There were two distinct *Hpa*II patterns evident. These were named D1 and D2. Pattern D1 had two bands, corresponding to fragments of approximately 470 and 480 bp, a triple group of bands corresponding to fragments of approximately 240, 250 and 265 bp, then a band corresponding to a fragment of 200 bp. Visible bands of lesser fragments corresponded to fragment sizes of 140, 115, 105 and 70 bp. The sum of fragments corresponding to these 10 bands exceeds 2 300 bp, suggesting that at least four different PCR products of 520 to 540 bp were present before digestion. The absence of any band corresponding to undigested product (in the range of 520 to 540 bp) suggests that all four genes have at least one *Hpa*II site. Further, the presence of two fragments exceeding 460 bp suggests that two of the genes may have only one *Hpa*II site, or two or more sites close to each other and close to one end of the fragment.

Pattern D2 had no bands evident for fragments greater than 300 bp long. There was a double band of fragments of approximately 250 and 260 bp, corresponding to the 240, 250, 265 bp triple band of pattern D1, and four bands of shorter fragments which appear identical to those of D1. In addition, a faint band was evident between the 67 bp and 34 bp bands of the marker.

The two patterns produced by all 11 isolates were distinct from those of VCS1001 (Figure 7.3c), which had one band at approximately 480 bp, and only two bands between the 190 bp and 331 bp markers, not three or four as is the case with the property F samples. The four bands below the 147 bp marker, however, are very similar in distribution in both VCS1001 and the two property F patterns.

Patterns D1 and D2 are similar to each other, particularly when compared to the pattern produced by VCS1001. The similarity of the two patterns within the flock suggest that the two strains may be related, one being derived from the other.

Table 7.3   PCR-RFLP patterns obtained from isolates collected in property F flock

Isolate number	Serogroup	Date collected	*Hpa*II pattern	*Sau*3A pattern
59	A	15 Dec 92	D2	DS1
60	I	15 Dec 92	D1	DS1
61	A	15 Dec 92	D2	DS1
62	A	15 Dec 92	D2	DS1
91	A	3 Mar 94	D1	DS1
92	A	3 Mar 94	D1	DS1
93	A	3 Mar 94	D1	DS1
100	A	19 Oct 94	D1	DS1
101	A	19 Oct 94	D1	DS1
102	A	19 Oct 94	D1	DS1
103	A	19 Oct 94	D1	DS1

Digestion with *Sau*3A produced eight bands, corresponding to fragment lengths of 495, 380, 325, 235, 190, 150 and 100 bp. The sum of all eight fragment lengths detected was 1 875 bp, again suggesting that at least four PCR products were produced, each with different *Sau*3A restriction sites, assuming that the four products all were approximately 520 to 540 bp, as indicated by the electrophoresis of the PCR products. With *Sau*3A digestion, it was not possible to discriminate confidently between the strains which had been differentiated by *Hpa*II (Figure 7.4). Additional bands from DNA fragments greater than 500 bp were visible after some reactions but these bands probably represented undigested product. The largest fragments visible in some lanes were approximately 530 bp, similar to the expected size of undigested product.

7.3.1.3.   Samples from property B, property W, property K and property T (Chapters 4 and 5)

In the studies described in Chapters 4 and 5, there was a continuity of infected sheep from the wether flock at property B, in the Monaro, which were derived from the breeding flock at property W, with the experiment at property K, near Tarcutta, and at property T at Holbrook.

It was desirable to show with as much certainty as possible that the isolates of *D nodosus* which were responsible for the outbreaks of intermediate footrot at property B and at property W were the same genetically as those associated with the outbreaks at properties K and T. It was considered highly probable that the same strains would be present at all sites but it was possible that some genetic diversity may be lost during the translocation of the sheep, either by chance, when the number of infected sheep was small, or because of an effect of the environment at the different sites being more suitable for some strains than for others.

Part of the aims of these studies was to evaluate PCR-RFLP of the *omp1* genes as a tool for molecular epidemiology. Consequently, 34 isolates of six different serogroups, collected at different times during the outbreaks and at different sites were subjected to PCR-RFLP. All PCR-RFLP *Hpa*II procedures on each isolate reported here were repeated at least once.

Nine patterns were identified; these were designated 1, 1a, 1b, 1c, 2, 4, 5, 6 and 7 (Tables 7.4, 7.5, Figures 7.5, 7.6).

The four patterns grouped together (1, 1a, 1b, 1c) were relatively similar to each other, in comparison to the other five patterns.

Again, there was no clear association between serogroup and RFLP pattern. The serogroup with the most isolates tested (serogroup H) produced seven patterns from 18 tests. Six isolates of serogroup C produced three patterns and five isolates of serogroup B produced four patterns. Two patterns were limited to specific serogroups; pattern 1a, which occurred only in serogroup A and pattern 7, seen only in five isolates of serogroup H. There were only two isolates of serogroup A subjected to PCR-RFLP.

The patterns seen most often were patterns 1 and 5 which occurred in isolates of three and two serogroups respectively. Patterns 2 and 7 were also common and occurred in three serogroups and one serogroup respectively (Table 7.5).

An isolate of serogroup H from a flock of sheep belonging to the owner of property F but managed at a different site was compared with the serogroup H isolates recovered from the Monaro-origin flocks. The pattern of this isolate (pattern 3, Figure 7.6) was distinct from all seven patterns of the H serogroup isolates from the Monaro flocks.

Fifteen isolates were subjected to PCR-RFLP with *Sau*3AI as well as *Hpa*II. The 15 isolates which yielded seven patterns with *Hpa*II were classified into five patterns (designated S1 to S5) with *Sau*3AI (Table 7.6). There was some consistency between the classifications produced by both REs. All three pattern 5 isolates were pattern S1 and there were no other S1 pattern isolates. Similarly, all S3 isolates were pattern 7, but one pattern 7 isolate was pattern S2. Interestingly, the isolate with pattern 1c, which differed from pattern 1 only by the absence of bands of approximately 460 and 300 bp (Figure 7.6), produced a unique *Sau*3AI pattern, S5, which differed from pattern S2 only by the absence of bands of 480, 190, 150 and 100 bp. In both cases, *ie* patterns 1/1c and patterns S2/S5, all visible bands which were present were in identical positions, implying that the isolate involved had one or, more likely, two fewer variant *omp1* genes than isolates with the 'parent' pattern.

There was continuity of isolates of particular patterns over the 26 months of the experiment and at all four sites. Patterns 5 and 7 was detected in isolates from property B in Spring 1992, property K in 1993 or 1994 and property T in November 1994 (Table 7.3). Pattern 2 was seen in isolates collected in October 1992, July 1993 and June 1994. Pattern 1 isolations covered a period from October 1992 to January 1994. The last pattern to be detected was pattern 6, from an isolate collected in November 1993 and seen again in an isolate from property T in November 1994. Patterns 1, 1a, 1b, 2, 4, 5 and 7 were demonstrable in the Monaro flock at home (property B or W) and in the flock after the move to the alternate sites (property K or T).

Table 7.4   Isolates collected from related flocks in 4 locations, with RFLP pattern. Isolates 81 and 82 were not recovered in the course of the study but from property W in a return visit to collect further isolates after the study area had moved to property K.

Isolate number	Serogroup	*Hpa*II pattern	*Sau*3AI pattern	Site of isolation	Date of isolation
26	H	5	S1	Property B	September 1992
27	H	5		Property B	September 1992
30	H	2		Property B	July 1992
34	C	4		Property B	October 1992
35	H	1	S2	Property B	October 1992
36	C	1		Property B	October 1992
37	B	2		Property B	October 1992
38	H	1c	S5	Property B	October 1992
39	C	4		Property B	October 1992
40	H	7	S2	Property B	October 1992
41	A	1a		Property B	October 1992
42	B	5		Property B	October 1992
43	B	1b		Property B	October 1992
53	C	2		Property B	December 1992
54	H	4	S2	Property W	December 1992
55	H	1		Property W	December 1992
56	H	1	S4	Property W	December 1992
63	G	1		Property W	December 1992
77	I	4		Property K	July 1993
78	A	1a		Property K	July 1993
79	H	2	S2	Property K	July 1993
80	H	7	S3	Property K	July 1993
81	H	1b	S2	Property W	August 1993
82	H	1b	S2	Property W	August 1993
83	I	6		Property K	November 1993
90	C	1		Property K	January 1994
97	B	5		Property K	June 1994
98	H	5	S1	Property K	June 1994

99	C	2		Property K	June 1994
104	B	6		Property T	November 1994
105	H	7	S3	Property T	November 1994
106	H	5	S1	Property T	November 1994
107	H	7	S3	Property T	November 1994
108	H	7	S3	Property T	November 1994

Table 7.5   Classification of PCR-RFLP patterns by serogroup for isolates from property B, property W, property K and property T outbreaks

RFLP pattern	A	B	C	G	H	I	Total
1			2	1	3		6
1a	2						2
1b		1			2		3
1c					1		1
2		1	2		2		5
4			2		1	1	4
5		2			4		6
6		1				1	2
7					5		5
Total	2	5	6	1	18	2	34

Table 7.6 Cross-classification of PCR-RFLP patterns with restriction endonucleases *Hpa*II and *Sau*3A

*Hpa*II pattern	*Sau*3AI pattern					
	S1	S2	S3	S4	S5	Total
1		1		1		2
1b		2				2
1c					1	1
2		1				1
4		1				1
5	3					3
7		1	4			5
Total	3	6	4	1	1	15

Figure 7.1 PCR-RFLP fingerprints of *omp1* genes of *Dichelobacter nodosus* VCS1001 using restriction endonucleases *Hpa*II (a) and *Sau*3AI (b) visualised on a 2.5% agarose gel.

A group of isolates of *D nodosus* strain VCS1001 were selected from the collection of the Department of Veterinary Clinical Sciences and cultured on hoof agar plates. Genomic DNA from cultures of each isolate was used as a template for a PCR using primers A and C (Moses 1993) specific for highly conserved regions of the multiple copies of the *omp1* gene which show internal sequence heterogeneity (see Chapter 2 for sequence of primers).

PCR product, which is a mixture of possibly four different, but similar length fragments corresponding to the four variably structured *omp1* genes, was digested with each restriction endonuclease, subjected to electrophoresis in a 2.5% agarose gel and stained with ethidium bromide. The consistent pattern of bands (genetic fingerprint) for the isolates of the same strain is shown in this figure for four of the isolates.

The reaction mixture consisted of PCR buffer, one unit of *Taq* DNA polymerase (Life Technologies, USA) and final concentrations of 3 mM $MgCl_2$, 0.4μM of each primer and 200μM of each of the four deoxynucleotide triphosphates. Sterile deionised water was used to make a final volume of 20 μl after the addition of DNA template, the volume of which was optimised for each bacterial sample. Each PCR reaction was performed in 20 μl capillary tubes. A touchdown PCR procedure (Don *et al*, 1991) was used. The amplification cycle commenced with denaturation at 94°C for 2 minutes followed by two cycles of 94°C for 5 seconds, 56°C for 5 seconds and 72°C for 30 seconds. The annealing temperature was decreased by 1°C every two cycles. At 50°C the cycle was repeated 20 times before a final extension at 72°C for 2 minutes.

Restriction endonuclease digests were carried out in solutions containing buffer solution, 2 μg of acetylated bovine serum albumin (BSA) and 1μl (10 units) of *Hpa*II or *Sau*3AI (Promega Corporation, Madison, Wisconsin). Fifteen μl of solution containing the PCR products (approximately 1 μg of DNA) was added and sterile deionised water was used to make a final volume of 20 μl. All reactions were carried out in sterile 0.5 ml Eppendorf tubes at 37°C overnight.

(a) *Hpa*II
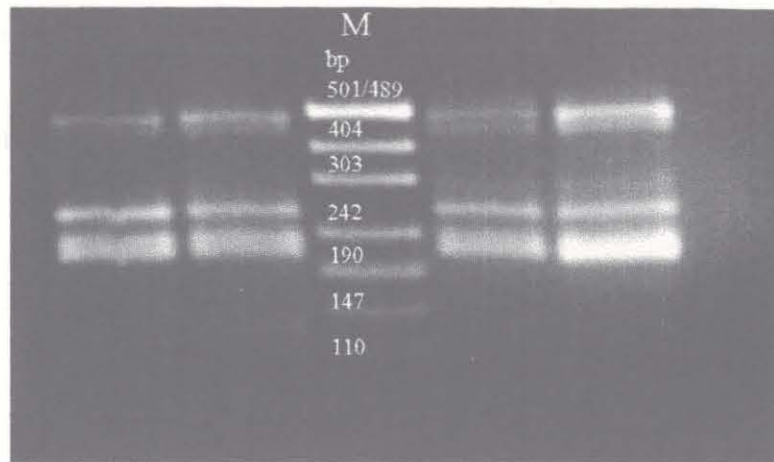
(b) *Sau*3AI
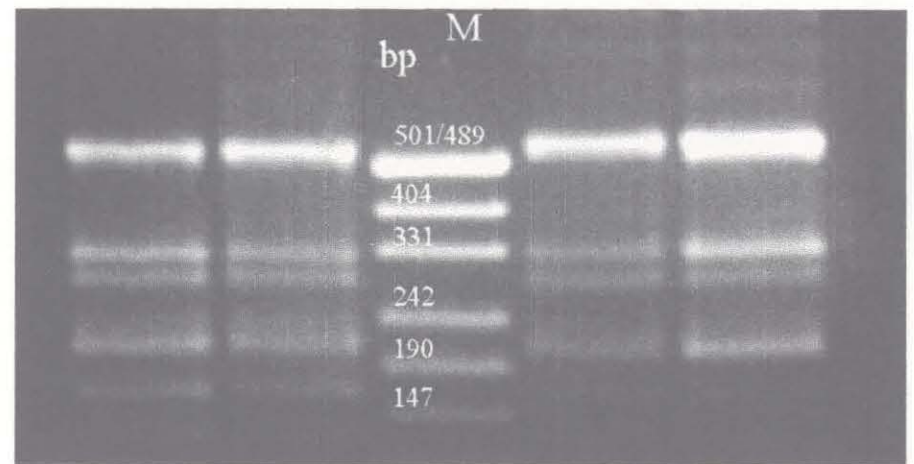
Figure 7.2   PCR product of *omp* gene of *D nodosus* isolates collected from property F. Genomic DNA from 11 isolates was subject to PCR with primers A and C, which amplify ~0.5 kb segments of the multiple copies of the *omp1* genes of *D nodosus*. The products were subject to electrophoresis in a 2.5% agarose gel. Lanes marked M contain the marker pUC19/*Hpa*II. The highest molecular weight band of the marker is for fragments of 489 and 501 bp. The 11 isolates tested all produced fragments which approximate 530 bp in size and no differences are evident between any of the isolates. See Figure 7.1 for a description of the method.

The 11 isolates were all of serogroup A except for isolate 60 in lane 2, which belonged to serogroup I.

(c)
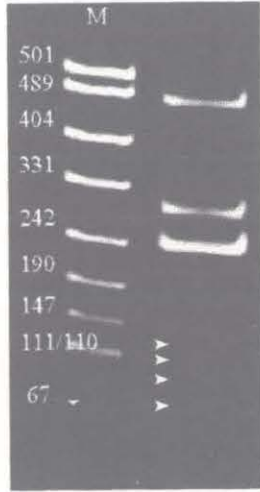
Figure 7.3 *Hpa*II PCR-RFLP patterns from *omp1* genes of *D nodosus* isolates collected from property F (a and b) between December 1992 and October 1994 and of *D nodosus* strain VCS1001 (c). Genomic DNA from *D nodosus* cells was used as a template for a PCR using primers A and C (Moses 1993) as described in Figure 7.1. PCR products were digested with *Hpa*II, subjected to electrophoresis in a 4-20% gradient polyacrylamide gel and stained with ethidium bromide. Lanes marked M contain the molecular weight marker pUC19/*Hpa*II and the fragment lengths, in bases, are shown at the left of each gel. The negative control lane is marked -ve. For the property F isolates (a and b), two patterns (D2 and D1) could be distinguished by the absence from pattern D2 of bands of approximately 470 and 480 bp, a double band at 250 bp, rather than a triple band as for D1, and a band faintly visible at approximately 40 bp (arrowed for isolate 59). Isolate 60 had a D1 pattern despite being of serogroup I, rather than A. The pattern for VCS1001 (c) had a band of 480 bp and two bands at 300 and 240 bp. Four bands between 67 and 120 bp were faintly visible (arrowed). The same pattern was evident for all 29 isolates of VCS1001 tested.

(a)

Isolate #	59	61	62	60	91	92
Serogroup	A	A	A	I	A	A
Pattern	D2	D2	D2	D1	D1	D1

(b)

Isolate #	93	100	101	102	103	-ve
Serogroup	A	A	A	A	A	
Pattern	D1	D1	D1	D1	D1	

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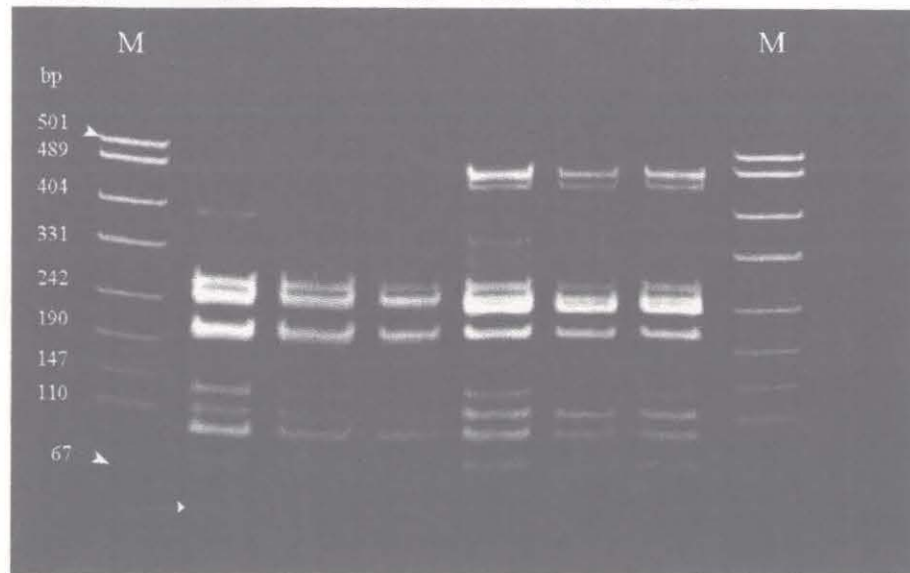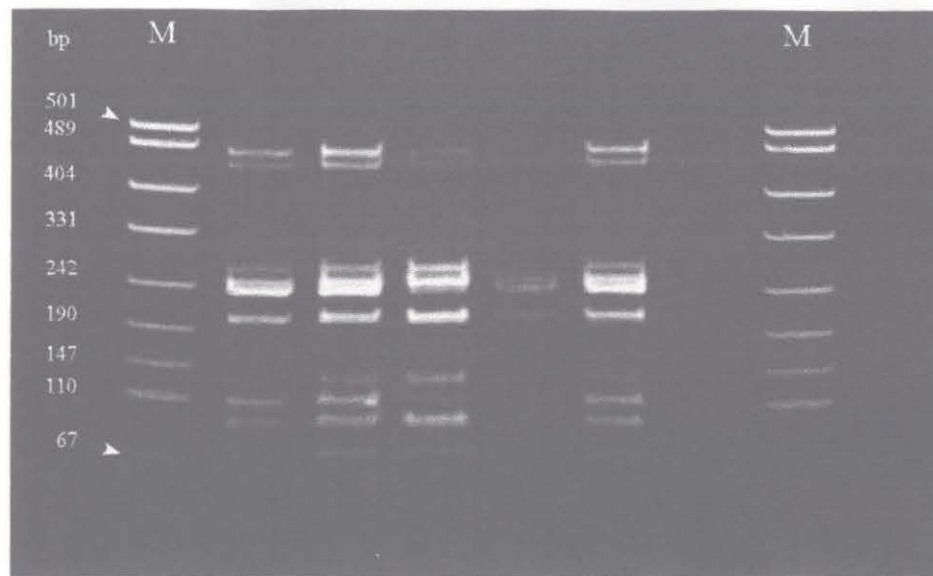| Isolate # | | 59 | 60 | 61 | 62 | 91 |
| Serogroup | | A | I | A | A | A |

bp   M

501
489
404
331
242
190
147
111/110
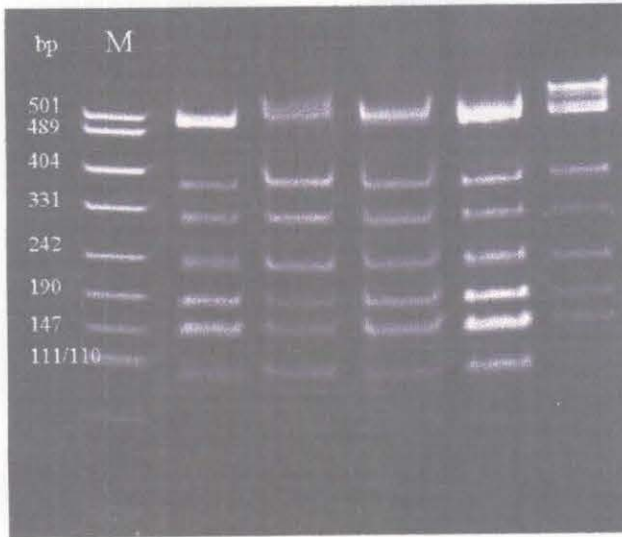
Figure 7.4   *Sau*3AI PCR-RFLP patterns from *omp1* genes of *D nodosus* isolates collected from property F between December 1992 and October 1994. The technique is described in the captions for Figures 7.1 and 7.3.

Isolates 59, 61 and 62 had different patterns by *Hpa*II from 60, 92 and all other property F samples (not shown) but cannot be readily differentiated with *Sau*3AI. Isolate 60 was a different serogroup from all other property F isolates. The patterns with *Sau*3AI were indistinguishable from all other property F isolates.

Isolate #	36	63	55	39	41
Serogroup	C	G	H	A	A
Pattern	1	1	1	1a	1a
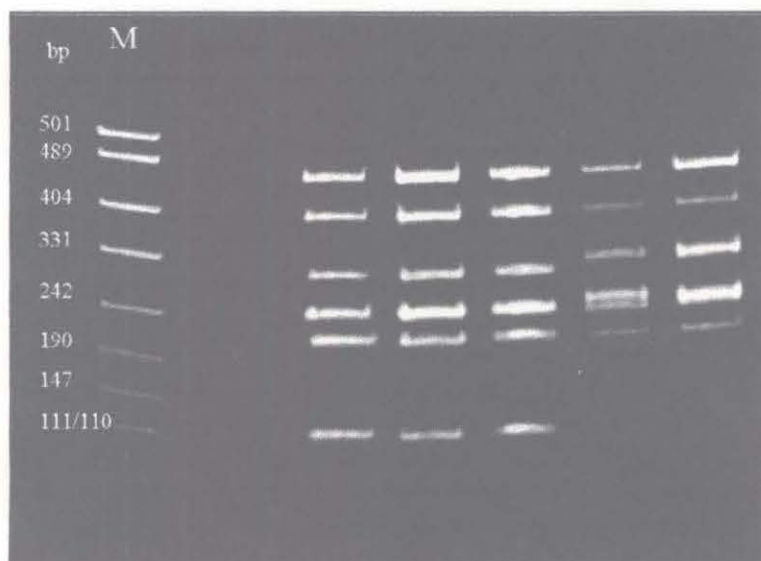
Figure 7.5 PCR-RFLP of *omp* gene (*Hpa*II) of isolates of three serogroups showing an identical pattern and two other isolates of serogroup A showing a similar, but distinctly different, pattern. All five isolates came from either property B or from property W (see Table 7.4), two flocks in slightly different locations in the Monaro which were operated by the same owner and between which sheep were exchanged from time to time.

The lane headed M contains the molecular weight marker pUC19/*Hpa*II and the fragment lengths in bases are shown at the left of the gel.

Pattern 1 was seen in two isolates of serogroup C, one isolate of serogroup G and 3 isolates of serogroup H. Other patterns were also observed for isolates of serogroups C and H but the only two isolates of serogroup A from these or related flocks had pattern 1a, and pattern 1a was seen only in these two serogroup A isolates (Table 7.5).

See Figures 7.1 and 7.3 for details of the method.

Isolate #	80	82	84	107	108
Pattern	7	1b	3	7	inc
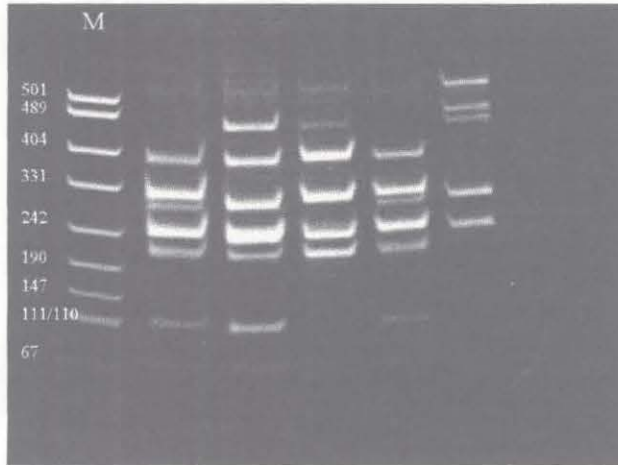
Figure 7.6   PCR-RFLP for selected H serogroup isolates of *D nodosus*, all but one (isolate 84) collected in the course of studies of related outbreaks of footrot at properties B, W, K and T. Patterns 1, 1b, 1c, 3, 4, 5 and 7 are illustrated.

Isolate 84 (pattern 3) was from an outbreak unrelated to any outbreak reported in this thesis but it was from a flock owned by the owner of property F, operated at another property not described here. It was included in this study of molecular epidemiology because it was of serogroup H and to ascertain if it could be differentiated from the seven patterns for the Monaro-sourced outbreaks and from the two patterns of the property F outbreak.

The digest for isolate 108 was incomplete. Lane headed M contains the molecular weight marker pUC19/*Hpa*II and the lengths (bp) of fragments of the marker are written beside the bands.
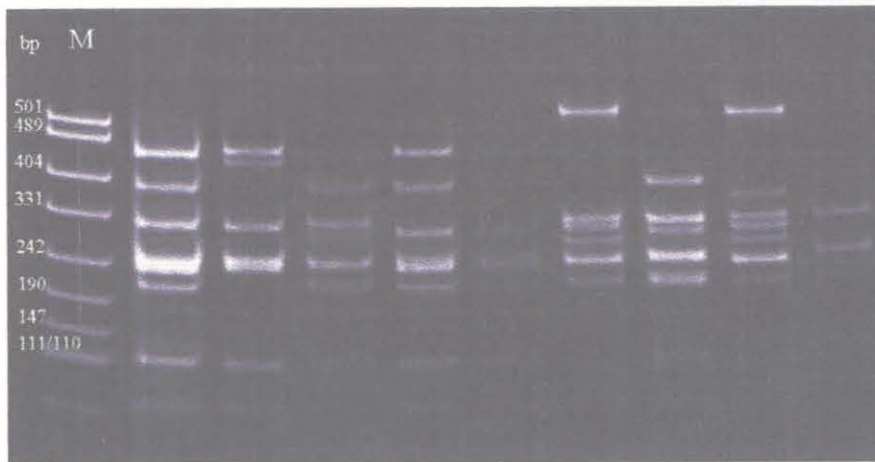
7.4.   Discussion

The objectives of the studies presented in this chapter were two fold. First, the molecular epidemiological technique of PCR-RFLP of the *omp* gene of *D nodosus*, using methods first described by Moses (1993) and later applied by Allworth (1995) and Ghimire and Egerton (1999), was to be further evaluated. Second, the technique was to be used to support the contention of continuity of *D nodosus* clonal lines between different geographic sites over a period of two years or more.

The Department of Veterinary Clinical Sciences' collection of *D nodosus* isolates presented an excellent opportunity to examine the stability of *omp* gene RFLP fingerprints over a period of years, during which a widely used prototype strain (VCS1001) was introduced artificially into sheep to initiate experimental footrot outbreaks then subsequently re-isolated from affected sheep, purified, identified on the basis of its serotype and virulence, replaced into the collection and nominated as the same strain. During these periods of infection in semi-natural conditions, there would have been many replicative events of the bacterium and opportunities for contact with other bacteria including other (undetected) strains of *D nodosus* which may have been present in the feet of experimental sheep.

This study has shown that the original *omp* gene RFLP pattern, using either *Hpa*II or *Sau*3AI as an investigatory tool, persisted. One pattern only was seen for all isolates. While this indicates genetic stability which encourages the use of the technique for epidemiological studies, it is also rather surprising, given the hindsight provided by the later studies of natural outbreaks reported here and by Ghimire and Egerton (1999). At no stage did the VCS1001 strain, as identified by serotype classification, adopt an alternate *omp* gene sequence, despite presumed exposure to *D nodosus* strains with different *omp* genes. Possibly the artificial nature of the outbreaks sufficiently restricted the chance of genetic exchange of fimbrial antigens between VCS1001 and other strains or perhaps the usual practice of elastase testing of isolates recovered from field studies ensured that only serotype A_1, elastase positive isolates were returned to the collection. Assuming that (1) the fimbrial genes which confer immunological classification are the mobile genetic elements and (2) that the only isolates inadvertently coming in contact with VCS1001 in the field studies were benign strains, alternate

omp gene RFLP fingerprints would have been discriminated against by virtue of their elastase-negative status.

Studies in two other 'flocks' also demonstrated that *omp* gene RFLP fingerprints persist in natural outbreaks. Eleven isolates collected between December 1992 and October 1994 from the flock at property F showed two *Hpa*II patterns. Thirty four isolates from flocks arising from the two related Monaro sites (property B and property W) showed nine *Hpa*II patterns, two of which occurred in isolates collected a number of times over a two year period (Table 7.4).

The stability of the VCS1001 isolates implies that clonal lines of *D nodosus* do not readily change *omp* gene RFLP fingerprints. Consequently, it seems likely that the nine patterns observed in the Monaro-origin flocks represent nine separate introductions into the flock of unrelated strains of *D nodosus* or, possibly, fewer than nine introductions followed by mutation of the *omp1* gene of one or more of the strains.

It is remarkable that the nine patterns occurred in six serogroup classifications, with three patterns occurring in three serogroups, three occurring in two serogroups, and three occurring in one only. This fact, plus the occurrence of seven patterns in the most widely identified serogroup (H), suggests very strongly that genetic exchange of either fimbrial genes or of *omp* genes occurs within a mixed population of *D nodosus*.

Alternatively, it could be hypothesised that the mixed population in the Monaro-origin flocks arose from 18 separate introductions, each of which was a unique serogroup x fingerprint type, and these 18 genotypes have remained relatively stable since introduction. If so, the restricted number of patterns (9) within the 18 genotypes suggests that either the technique has poor discriminatory powers or that there is a limited number of *omp* gene types within the *D nodosus* population. Although this latter has not been disproven, evidence from the four unrelated sources described in this thesis and from 12 flocks studied by Allworth (1995), suggests that isolates from unrelated flocks have unique patterns.

It is clear from these studies that it is essential to characterise a large number of isolates in order to describe the full range of RFLP fingerprints likely to be found in field outbreaks. While the range of patterns detected at property F appeared to be limited (only two patterns were identified with _HpaII_), nine patterns were found in the flocks originating from property B and property W. While it is possible that one of these (pattern 6) was introduced inadvertently by flock 2 (Chapter 4), all eight other patterns were found at property B or property W. Although all eight patterns were found in the ten isolates of four serogroups recovered from property B in October 1992, one would normally expect that more than ten isolates would be necessary to discover eight different patterns, if only nine existed. It is, in retrospect, surprising that only one new pattern was found after eight had been identified in the first thirteen isolates examined (Table 7.4).

Two different restriction endonucleases were used in these studies; _HpaII_ and _Sau_3AI. Both REs were predicted to produce similar numbers of fragments from the four _ompl_ genes of VCS1001 (12 and 10 respectively, see 7.3.1.1.), and _Sau_3AI had been used in the initial studies by Moses (1993). In my studies, _Sau_3AI was less able than _HpaII_ to discriminate between strains. With the property F isolates, two _HpaII_ patterns were found, but both appeared identical with _Sau_3AI (Table 7.3). With the Monaro-sourced isolates, seven _HpaII_ pattern isolates tested with Sau3AI produced only five _Sau_3AI types (Table 7.6). Another additional factor which favours the use of _HpaII_ over Sau3AI was the frequent occurrence with _Sau_3AI of a band corresponding to a fragment greater than 500 bp long, similar to the size of the undigested product. This was expected with VCS1001, from the work done by Moses (1993). This occurred in fewer isolates with _HpaII_. The advantage lies in the ability to distinguish between a band greater than 500 bp which should be present after digestion, because one gene variant has no restriction site, and a band which is present because the restriction endonuclease has failed to properly digest all of the PCR products.

Some attempt was made to relate the fingerprints found in the studies described here to the findings published by other authors, particularly Moses (1993). The first major discrepancy discovered was in the apparent size of the amplified products of VCS1001, which exceeded 500 bp consistently and was not 465 bp to 489 bp, as predicted by Moses (1993). It is common experience in our laboratory for products amplified from VCS1001 with primers A

and C to exceed 500 bp (O Dhungyel, personal communication). The second major discrepancy was in the apparent consistency of product size across a range of isolates. Electrophoresis of part of the PCR reaction mixtures for isolates covering a large range of *Hpa*II patterns produced bands consistently slightly larger than 500 bp, as in Figure 7.2. This was not expected from Moses' limited work with a range of isolates (Moses 1993).

Reasons for the discrepancy are not clear, however, in the case of VCS1001, the difference could have arisen as a result of laboratory adaptation of the strain used by Moses (1993), leading to a genetic change away from the isolates in our collection, which are generally closer to field isolations. This proposition, however, is at odds with the stability of RFLP patterns found with in Department collection.

It was not the purpose of the studies described in this thesis to explain the molecular basis of the differences in *Hpa*II patterns, rather it was to explore the usefulness of the technique. It is clear, however, that further studies to better understand the *omp1* region of a variety of *D nodosus* isolates would significantly enhance our understanding of the application of *omp1* gene RFLP fingerprinting to epidemiological studies.

Further study of the mechanism(s) involved in the exchange of genetic material between strains of particular *omp* types would also assist an understanding of the complex interactions occurring within mixed infections - particularly when vaccination is used as a control measure. Possibly one of the most useful relationships to understand is that between *omp* type and virulence. If, as suggested in this and other discussions (*eg*, Mattick *et al* 1991; Ghimire and Egerton 1999), genetic exchange involves fimbrial genes and not other genomic regions, *omp* genes and virulence related genes will maintain their association even when serogroup changes. If so, the objects of eradication in footrot control within flocks will become a particular *omp* type, because of its association with virulence, rather than eradication of a particular serogroup. It is relevant that, in their studies with Nepalese footrot, Ghimire and Egerton (1999) described 11 *omp* gene RFLP fingerprints but only two which occurred in virulent isolates.

There was the suggestion in the description of RFLP fingerprints described in 7.3.1.2 and 7.3.1.3 that some patterns within flocks may be related to each other. For example, the two

*Hpa*II patterns found at property F, and patterns 1 and 1c at property B, which were pattern S2 and S5 with *Sau*3AI, showed similarities which could have been explained by the derivation of one strain from the other strain, with the loss of some genetic material. A better understanding of the relationship between these pairs of isolates at the molecular level would also assist in our understanding of the reliability of PCR-RFLP of the *omp* gene as an epidemiological tool.

The second objective of the molecular genetic studies was to provide support for the fact that the same strains of *D nodosus* were present at property B and property W as were present at property K and property T. This was achieved in that, of the 16 isolates of seven *Hpa*II RFLP patterns from property K and property T which were *omp* gene typed, only one pattern did not occur which had occurred at property B (pattern 1c) and only one 'new' pattern (pattern 6) was detected. This conclusion is supported by the occurrence of a similar range of serogroups, dominated by serogroups H, B and C in both the Monaro and on the south-west slopes environments.

7.5.   Summary

The technique of PCR-RFLP of the *omp* gene region of *D nodosus* was evaluated and used as a molecular epidemiological tool. The method gave consistent, repeatable results when applied to a series of related isolates and offers the ability to accurately identify strains of *D nodosus* through repeated cycles of field infection and laboratory isolation and storage. For this reason, it seems that the technique could be applied to field investigations to demonstrate the relatedness of outbreaks.

It has also been shown that natural field outbreaks of footrot can involve multiple strains of *D nodosus* and multiple isolations are necessary to identify all, or nearly all, of the *omp* gene types present. Multiple isolations must be a feature of any epidemiological study where two outbreaks may have a limited amount of contact, for only a small number of *omp* gene types may be transmitted between flocks.

Studies reported in this thesis support the view that PCR-RFLP of the *omp* gene region will be a useful and valuable epidemiological tool. Further study to confirm its usefulness should aim to demonstrate the variability of *omp* gene types which occur with the *D nodosus* population, so that the probability of finding similar patterns from unrelated outbreaks can be estimated.

Of the two restriction endonucleases used in these studies, *Hpa*II appears more useful than *Sau*3AI, but there are probably occasions when both REs could be used to provide extra stringency to differentiate between isolates.

The hypothesis that there is exchange of genetic material determining antigenic classification between strains of *D nodosus* occurring together in footrot outbreaks gained further support from the studies reported here. This, and a clearer understanding of the stability of *omp* gene types and virulence factors within strains, warrants further study.

CHAPTER 8

CONTENTS

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GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1.   Diagnosis of IFR

This thesis has reported the results of studies into the epidemiology of intermediate footrot (IFR). Two natural outbreaks were studied, one in a flock near Yass, in the southern Tablelands of NSW and one near Cooma, in the Monaro district. The observations in the second flock were continued for two additional years when a portion of the infected flock were moved to a property near Tarcutta, in the south-west slopes region of the state.

The observational studies showed that IFR typically causes up to 6% of a flock of exposed sheep to develop a maximum footscore of 4, and up to 25% to develop a maximum score of 3. During prolonged periods of non-transmission and while outbreaks are developing, the proportion of sheep with maximum footscores of 2 exceeds the proportion with more severe lesions by a factor of at least two (Figures 5.3 and 5.4) but, as outbreaks wane or if conditions are not fully favourable, the proportion of the flock with a maximum score of 2 declines and the proportion with more severe lesions increases, before declining as a result of persisting unfavourable conditions.

Methods to distinguish IFR from VFR based on the relative proportions of severely affected sheep in infected flocks have been widely used and recommended since the early 1980s (Stewart *et al* 1984; Egerton 1989; Anon 1995; Allworth and Egerton 1999). The need to examine a large and randomly selected part of an affected flock has been stressed (Egerton 1989) . The effect of the environment and the timing within the outbreak on the proportion of the flock with scores of each category have not, however, been properly examined.

It has been shown in these studies that assessment of the form of footrot should be made with careful consideration of the relationship between environmental conditions and footrot and the changing ratio of maximum footscores over the course of an outbreak.

Two important observations relevant to this diagnostic approach arose from these studies. First, environmental conditions which favour the transition of a footrot lesion from inapparent to a detectable state and from score 2 to score 3 or 4, are less stringent than those required for transmission between sheep. Thus, if transmission has occurred to a high degree, such as a flock prevalence of 70% or more would indicate, it is reasonable to assume that the disease has had the opportunity to express itself fully, or nearly so. It cannot be stated that the disease would appear worse, in terms of the proportion of the flock with severe lesions, if it were transmitted to sheep in a more favourable environment. Second, conditions which do not favour transmission may, at least for a short time, exacerbate some of the lesions and increase the proportion of the flock with severe infections beyond that which occurred when the outbreak was active or if the sheep were grazed in a more favourable environment.

In summary, calculations of the proportions of the flock affected with lesions of each score should use the number of sheep *at risk* as the denominator, rather than the number affected with footrot, as suggested by Allworth and Egerton (1999). This step would partly avoid overstating the severity of the disease, but interpretation of environmental factors is still essential to make a correct diagnosis.

8.2. Environmental conditions favouring transmission of IFR

Graham and Egerton (1968) found that transmission of virulent footrot begins when mean daily temperatures consistently exceeds 10°C, following a period of sustained high rainfall. The studies reported in this thesis confirmed these predictions for IFR. In the outbreaks of IFR reported in these studies, rainfall exceeded 60 mm on average for the five months preceding and including the month in which the outbreak occurred although rainfall did not consistently exceed 50 mm in each month. Outbreaks of IFR began in late August or early September in the southern Tablelands and the western slopes. In the Monaro, outbreaks began in October, approximately six weeks later than in the warmer districts.

8.3. Eradication of IFR

IFR can be eradicated with methods recommended for VFR. Repeated inspections of sheep during a non-transmission period may be more important with IFR than with VFR, because latent infections occur with IFR and may be more important in the epidemiology of IFR than VFR. The only other report that eradication of IFR could be successful (Allworth 1995) involved repeated inspections and removal of affected sheep and that requirement was emphasised by my findings. From the observations made in the flock at property F, these inspections should commence in mid to late summer and continue into the autumn and, possibly, early winter. Conditions in late autumn and winter may encourage the expression of latent infections but not lead to transmission to unaffected sheep.

Intermediate virulence covers a spectrum of virulence from a low grade, similar to benign footrot, to a high grade, which merges into VFR. While it is clear that IFR of high grade virulence can be eradicated as described, it is probable that eradication of lower grade strains, like benign strains, is more difficult (Allworth 1995). Strains of low grade virulence may be more likely to develop latent infections which can persist for much longer periods without being eliminated from the sheep's foot or without becoming an apparent infection compared to strains which cause higher grade IFR or VFR.

8.4. Antibiotic use with IFR

Antibiotics, while quite effective in the control of IFR, are contra-indicated in attempts to eradicate the disease by methods based on inspection and culling. Their use leads to the development of a persistent carrier state in feet which have, before treatment, either active and visible lesions or mild, inapparent infections. The suppression of the disease can be prolonged, up to several months, and is followed by recrudescence of infection, at least in some cases. The suppression of the disease to an undetectable state for such long periods is not induced by vaccination or topical zinc sulphate solution, nor does it occur naturally with IFR. It is proposed that antibiotic treatment may remove the bacterial flora which support a footrot lesion but not eliminate all *D nodosus* organisms, allowing them to persist in the interdigital skin in a dormant state, as recorded by Egerton and Parsonson (1969), until environmental conditions

again stimulate the complex infection and inflammatory process which enables *D nodosus* multiplication. Vaccination appears not to lead to the same consequences, probably because it specifically targets *D nodosus*, rather than the mixed bacterial flora which necessarily accompany a footrot lesion.

8.5.   The economic cost of IFR

IFR causes less severe depression of wool production and bodyweight than VFR because it causes fewer severe foot infections and, therefore, lower overall severity scores. In addition, most sheep affected with IFR recover promptly when environmental conditions become unfavourable for disease transmission and expression.

The predicted loss in annual wool production with IFR is around 2%. Assuming the gross value of a sheep's fleece to be around $25, the predicted cost of IFR on wool productivity is around $0.50 per sheep in each year that an outbreak occurs. As well as losses of wool production, IFR will also reduce the average bodyweight of an affected flock by about 2.5 kg at the peak of the outbreak, with economic consequences for reproduction and sale price. If these losses amounted to a further $0.50 per head, averaged across the flock, the total cost of IFR in an unaffected flock is expected to be approximately $1.00 per head.

The cost of disease control measures, such as vaccination or repeated footbathing, are expected to exceed $1.00 per head so it unlikely that a cost:benefit study would reveal any nett benefit to a control program. On the other hand, eradication of IFR has been shown to be possible and, although the initial cost of eradication is higher than the cost of control measures, the benefits will continue to accumulate with no further cost once eradication is achieved.

Consequently, eradication of IFR may be a rational objective for flock owners but careful consideration is necessary. The successful eradication program reported in Chapter 3 occurred when there was an extended non-transmission period over summer and autumn which provided good opportunities to identify and cull affected sheep. These conditions will not exist in every environment where IFR exists and for owners of flocks in some environments, attempts to eradicate this form of the disease may not be financially rational.

The results of this study suggest that IFR should not be a target for eradication in compulsory statewide programs because it cannot be justified on economic grounds. Although the disease can be eradicated in at least some environments, the cost of the disease does not justify the expense of eradication.

Further, compulsory eradication cannot be justified on the basis that IFR will cause more severe footrot if it is transferred to a more favourable environment. Observations reported here show that this is not the case.

Control within a flock, aimed at minimising the cost of the disease, should focus on two activities; first, low cost treatments, such as footbathing treatment coinciding with other husbandry procedures which require that sheep are yarded and, second, culling of the worst-affected sheep because these sheep are likely to remain as the most susceptible sheep and suffer the most serious losses of production in any future outbreaks. This latter approach could also lead to the development of breeding programs incorporating resistance to footrot. Genetic resistance to footrot may have much to offer in limiting the effect of IFR.

8.6.   PCR-RFLP of the *omp* gene

The technique of PCR-RFLP of the *omp* gene of *D nodosus* has a role as an epidemiological marker. The use of the technique highlighted the complex nature of mixed infections in natural outbreaks of footrot but was able to provide evidence that particular *D nodosus* genotypes persisted over time. Evaluation of the technique with laboratory isolates of one strain (VCS1001) showed that PCR-RFLP patterns remained constant in one strain over the course of many years, frequent sub-culture and re-isolation from field infections.

The technique also provided further evidence that genetic exchange of fimbrial genes between strains of *D nodosus* occurs in natural outbreaks. This event has implications for eradication programs based on the use of serogroup-specific vaccines and should be addressed in further research.

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To accompany Table 4.6

Prevalence of footrot, flock 1

with details of missing sheep

Week	Infected sheep removed	Prevalence of footrot cases Number of sheep present shown in brackets				
		Control	**Footbath**	**Antibiotic**	**Vaccine**	**Total**
0		15 (100)	20 (101)	11 (101)	20 (101)	66 (403)
5		24 (99)(1)	14 (100)(2)	5 (101)	10(100)(3)	53 (400)
10	57	24 (97)(4)	16 (101)	4 (100)(5)	13 (97)(6)	57 (395)
13	8	Prevalence not recorded; only previously unaffected sheep inspected				
16	13	21 (96)(7)	15 (98)(8)	11(101)	11(93)(9)	58 (388)

(1)   one Control missing; had been affected at Visit 1. Was dead (Tag 300)
(2)   one Footbath sheep missing; had been unaffected at Visit 1. Returned Visit 3.
(3)   one Vaccinate missing; had been affected at Visits 1. Was dead (Tag 385)
(4)   two more Controls dead (88, 396). 1 had been affected, 1 unaffected, at Visit 2.
(5)   one Antibiotic sheep missing; had been unaffected at Visits 1 & 2 . Returned Visit 4
(6)   three Vaccinates missing; 1 had been affected, 2 unaffected, at Visit 2. All dead (42,
       357, 400)
(7)   one Control missing; previously unaffected; returned Visit 6
(8)   three Footbath sheep missing; previously unaffected; returned Visit 6
(9)   two Vaccinates dead (141, 399); one unaffected, one affected at Visit 3; two missing,
       previously unaffected, returned Visit 6

To accompany section 4.3.1.13.

Geometric mean agglutinin titres to H and B antigens of a sample of vaccinates and controls

Week	Vaccinates			Controls		
	n	Titre H	Titre B	n	Titre H	Titre B
0	25	104	25	26	82	20
5	25	2051	581	25	49	14
10	24	33 270	288	25	43	14
13	22	8203	108	18	64	18
16	19	8853	157	18	80	35

To accompany section 4.3.2.1.

Distribution of sheep categorised by maximum footscore, flock 2

| Week | Number present | Prevalence | | |
		Score 2	Score 3	Score 4
24	300	30%	17%	3%
28	298	66%	20%	2%
41	274	64%	22%	3%

To accompany section 5.3.8.

Geometric mean aggltinin titres in a sample of sheep in groups CON, INT and VIR. Vaccination with serogroups A, B and H given to group CON, and with serogroup A to group INT in weeks 6, 12 and 21. Tests of significance (analysis of variance) applied to groups within serogroups.

Week	Significance	Group VIR			Group INT			Group CON		
		Titre A	Titre B	Titre H	Titre A	Titre B	Titre H	Titre A	Titre B	Titre H
6	ns	104	58	59	82	39	49	95	50	57
12	p < .005	63	16	99	2268	17	87	3880	222	6640
18	p < .005	86	24	73	15 629	55	55	22 724	1178	15 847
25	p < .005	117	37	166	47 051	97	128	38 217	3497	44822
34	p < .005	127	36	118	21 350	87	157	19 109	1874	29 984
46	p < .005	87	52	108	16 703	98	142	12 289	1190	9519

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