The role of fimbrial antigens of *Dichelobacter nodosus* in diagnosis and pathogenesis of footrot.

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A thesis submitted for the degree of

Doctor of Philosophy

of the University of Sydney

Australia

March 2002

Faculty of Veterinary Science
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Declaration

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

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Dedication

This thesis is dedicated to my mother late **Mrs. Chandrakala Dhungyel** whose dedication for the welfare of animals led me into veterinary education, and to **my parents and to one and all members of my large family** who have been the main source of inspiration in my endeavour.

Acknowledgments

I am profoundly indebted to my supervisor, Emeritus Professor John Egerton and Associate supervisor, Professor Richard Whittington for their thorough guidance and help in every aspect of this study. Their dedicated and friendly help was one of the main sources of inspiration for me to complete this study on time.

I am grateful to Dr. Peter Cox of Sydney Waters for his initial help with the design of serogroup specific PCR primers and providing me with the fimbrial sequences. My grateful thanks to Professor Julian Rood and Dr. Ruth Kennan of Monash University for the collaboration in one of the studies presented in this thesis.

I am grateful to Assoc. Prof. Garry Cross for helping me with the photographs and all the other computer problems. My sincere thanks to Prof. Herman Raadsma, Assoc. Prof. Robert Love, Prof. Frank Nicholas, Dr. Kym Abbott, Dr. David Jordan and Dr. Shiva Ghimire for their suggestions and friendly moral support all along the period of my study.

My special thanks to my friends and colleagues at Camden, Craig Kristo, Marilyn Jones, Gina Attard, Dr. Alison Collins, Dr. Imke Tammen, Julie Cavanagh, Natasha Ellis, Dr. Bill Blackwell, Colin Cavanagh, Suresh Khatkar, Jiri Tasler, Juha Virolainen, Quynh Vu, Helen McGregor, Sandra Saville, Eileen Risby, Ron Henderson, David Palmer, Mathew VanDjick, Marie Wildrige for their friendship and help at one stage or the other during my study. Thanks to Mehar Singh Khatkar for helping with proof reading.

Thanks to Venessa Saunders, Ian Marsh, Leslie Reddacliffe, Dot Thompkins, Joe Kormos and Steve Pitt of EMAI for helping me with the trial works. They had a few difficult days in the field with the feral goats used in our trial. Many thanks to Dr. John Seaman of NSW Agriculture and Caroline Gadon of Cicerone Project for helping me to get the trial animals and providing me with the footrot data for the first 2 phases of the trial at Uralla and Toogong. Thanks are also due to Dr. B R Joshi, Mr. H D Joshi and all the laboratory staff at Lumle Agricultural Centre, all the porters and

flock owners of Ghanpokhara and Siklis for helping with the anamnestic trial work in Nepal.

The study presented in this thesis was performed in the Faculty of Veterinary Science, The University of Sydney, Camden, Australia. I thank the Faculty and the University for providing me with the facilities for my study.

I am grateful to my beloved parents, all my brothers and sisters, to Om bhai and family whose unconditional support to me and my family has led me to achieve my goals. Their hardships as refugees have been a source of inspiration for me. Thanks to my Nepali friends in Sydney who have given moral support all along.

Finally, my sincere and grateful thanks to my wife Pabitra, daughters Sita and Geeta, and son Ramesh who had been there always to inspire me all along my studies. I am proud to achieve this for all of them.

Summary

Studies presented in this thesis looked at developing new methods for the diagnosis of virulent footrot (VFR) in sheep and identification of serogroups of *Dichelobacter nodosus*, the principal causative agent of footrot.

Earlier studies had shown that immunological memory response in sheep recovered from VFR can be aroused by natural or recurrent infection or by injection of outer membrane protein (OMP) antigens to be used as a retrospective diagnostic test for VFR. But OMP antigen was found to be non-specific in older animals. To overcome this non-specificity of OMP antigen in anamnestic response, pilus antigen was evaluated in a trial at Camden.

The results of this trial indicated that antibodies to pilus antigen can be detected over time in a manner similar to OMP antibodies so a retrospective assessment of VFR status can be made by anamnestic test with pilus antigens. The anamnestic response to pilus was similar in character to OMP antigen but unlike OMP was highly specific. The response to anamnestic challenge with pilus was determined by severity of the lesions they had expressed, with severe lesions triggering the greater responses. However, there was variation between individuals, with some (6 of 46 with severe lesions) failing to respond. This individual variation is probably mediated genetically as is response to vaccination.

This anamnestic test was tested in flocks of sheep in Nepal that had a history of VFR which had apparently been eradicated. That assessment, based on clinical findings, was confirmed by the uniformly negative results in the pilus anamnestic test applied to a representative sample of the population. This allowed a conclusion that the virulent strains of *D. nodosus* involved had been eliminated from these flocks.

As mentioned in the preceding study pilus antigen was found to be very specific and ideal for retrospective diagnosis of virulent footrot with an anamnestic challenge ELISA test. However, serogroup specificity was seen as a disadvantage of using pilus antigen for the anamnestic test. The possibility of using multivalent pilus antigens was tested in another trial. These animals had been involved in a clinical expression

experiment conducted by another research group and had a clinical and bacteriological history extending over more than 12 months. After these initial trials all these animals were treated for footrot and managed for 5 months as a single flock at Camden. These were then challenged with multivalent pilus antigen (serogroup A – I) as a single injection.

The results obtained indicate that multivalent pilus anamnestic ELISA is equally effective as monovalent pilus. This has the added advantage that prior knowledge of the serogroups present in the flock is not required. It has the possibility of being used as an indirect test to check the presence of serogroups in a flock without doing the bacterial cultures. This test can identify most animals with pre-existing underrunning lesions (Scores of 3 or higher). However, the sensitivity and specificity of this test need to be tested extensively in flocks of known clinical history before it could be adopted as a routine test.

As a key component of a larger study to determine the role of fimbrial genes (*fimA* and *fimB*) of *D. nodosus* in the pathogenesis of footrot using allelic exchange to disrupt these genes of a strain (serogroup G), the study presented in this thesis contributed a detailed characterisation of the resultant mutant and the wild strains and tested these strains for virulence in sheep. The results presented provided the first definitive evidence that the *fimA* gene is essential for virulence of *D.nodosus* in sheep. *In vivo* virulence testing of two *fimA* mutants showed that they were not able to establish any footrot whereas the wild type of the same strain produced virulent footrot in the same trial conducted under similar conditions. These mutant bacteria were not re-isolated from interdigital skin after *in vivo* challenge. This indicated that *fimA* mutant strains could not colonise the ovine foot, and the simplest and most likely explanation for these results was that colonisation of the interdigital skin and subsequent penetration of the *stratum corneum* requires the adhesive activity of type IV fimbriae. However, since these mutants also had altered ability to secrete extracellular proteases, and perhaps other as yet unknown extracellular proteins, the possibility of the involvement of these factors as major determinants of host colonisation or invasion cannot be excluded.

Current methods for the identification of the serogroup of *D. nodosus* present in the bacterial population requires isolation of the organism and after purification by subculture, antigenic analysis with agglutination tests. This usually takes at least 3 to 4 weeks. With the objective of developing a rapid serogroup specific PCR assay, the basis of serogroup variation in *D. nodosus* localised in the fimbrial gene region was exploited. A common forward primer and 9 serogroup specific reverse primers were selected from the fimbrial gene sequences of the prototype strains. Analytical sensitivity of the serogroup specific primers on chromosomal DNA was similar to PCR tests in other bacterial species reported before. Immuno-magnetic bead capture PCR method was able to detect 5 to 10 cells in cell lysates. Specificity within and between the serogroups of *D. nodosus* was tested with all the prototype strains. They were found to be very specific to each serogroup and specific only to *D. nodosus* when tested with 84 commonly found bacterial strains or strains related to *D. nodosus*.

To overcome the time delay in conducting 9 different amplifications to find out the prevalence of all possible serogroups in a flock multiplex PCR reactions with common forward primer and groups of 3, 4 and 5 reverse primers were successful in reducing the number of reactions to 2 (with groups of 4 and 5) or 3 (with groups of 3) primers. A drawback of the multiplex reaction was that if a template was 1000 times less concentrated that the others in the mixture it was not amplified but the margin for difference is very high.

The main aim of developing rapid serogroup specific PCR was to apply these tests directly on footrot lesion samples to make it a rapid diagnostic test for field samples. The sensitivity of the test on lesion samples was found to be very low. To try and improve the sensitivity an overnight or four days old pre-enrichment culture in broth was tested but was found to be no better than direct PCR. The immuno-magnetic capture method which improved the sensitivity of pure culture samples by 10 –100 fold also had very low sensitivity with lesion samples.

However, this drawback can be overcome by picking up colonies from 4 days old lesion cultures on hoof agar (HA) plates for serogroup specific / multiplex PCR. If the colonies are too small/ too few on the lesion cultures these can be sub cultured onto a quarter of 4 % HA plates and then used for the PCR test which also reduces the time

taken for serogrouping at least by 2 weeks. The other advantage is that individual colonies do not need to be isolated. A PCR test can be done on pooled colonies just as well and can be used to identify all serogroups present in the sample.

Serogroup specific PCR is much faster and is more sensitive and accurate than slide agglutination tests which take 3 to 4 weeks to complete. Multiplex PCR makes it easier to detect different serogroups in a sample with a maximum of 3 PCR tests. Serogroup specific multiplex PCR will be a useful tool for footrot control based on specific vaccination. The difficulty in using the test on direct lesion swabs needs to be further looked into. There may be future advances in the application of PCR tests to clinical samples.

Publications

1. **Dhungyel, O.P.,** Whittington, R.J., Ghimire, S.C. and Egerton, J.R. (2001). Pilus ELISA and anamnestic test for the diagnosis of virulent ovine footrot and its application in a disease control program in Nepal. *Veterinary Microbiology*, **79**: 31-45.

2. **Dhungyel, O.P**., Whittington, R.J., and Egerton, J.R. (2001). Serogrouping of *Dichelobacter nodosus* by PCR method. In Proceedings of 2001 Australian Society of Microbiology Annual Scientific Meeting and Exhibition, Perth.

3. **Dhungyel, O.P**., Whittington, R.J., and Egerton, J.R. (2002). Serogroup specific single and multiplex PCR with pre-enrichment culture and immuno-magnetic bead capture for identifying strains of *D. nodosus* in sheep with footrot prior to vaccination. (*Molecular and Cellular Probes* **16**: 285-296).

4. Kennan, R M., **Dhungyel, O P**., Whittington, R J., Egerton, J R. and Rood, J I. (2001). The type IV fimbrial subunit gene (*fimA*) of *Dichelobacter nodosus* is essential for virulence, protease secretion and natural competence. *Journal of Bacteriology* **183 (15)**: 4451-8.

5. Kennan, R M., **Dhungyel, O P**., Whittington, R J., Egerton, J R. and Rood, J I. (2002). Transformation-mediated serogroup conversion of *Dichelobacter nodosus*. (In preparation)

6. Ghimire, S.C., Whittington, R.J., **Dhungyel, O.P**. and Egerton, J.R. (2001). Diagnosis of footrot in goats: application of ELISA tests for response to antigens of *Dichelobacter nodosus*. Submitted to *Veterinary Microbiology*.

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Abbreviations

DNA	Deoxyribonucleic acid
D. nodosus	*Dichelobacter nodosus*
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
HA	Hoof agar
IDS	Interdigital skin
KSCN	Potassium thiocyanate
OD	Optical density
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
VFR	Virulent footrot

CHAPTER 1

Literature Review

Contents

1 Introduction

1.1 General description

Footrot is a contagious disease of feet of ruminants commonly seen in sheep and goats and a milder form in cattle (Egerton et al., 1969). It occurs world wide. It is a complex disease resulting from bacterial infection in which *Dichelobacter nodosus* (Dewhirst et al., 1990) is the essential transmitting agent (Beveridge, 1941; Egerton et al., 1969). *D. nodosus* is a Gram-negative obligate, anaerobic bacillus.

Footrot in sheep can result in a debilitating lameness with marked loss of productivity thereby causing economic loss. The disease is a chronic bacterial infection causing inflammation of the epidermal tissues of the hoof with underrunning of the horn progressing from an initial interdigital dermatitis. Footrot lesions result from a combined infection by *D. nodosus* and *Fusobacterium necrophorum*, although other bacteria like spirochaetes subsequently invade the primary lesions. *D. nodosus* is the essential transmitting agent and in its absence footrot does not develop (Egerton et al., 1969). *D. nodosus* lives only in the diseased feet of the animals and survives for 7 to 14 days in faeces, soil or pasture (Stewart and Claxton, 1993). Healthy animals acquire *D. nodosus* from affected animals which contaminate the environment (Stewart and Claxton, 1993; Whittington, 1995b).

1.2 Predisposing causes

Healthy, normal skin is not susceptible to infection by *D. nodosus* (Egerton et al., 1969). Invasion by this organism requires some prior damage to the interdigital skin. Hydration resulting from constant exposure to wet or moist conditions facilitates invasion by *F. necrophorum*. This primary infection and tissue damage enables infection by *D. nodosus*. If *D. nodosus* is not present the superficial dermatitis caused by *F. necrophorum* resolves (Egerton et al., 1969).

Transmission of footrot occurs only in constantly warm and wet environments. Isolated and short wet periods in an otherwise dry season do not allow transmission of footrot (Graham and Egerton, 1968). Outbreaks of footrot are usually confined to

regions that have sufficient annual rainfall to produce a lush green pasture during the warmer and humid months. Transmission of footrot stops when the environment dries out with the consequent dehydration of the interdigital skin.

Transmission only occurs when the mean daily ambient temperature is consistently above 10^0 C. However, established infections can persist through cold and hot temperatures (Graham and Egerton, 1968).

In brief the transmission of footrot to susceptible animals and the development of new cases requires the presence of:

(i) *D. nodosus* from infected sheep or recently contaminated pasture or soil

(ii) sufficiently wet conditions to allow invasion of the interdigital skin by *F. necrophorum* ; and

(iii) mean daily temperature above 10^0 C.

1.3 Clinical description

The clinical manifestation of footrot in sheep is determined by virulence of the strain of *D. nodosus* involved (Egerton and Parsonson, 1969; Stewart et al., 1984), environmental conditions (Graham and Egerton, 1968), and the innate and acquired resistance of the host (Bulgin et al., 1988; Skerman et al., 1988; Raadsma et al., 1993). Interactions between these factors determine the severity of the disease, which is expressed as a continuous spectrum of clinical entities ranging from benign to highly virulent footrot (Egerton, 1986; Stewart et al., 1986c; Egerton and Raadsma, 1991). Three clinical forms of footrot have been accepted for descriptive purposes and they are: virulent, intermediate and benign (Egerton, 1986; Stewart, 1989). These terms are also being used to describe the strains of *D. nodosus* which are isolated from and have the potential of causing respective clinical forms in exposed, susceptible animals.

1.3.1 Virulent footrot (VFR)

Virulent footrot in sheep is characterised by initial inflammation of the interdigital skin which is followed by a break in the skin-horn junction on the axial aspect of one

or both digits. This inflammatory disruption progresses into the epidermal matrix of the hoof. This results in extensive underrunning, extending up to the anterior and abaxial wall of the hoof horn. On feet which have been affected for several weeks or months, the hoof becomes long and misshapen and lameness is severe. Lesions of VFR have a distinctive foul smelling odour. Usually a high proportion of susceptible animals and their feet are affected (Beveridge, 1941). Clinically footrot is considered virulent if more than 10% of affected animals have underrunning of hard horn in at least one foot (Egerton, 1989c). More recently Abbott (2000) has argued that diagnosis should be based on animals at risk rather than using affected animals as the denominator. In the absence of treatment or control measures, VFR persists in chronic form in a high proportion of affected sheep (Beveridge, 1941). VFR causes a very significant economic loss to the sheep industry in Australia (Stewart, 1989; Egerton and Raadsma, 1991).

1.3.2 Benign footrot (BFR)

Benign footrot is often erroneously called footscald. The main lesion of BFR is interdigital dermatitis with little or no underrunning of the hoof (Egerton and Parsonson, 1969). It causes only mild lameness and rapidly regresses after topical treatment or after the advent of dry environmental conditions. However, in some countries in South America BFR is complicated by myiasis leading to secondary bacterial infections, severe lameness and economic loss (Egerton and Raadsma, 2000). In BFR a large proportion of the flock can be affected but only under conditions favourable for spread of the disease. Clinically BFR is diagnosed if less than 1% of the affected sheep have underrunning of the hard horn of hoof (Egerton, 1989c). Usually the economic losses due to BFR are minimal (Stewart, 1989; Egerton and Raadsma, 1991).

1.3.3 Intermediate footrot (IFR)

The intermediate form of footrot lies between virulent and benign forms with both lesion severity and the production losses observed (Stewart, 1989; Egerton and Raadsma, 1991). Severe lesions may be seen in a few sheep. A variable percentage of the flock may be affected depending on the climatic conditions. Only a small

percentage, less than 10% of affected sheep have underrunning lesions (Egerton, 1989). A majority of the animals in the flock have lesions either restricted to the interdigital skin or underrunning of only the soft horn of heel and sole (Stewart et al., 1982; 1984). The degree of necrosis and hoof damage of the severe lesions of IFR is markedly less than that seen in outbreaks of VFR, except in a few animals (Abbott, 2000).

1.4 Scoring of footrot lesions

It is important to describe as objectively as possible the lesions in affected animals in order to study the pathogenesis, epidemiology, treatment, control, and production losses due to footrot.

In the method of Egerton and Roberts (1971) inflammation confined to the interdigital skin is scored 1, or 2 if it is severe; if the soft horn of the hoof is underrun it is scored 3, or 4 if the underrunning extends to the tissues under the hard hoof. This system was modified by Stewart et al (1982; 1985) who subdivided score 3 lesions into 3a, 3b and 3c according to the degree of underrunning. These methods are used by most research groups although further modifications have been made. In an extensive study by Whittington and Nicholls (1995) sixteen methods of grading the lesions of ovine footrot were assessed on the basis of the effect of the lesions on the humoral immune response of the host to the causative agent *D. nodosus*. The results of this study found that the methods that allowed for qualitative and quantitative differences in lesion scores between sheep were the best predictors of host response, and methods that assessed the lesions in each of the eight digits were more efficient than methods that did not grade the digits within feet. This study also found that weighting the scores for lesions that involved underrunning of the hoof provided a powerful means of predicting host response. They proposed total weighted footscore, which is the sum of the footscores of the four feet weighted for underrun lesions as a simple and effective grading system for sheep with lesions of footrot. However, the original method of Egerton and Roberts (1971) remains as the simple and most commonly used method for most footrot scoring work.

1.5 Pathogenesis

Beveridge (1941) was the first to conclude from his studies that *D. nodosus* was the primary causal organism of footrot in association with *Spirochaeta penortha*. His studies could not show a significant role for *Fusobacterium necrophorum* in pathogenesis and he considered it a secondary invader. Thomas (1962) considered that only *D. nodosus* was capable of causing severe footrot with progressive separation of the horn of the hoof. Egerton et al. (1969) with their experiments were able to demonstrate that *D. nodosus* alone did not induce footrot in sheep in a dry environment. From their studies it was concluded that following colonisation of macerated interdigital skin, *F. necrophorum* facilitated infection with *D. nodosus*, by inducing hyperkeratosis and parakeratosis, resulting in crevices and facilitating anaerobic bacteria to survive and multiply. It was suggested in this study that footrot was caused by a synergic association of the obligate parasite, *D. nodosus*, in conjunction with *F. necrophorum*. The latter is present in the normal flora of the ovine gastro-intestinal tract. The disease started to develop only when the presence of *D. nodosus* coincided with warm and wet conditions, and faecal contamination facilitating colonisation of the interdigital skin by *F. necrophorum* (Egerton et al., 1969). *D. nodosus* had less inflammatory and destructive capacity but started the invasion and extension of the lesion into the matrix of hoof. It had the capacity to remain in lesions for long periods, thereby maintaining the infection (Roberts and Egerton, 1969).

1.5.1 Pathology

In the early studies of Beveridge (1941) he considered that the inflammatory process of footrot caused progressive accumulation of exudate in the intercellular spaces producing cavities, progressive cellular atrophy, and detachment of the horn from epithelium. In 1955, Deane and Jensen identified the degenerative (affected) layer of the epithelium as the *stratum granulosum* and superficial layers of the *stratum spinosum*. In their view there was hydropic degeneration, necrosis of epithelial cells, and formation of larger vacuoles with infiltration of leucocytes in the early lesions. In advanced lesions there was extensive liquefactive degeneration and necrosis of the

epithelial cells at the edge of the lesion in *stratum granulosum* and superficial cells of *stratum spinosum*. In contrast to the view of Beveridge (1941) they suggested that necrosis in this region was responsible for detachment of the hoof from the underlying epithelial structures of the foot. These studies did not establish the direct association between the pathological changes and the presence of *D. nodosus*. Thomas, (1962) in his study was able to demonstrate abundant *D. nodosus* invading the *stratum lucidum* beyond the point of cleavage of the epidermis but *D. nodosus* were not observed to penetrate *stratum granulosum* and *stratum spinosum* even though the cells in these regions showed cytoplasmic degeneration. The changes like degeneration, detachment, and liquefactive digestion of partially keratinised cells of the *stratum lucidum* and *stratum spinosum,* as well as formation of sinuses and necrotic foci in the horn, were thought to be directly related to the presence of *D. nodosus* and were not associated with other bacteria (Thomas, 1962). This damage was considered to be consistent with digestion of keratolytic proteases of *D. nodosus* (Thomas, 1962; 1964).

However, the results of Egerton et al (1969) questioned these findings. Their study concluded that the cause of separation of horn was due to disruption of epidermis by diffuse inflammation and cellular infiltration and was not associated with immediate presence of bacteria or keratolytic activity. So they considered that there was no evidence of separation due to direct bacterial attack on the horn itself. However, the study of Stewart and Parsonson (Stewart, 1989) and Hine (1984) reconfirmed the findings of Thomas (1962), suggesting that the separation of hoof was due to direct bacterial attack. In their histological study they have shown consistent association of *D. nodosus* with the point of cleavage and the plane of separation located in the interface zone between *stratum lucidum* and *stratum granulosum*. Even though *D. nodosus* did not have keratolytic activity either *in vivo* or *in vitro*, proteases or other factors were thought to be involved in separation of epidermis since the lesion did not involve keratinised tissue (Stewart, 1989). The intensity of the inflammatory response which usually extended from the dermis to the epidermis was a common feature of footrot. Occasional phagocytosis of *D. nodosus* by neutrophils was noted in the epidermis where there were large numbers of monocytes and neutrophils (Hine, 1984).

1.6 Immunological memory

Immunological memory is the capacity of immune system to mount an accelerated and more prolonged response on re-exposure to a previously exposed antigen than that occurs after the primary stimulus (Adelstein et al., 1990). Memory is manifested by a sharp rise in serum immunoglobulin (Ig) following a second dose of the same antigen. It is a response mediated by both B cells and T cells. A typical anamnestic B cell response has been demonstrated by vaccine administration for ovine footrot (Egerton and Thorley, 1981; Stewart et al., 1983). In ovine footrot vaccination studies it has been demonstrated that the duration of immunological memory following vaccination can be very long and an optimum secondary response can be achieved by extending the inter vaccination interval to at least 1 year (Schwartzkoff et al., 1993; Ghimire, 1997).

Immunological memory responses in sheep recovered from footrot can be aroused by natural recurrent infection or by subcutaneous injection of OMP or pilus antigens of *D. nodosus* (Whittington and Marshall, 1990; Whittington and Nicholls, 1995b; Whittington, 1996). The magnitude of this anamnestic immunological response is an indicator of the primary immune response of the animal, and is directly correlated with the highest antibody response attained during infection, which in turn is determined by the severity of foot lesions. The memory response lasts for at least one year after recovery from footrot. The memory response to OMP antigen is not very specific (Whittington, 1996; Whittington et al., 1997) but for pilus it is specific and this test has been used for the retrospective diagnosis of virulent footrot.

1.7 Characteristics of virulence

Virulence of *D. nodosus* has been correlated with various *in vitro* characteristics like colony morphology, fimbriation, twitching motility, agar corrosion and diffuse polar material on the cell surface amongst others and these are regarded as potential virulence factors. Virulence associated gene regions also have been studied (Katz et al., 1991; Rood et al., 1996; Whittle et al, 1999, see below). However, the virulence of

an isolate is not indicated by any one of these characteristics (Egerton and Raadsma, 1993). The full components of virulence have not been defined.

1.7.1 Fimbriation

In a variety of bacteria, fimbriae are associated with virulence as they are the organelles for surface attachment (Johns and Isaacson, 1983; Klemm, 1985; Moore and Rutter, 1989). In *D. nodosus* the role of fimbriae in attachment and colonisation of skin has not been demonstrated but the degree of fimbriation is correlated with virulence of an isolate (Stewart, 1973; Thorley, 1976; Stewart and Egerton, 1979; Skerman et al, 1981; Every and Skerman, 1983). However, this correlation is not absolute as a few strains known to be benign were shown to have as many fimbriae as virulent strains (Depiazzi and Richards, 1985; Stewart et al, 1986). Fimbriation was determined by electron microscopy in most of the above studies, rather than by quantification of purified fimbrial protein from a known number of cells. It is known that *D. nodosus* fimbriae can easily detach from cells in the process of preparation for microscopy (Mattick et al, 1984). So it is not known whether the results of the above studies were accurate. Fimbriae play a definite role in the virulence of *D. nodosus* (Kennan et al, 2001; and see chapter 5 of this thesis).

1.7.2 Twitching motility

Depiazzi and Richards (1985) have shown that twitching motility is more common in virulent isolates than benign, and this relationship was shown to be better in isolates subcultured a few times. However, this is a highly variable characteristic (Depiazzi et al, 1990). Twitching motility alone cannot differentiate strains of benign and intermediate virulence (Depiazzi and Richards, 1985). Depiazzi et al, (1990) found that a laboratory derived avirulent strain was highly motile. So this characteristic may only be useful as a supplementary test to other virulence associated factors.

1.7.3 Agar corrosion

Corrosion of agar under the colony is caused by *D. nodosus* grown on solid agar media (Beveridge, 1941; Egerton and Parsonson, 1966; Stewart et al., 1986).

Whittington (1994) suggested that this corrosive action could probably be ue to the production of polysaccharide degrading extracellular enzymes. This corrosive property was found to be highly correlated with the virulence of *D. nodosus* by Stewart et al. (1986). However further investigation on this characteristic has not been carried out.

1.7.4 Extra cellular proteases

Secretion of extra cellular proteolytic enzymes by *D. nodosus* was first reported by Thomas (1964 a, b). These enzymes were shown to degrade substrates like casein, azocasein, elastin, hoof powder (Thomas, 1964a), gelatin, fibrin (Thomas, 1964b; Broad and Skerman, 1976), hide azure powder (Broad and Skerman, 1976; Kortt et al., 1982), collagen I, collagen III and α-keratin (Green, 1985). These enzymes lose 85% of their activity when heated at 50^0 C and are completely inactivated in 30 mins at 70^0 C (Thomas 1964b). Various characteristics of these protease enzymes have been widely used to characterise *D. nodosus* isolates (Egerton and Parsonson, 1969; Depiazzi and Richards, 1979; Stewart, 1979; Kortt et al., 1982; Depiazzi et al., 1991; Palmer, 1993; Links et al., 1995). In a review of these protease based tests Whittington (1994, 1995a) found a good correlation between virulence of *D. nodosus* strains and positive test results but suggested further verifications. He also noted inconsistencies between different tests, and between clinical diagnosis and laboratory test results indicating a false positive rate of 20 –25 %. He suggested that factors, other than proteases may be involved in virulence of the organism.

The genes encoding proteases of *D. nodosus* have been identified and characterised (Lilley et al., 1992; Kortt et al., 1993; Riffkin et al., 1993; 1995; Katz et al., 1991). Monoclonal antibodies specific to virulent and benign strains have been developed (Riffkin et al., 1995) and were used in ELISA to screen the presence or absence of particular proteases in different isolates. Monoclonal antibody ELISAs on pure cultures were able to differentiate virulent and benign isolates at a similar level to conventional tests but on lesion samples the results were less consistent (Links et al., 1995). The correlation between accuracy of this ELISA and conventional tests like elastase and gelatin gel tests were also limited.

1.7.5 Virulence specific gene regions

A genomic region, which was shown to occur at a high frequency in virulent isolates of *D. nodosus* but at a low frequency in benign isolates, was identified by using a recombinant plasmid pJIR318 (Katz et al., 1991; 1992) as a probe in dot blot hybridisations. Because of its apparent association with virulence this region was designated as the *vap* (virulence-associated protein) region (Katz et al., 1992). This region seems to have arisen by the integration of a genetic element, the *intA* (*vap*) element into a tRNA gene (Katz et al., 1991; Rood et al., 1996). Multiple copies of the *intA* element, known as the *vap* regions 1, 2 and 3 were shown to be present in a virulent prototype strain of *D. nodosus,* A198 (Katz et al., 1994). Regions 1 and 3 form part of the contiguous 11.9 kb virulence-associated region (Cheetham et al., 1995). Region 1 is integrated into a tRNA-ser_{GCU} gene close to the aspartokinase (*askA*) gene, while region 2 is integrated into a different tRNA-*ser* gene, tRNA-ser_{GCA}, next to the polynucleotide phosphorylase gene (*pnpA*) (Bloomfield et al., 1997).

The genes that have been identified in *vap* regions of virulent strain A198 of *D. nodosus* have been designated *intA*, *vap* A- H, and *toxA* (Katz et al., 1992; Cheetham et al., 1995; Bloomfield et al., 1997). The product of *intA* gene is an integrase belonging to the lambda family of site specific recombinases (Cheetham et al., 1995). VapA and ToxA of *D. nodosus* were thought to have similar function to HigA and HigB proteins from the killer plasmid Rts1 in *Escherichia coli* (Bloomfield et al., 1997). The *vapB* and *vapC* genes were similar to *vagC* and *vagD* from virulence plasmid of *Salmonella dublin* (Pullinger and Lax, 1992). VapD and vapE were similar to the products of plasmid borne genes of unknown function from a variety of bacterial species (Katz et al, 1992; Cheetham et al., 1995) and no known similarities have been found to other genes, *vapF*, *vapG* and *vapH*.

A native plasmid, pJIR896, containing *vap* region 1 and an insertion sequence (IS1253) has been isolated from a strain of *D. nodosus* and this is thought to be the origin of the *vap* sequences found in the chromosomal region of other strains (Billington et al., 1996). IS1253 has not been found in the *vap* regions of the virulent

strain A198, instead a copy has been found near the outer membrane protein (*omp*) gene cluster (Moses et al., 1995).

The *intB*, a second genetic element, which is thought to be a part of a prophage or a conjugative transposable element has been found integrated next to *vap* region 3 in strain A198 (Bloomfield et al., 1997). A regulatory gene, *regA,* and three genes of unknown function, *gepA*, *gepB* and *gepC* were found at the end of this element, *intB*.

A new element, the *intC* element was found in strain C305 of *D. nodosus* (Whittle at al., 1999). This element is integrated into tRNA-ser_{GCU}, next to *askA*. In that study they have demonstrated loss of thermostable protease activity with loss of this element from a virulent strain of *D. nodosus*. Thermostability protease activity is used as a measure of virulence in *D. nodosus* isolates. So it is suggested that loss or gain of *intC* element from *D. nodosus* strains in natural infections may allow switching from the virulent to benign phenotype and vice versa, which could have a selective advantage (Whittle et al., 1999). However, this has not been proven.

A 27 kb DNA sequence known as virulent-related locus or *vrl* was first identified in a virulent strain of *D. nodosus*, A198 (Haring et al., 1995), using recombinant plasmid probes pJIR314B and pJIR313 (Katz et al., 1991). In a study of Rood et al (1996) using 771 isolates of *D. nodosus* the presence of *vap* and /or the *vrl* region (s) was shown as a reasonable predictor of virulence irrespective of the virulence determined by clinical evidence, supported by laboratory tests like elastase activity, protease thermostability, zymogram pattern or colony morphology. In this study they have classified isolates into 3 major categories, category 1 isolates contained both *vap* and *vrl* regions, category 2 contained only *vap* region and category 3 contained neither loci. None of the isolates had the *vrl* region only. They suggested that the presence of the *vap* region is essential either for insertion or maintenance of the *vrl* region.

1.8 Diagnosis of footrot

1.8.1 Clinical diagnosis

As in diagnosis of any disease the information on the history of the flock, including their origin, contact with other flocks, and the environment is which they have been

kept is important in making a diagnosis of footrot. On the basis of percentage of feet with score 4 lesions the disease can be classified as virulent if greater than 10%, as intermediate if between 1-10%, and benign if less than 1%. For 90% confidence limits of the estimate of score 4 lesions it is determined by the number of animals examined (Egerton, 2000).

Clinical differentiation of different forms of footrot is more difficult in the early stages of the disease when there are only the interdigital lesions. In such a situation consecutive visits a few weeks apart are required to examine the progression of lesions in previously identified animals after a period in the paddocks or after placing them in pens on wet mats (Egerton, 1989).

1.8.2 Differential Diagnosis

A number of other viral, bacterial, parasitic, physical and metabolic diseases can cause lameness in sheep and other ruminants so a differential diagnosis between these diseases is very important in the diagnosis of footrot. Viral diseases causing lameness in ruminants are foot and mouth disease (FMD), contagious pustular dermatitis (scabby mouth or orf), blue tongue and ulcerative dermatosis. Bacterial causes of lameness are footrot, ovine interdigital dermatitis (OID), foot abscess, erysipelas, toe abscess, infectious arthritis, and strawberry footrot. A physical cause is trauma. Parasitic infestation like strongyloides and trombicula, and metabolic causes such as laminitis and osteodystrophy may also cause lameness.

A review of the differential diagnosis of mixed bacterial infections causing lameness that are commonly seen in sheep (Egerton, et al., 1969; Stewart, 1989) is presented in Table 1.1.

Table 1.1. Differential diagnosis of mixed bacterial infections causing lameness in sheep under wet conditions[1] (adapted from Egerton, 1969 and Stewart, 1989*) A. pyogenes = Actinomyces pyogenes; D. nodosus = Dichelobacter nodosus; F. necrophorum = Fusobacterium necrophorum*

Disease	**Animals affected**	**Tissue affected**	**Bacterial flora**	**Clinical signs**
Ovine interdigital dermatitis (OID, 'Scald')	All Classes	Interdigital Skin	*A. pyogenes, F necrophorum* And *diptheroids*	Transient lameness in a few affected sheep. Disappears when pastures dry out. May be present in all feet.
Benign footrot ('Scald', non progressive footrot)	All Classes	Interdigital Skin	OID flora. *D. nodosus* of low virulence, *spirochaetes* and motile fusiform bacteria	Low to high morbidity rate. May be present in all feet. Mild lameness in proportion of affected sheep. Cases resolve without treatment when pastures dehydrate but recur in next wet, warm season. Minimal production loss.
Intermediate Footrot	All Classes	Interdigital skin, Matrix of horn	OID flora. *D. nodosus* of intermediate virulence, *spirochaetes* and motile fusiform bacteria	Small proportion of flock affected with severe (underrunning, score 4) lesions. <10% . May be present in all feet. Lameness commensurate with severity of lesion. Persists in a few sheep in the absence of treatment. Minimal to moderate production loss.
Virulent Footrot	All Classes	Interdigital skin, Matrix of horn	OID flora. Virulent *D. nodosus spirochaetes* and motile fusiform bacteria	High proportion of flock affected. May be present in all feet. Causes marked lameness. Persists in the absence of treatment. Marked production loss in chronically affected sheep.
Foot abscess	Rams, ewes in late pregnancy	Subdermal tissues and distal interphalangial joint. Sinuses opening at the coronet	*A.pyogenes* and *F.necrophorum*	Low proportion of flock affected (usually less than 10%). Occurs most commonly in hind feet. Causes acute lameness. Chronic cases characterised by marked swelling and discharging sinuses
Toe 'abscess' (Lamellar suppuration)	All Classes	Sensitive laminae of toe region. Sinuses may discharge above coronet	Various bacteria including *F.necrophorum*	Low proportion of flock affected. Front feet most commonly involved. Causes acute lameness.

[1] Growth of aerobic bacteria on the surface of the wet interdigital skin provides suitable conditions for invasion of the epidermis by *F.necrophorum.* This invasion results in OID. In flocks where *D. nodosus* is present invasion of OID by this organism results in benign, intermediate or virulent footrot depending on the virulence of the strain present. Other bacterial invasion beyond the epidermis results in foot abscess. Toe abscess does not arise from a primary OID but through a break in the continuity of the horn. Table reproduced with kind permission of J. R. Egerton.

1.8.3 Laboratory diagnosis

The present routine laboratory diagnosis of footrot is an elaborate and time-consuming process which is complicated by the fastidious growth requirements and slow growing nature of *D. nodosus*. Provisional diagnosis is based on the clinical examination and characteristic cellular morphology of the organisms observed by microscopic examination of lesion smear. The organisms can be recognised as Gram-negative straight or slightly curved large rods with terminal knobs (Skerman, 1989).

In *in vitro* culture on hoof agar media (Thomas, 1958) *D. nodosus* colonies display some typical morphological features, which enable them to be differentiated from other bacterial colonies (Thorley, 1976; Skerman et al., 1981), and to a lesser extent to estimate the virulence of the strain on the media (Stewart et al., 1986). The twitching motility of *D. nodosus* in stab-inoculated agar cultures can also provide some information on virulence of the organism (Depiazzi and Richards, 1985). However, the *in vitro* cultural characteristics of *D. nodosus* tend to be variable and not very precise and this limits the usefulness of this method.

1.8.4 Immunoassays

The agglutination test using rabbit antisera against whole cell or fimbrial antigens is used for classification of strains of *D. nodosus* (Claxton et al., 1983). Immunological tests using various preparations of antigens have been reported for the detection of *D. nodosus* bacteria and for surveillance of the infection status or antibody responses of animals with footrot (Stewart et al., 1990; Whittington and Marshall, 1990; Whittington and Egerton, 1994; Whittington, 1996; Dhungyel et al., 2001). The use of antigens common to all the serogroups such as outer membrane proteins (OMP) is useful in detecting heterologous serogroups but is non-specific in older animals (Whittington, 1996). This non-specificity can be overcome by using fimbrial antigens, and anamnestic tests using these antigens seem to be good epidemiological tools for VFR (Dhungyel et al., 2001). However, the question of serogroup specificity with these antigens need to be evaluated.

1.8.5 Polymerase chain reaction (PCR) assays

Sensitive PCR assays based on the specific ribosomal RNA sequence (La Fontaine et al., 1993) and fimbrial gene sequence (Cox, 1992) have been developed for the detection of *D. nodosus*. In principal these tests can detect down to one cell of the organism, are capable of direct diagnosis from lesion materials and have the potential of eliminating the need for time-consuming culture techniques. A PCR assay based on the ribosomal RNA sequence of *D. nodosus* has been extensively evaluated by Egerton and Rood (1996). That study with lesion samples demonstrated the high specificity of the test but it was not sensitive enough for practical application. The main limitation for using this assay on direct lesion samples was an inhibition problem due to extraneous materials in the samples. This has been reported as the common problem with biological samples in PCR assays (Egerton and Rood, 1996). However, progress has been made in modifying the procedures of direct PCR assays on biological samples (Widjojoatmodjo et al., 1992; Millar et al., 1995, Mason et al., 2000).

The possibility of developing serogroup specific PCR assays for the diagnosis of specific *D. nodosus* was highlighted by Cox (1992). Even though, similar tests have been tried with limited numbers of laboratory cultures of *D. nodosus* in America (John et al., 1999) and New Zealand (Zhou et al., 2001) there has not been extensive evaluation of this test with field samples. The potential use of such a test if developed with the modified procedures for sensitive application on field samples could be valuable.

1.8.6 Virulence determination of *D. nodosus* strains

Virulence determination of *D. nodosus* strains is done to supplement or to confirm the clinical diagnosis of footrot. Based on the quantitative activity of protease enzymes produced by *D. nodosus* a prototype elastase test was first reported by Egerton and Parsonson (1969). Over the years this test has been refined (Stewart, 1979). The time from inoculation of the isolate onto a elastin agar plate to the detection of elastase activity is now used to classify the virulence of that isolate. This test provides a useful classification of *D. nodosus* in general agreement with severity of clinical

characteristics (Stewart, 1979). Besides the quantitative differences, proteases produced by virulent and benign isolates of *D. nodosus* also show different thermostability (Egerton and Parsonson, 1969; Depiazzi and Richards, 1979). This difference in thermostability of protease enzymes was used for testing the virulence of isolates using hide powder azure as a substrate (Depiazzi and Richards, 1979). Refinement of this test has led to the development of a gelatin gel test (Palmer, 1993; Liu and Young, 1993).

An electrophoretic zymogram test (Every, 1982; Kortt et al., 1983; Gordon et al., 1985) was also used to test the virulence of *D. nodosus* but because of the technical difficulties is not commonly used.

A monoclonal antibody based ELISA was shown to be able to differentiate strains of *D. nodosus* causing different clinical forms of footrot (Stewart et al., 1990). The performance of this antibody test has not been assessed extensively against other laboratory tests.

All these above mentioned tests were designed to test the virulence of *D. nodosus* based on phenotypic expression so these were likely to be influenced by factors that affect the growth and thus the phenotypic expression of the organism. To overcome this drawback the ability to determine pathogenic capacity of strains of *D. nodosus* at the genetic level were looked at. Three virulence-associated gene probes with specificity for virulent strains were developed by Katz et al (1991). Further development of this test to identify virulent, benign and intermediate strains was done by Liu and Young (1993) and Liu (1994). The initial assessment of these gene probe based dot-blot hybridizations using a collection of 96 isolates have shown a general agreement with elastase and gelatin gel tests and were correlated with clinical manifestations of footrot (Liu, 1994).

For improved determination of the virulence of *D. nodosus* a PCR assay was developed based on the nucleotide sequence gene probes developed for dot-blot hybridization test (Liu and Webber, 1995). Virulent and benign specific PCR assays were developed and tested and have shown to be sensitive, specific and fast for virulence testing (Liu and Webber, 1995). However, there were discrepancies between

the phenotypic and genotypic classification of isolates by this method (Rood et al., 1996).

Hence, these more recently developed diagnostic tests need to be extensively assessed against the existing tests for practical routine use in diagnosis of footrot. Also these tests may need to be further modified and developed in accordance with the recent advances made in PCR technology.

1.9 Antigenic classification of *D. nodosus*

Antigenic variation among strains of *D. nodosus* was first observed by Beveridge (1941). Egerton (1973) described surface (K) and somatic (O) antigens that were detected using a slide agglutination test. Of the two antigens, one was heat-labile, responsible for the coarse K-type agglutination and could be removed by washing the cells. The second antigen was heat-stable and could cause fine agglutination. Short et al., (1976) showed strong evidence that the K-type agglutination was due to a fimbrial reaction and the other one due to surface antigens.

Fimbriae of *D. nodosus* were first identified by Stewart (1973) and Walker et al., (1973) and were shown to be antigenic and able to mediate the K-agglutination reaction. They were shown to have a strong relationship with antigenicity and protection against challenge (Egerton, 1974; Egerton, 1978; Egerton, 1984; Stewart, 1978a and 1978b).

1.9.1 Australian system of classification

Egerton (1973) classified 33 of the 46 isolates of *D. nodosus* held at McMaster collection into serogroups A, B or C by K-agglutination. Studies were carried out to investigate the heterogeneity of fimbrial antigens in the *D. nodosus* population with the aim of devising a practical system to screen many isolates and to classify them into different serogroups by slide agglutination (Claxton et al., 1983). Initially field isolates were screened against the three prototype antisera of Egerton (1973). Those isolates which did not react with the available antisera were screened by raising antisera against them. By this method Claxton et al., (1983) classified 1260 of 1267

isolates into eight serogroups (A-H). The remaining seven isolates were later classified into the ninth serogroup, I (Claxton, 1986).

This system of serogrouping by slide agglutination has since been extensively used in Australia and New Zealand to classify the field isolates of *D. nodosus*. Claxton (1981) also found that cross-tube agglutination gave titres reflecting the slide agglutination test results across serogroups. Isolates of heterologous serogroups gave titres of <80 with prototype antisera. Isolates within serogroups were further subdivided into serotypes by serotyping with tube agglutination. A total of 18 serotypes have been identified in different serogroups, and all the isolates of Australia, New Zealand and UK have been classified by this method (Hindmarsh and Fraser, 1985; Kinsley et al., 1986).

The validity of this serotype classification was tested for serogroups A and B by cross-tube agglutination using absorbed and unabsorbed antisera (Claxton, 1981). Absorption of antiserum with homologous antigen removed all the agglutinating activity whereas absorption with heterologous antigen removed only part of that activity. This suggested the existence of two epitopes, one serogroup specific and the other serotype specific. This was further confirmed by carrying out cross-agglutination tests with these 2 antigens and antisera against them absorbed with a number of other serogroup A antigens (Claxton, 1981).

The protection after vaccination was found to be restricted to components of a fimbrial serogroup (Egerton et al., 1972; Egerton, 1974). Cross-protection trials within a serogroup were conducted in parallel to the agglutination studies for serogroup A which did show protection against serotypes within serogroup (Claxton, 1981).

1.9.2 Other classification systems

Thorley and his team in the UK developed their classification based on cross tube agglutination with absorbed rabbit antisera and a total of 17 serotypes (A-H and J-R) were identified (Thorley and Day, 1986). Considerable cross reaction was observed

between serotypes even though absorption of antisera removed shared agglutinating epitopes between isolates.

An American system of classification has been developed at the Oregon State University. Tube and/or cross tube agglutination tests were used to classify the isolates and those reacting within a dilution of homologous reaction were considered to be of one serotype (Schmitz and Gradin, 1980). By this method a total of 21 serotypes designated as I- XXI have been identified (Gradin et al., 1993). Attempts have been made to regroup the American serotypes by the Australian classification and seven serotypes have been found to belong to serogroup B, 3 each to serogroup A and H, and one each to serogroups C, D, E, F and G. American serotypes III, IV and XVIII were not represented by Australian serogroups, and a counterpart of serogroup I has not been found among American isolates (Gradin et al., 1993).

The Australian system gives a basis for understanding the serogroup specific nature of vaccine protection whereas the other systems may have less relevance to this.

1.10 Type IV fimbriae

1.10.1 General characteristics:

Fimbriae are filamentous non-flagellar appendages on the surface of bacteria (Ottow, 1975). Compared to flagella they are less rigid, thinner, straight and more numerous. They are also referred to as pili (Elleman, 1988).

The fimbriae of *D. nodosus* have been classified as type IV, which include the fimbriae of *Moraxella bovis, Neisseria gonorrhoeae and Pseudomonas aeruginosa* among others (Ottow, 1975; Dalrymple and Mattick, 1987). Type IV fimbriae have a number of characteristics in common, like a polar location on the cell, enabling twitching motility and certain distinctive features of the fimbrial subunit (Ottow, 1975; Henrichsen, 1983; Mattick et al., 1993). These features include a short, positively charged leader sequence in the primary translation product, N-methylphenylalanine as the first residue in the mature protein, highly hydrophobic and highly conserved amino terminal domain, and other similarities in the protein

(Dalrymple and Mattick,1987; Mattick et al., 1993). Type IV fimbrial subunits range from about 145 to 160 amino acids in length. Variation between and within the species of bacteria occurs primarily in the carboxy-terminal 70% of the protein (Dalrymple and Mattick, 1987).

On the basis of general sequence conservation across species, three distinct regions can be identified in the type IV fimbriae (Figure 1.1) (Dalrymple and Mattick, 1987). Region I comprises of the highly conserved amino terminal one third of the protein. The highly variable carboxy terminal two third of the protein has been divided into regions II and III. Region I is further subdivided into Ia, the leader peptide, Ib, the highly conserved and hydrophobic amino terminal region between the first and the 27^{th} residue in the mature protein which is conserved across species; and region Ic which extends from residue 28 to glycine at residue 54 (55 in *N. gonorrhoeae*) of the mature protein. This region is almost totally conserved within bacterial species and fairly conserved between species (Figure 1.1). In addition the number and positions of cysteine residues, and thus the presumptive disulfide loop is also variable between type IV fimbriae (Dalrymple and Mattick, 1987).

1.10.2 General characteristics of *D. nodosus* Fimbriae

Fimbriae of *D. nodosus* have a diameter of about 6nm and may extend to 10 μm in length (Stewart, 1973). Fimbrial subunit proteins of different strains of *D. nodosus* vary in sizes from 16.5 to 19 KDa (Mattick et al., 1984). Although the exact function of *D. nodosus* fimbriae have not yet been clearly defined, they appear to play a central role in the invasion by the bacterium of the epidermal matrix of the hoof (Mattick et al., 1985a). Virulent isolates of this organism contain large numbers of fimbriae whereas those with few or no fimbriae are relatively avirulent or benign (Short et al., 1976; Skerman et al., 1981; Every and Skerman, 1983; Depiazzi and Richards, 1985; Stewart et al., 1986). It has been established that *D. nodosus* fimbriae represent primary serological and immuno protective antigens (Egerton, 1973; Schmitz and Gradin, 1980; Claxton et al., 1983). Animals vaccinated with highly fimbriated cells (Thorley and Egerton, 1981) or purified fimbriae (Stewart, 1978a; Every, 1979; Lee et al., 1983) developed a high agglutinin titre and were protected against homologous

infection, whereas animals vaccinated with less-well fimbriated cells had low levels of protection (Thorley and Egerton, 1981). The role of fimbriae as a major immunogen was further confirmed when animals vaccinated with recombinant DNA fimbriae expressed in a surrogate host (Elleman et al., 1986; Mattick et al., 1987) were protected against homologous challenge (Egerton et al., 1987; Stewart and Elleman, 1987).

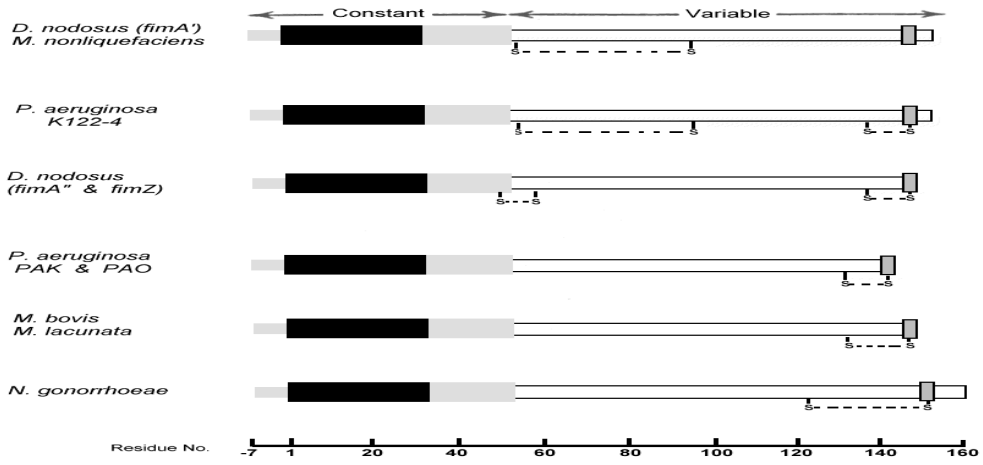


Figure 1.1 Schematic comparison of fimbrial subunit sequences of some type IV fimbriate bacteria

Source: Mattick *et al.* (1993). Lightly shaded thin block in the constant region represents the leader sequence, 6-7 amino acids long, which is cleaved in the mature protein. Darkly shaded block represents the highly conserved sequence which includes the first 32 amino acids of the mature protein and is highly hydrophobic. Lightly shaded thick block represents the area (residue 33 to 54/55 of the mature protein) which is highly conserved within species but has some divergence between species. Limited homology is observed between species in the variable region except for a limited part near the carboxy terminal (lightly shaded block). Dotted lines join pairs of cysteine residues.

1.10.3 Classification of *D. nodosus* fimbrial subunits

The gene encoding the fimbrial subunit *fimA*, has been well characterised from a number of *D. nodosus* isolates representing different serogroups and serotypes. On the basis of general sequence homology, *D. nodosus* fimbriae have been classified into 2 distinct fimbrial subunits, class I or A set, *fimA′* and class II or D set, *fimA″* (Elleman

1988; Mattick et al., 1991). Class I comprises of serogroups A, B, C, E, F, G, and I, and class II serogroups D and H. The fimbrial subunit of 'serotype' M (Day et al., 1986) has also been found to have the features of class I subunits and is suggested as a 'serogroup' M rather than a serotype (Ghimire et al., 1998). The two classes are also distinguished by a different physical and genetic organisation of the fimbrial gene region (Hobbs et al., 1991; Mattick et al., 1993). Both class I and class II fimbrial subunits show absolute conservation in protein sequence in the 7-amino-acid leader sequence and up to residue 18 of the mature protein, which includes most of the hydrophobic amino-terminal region (Mattick et al., 1993), and these are common features of the type IV fimbrial subunits in general (Dalrymple and Mattick, 1987).

This close similarity extends up to residue 34, with conservative or semiconservative substitutions that are largely but not absolutely class specific. Downstream of this point the sequences of the two classes of subunits diverge dramatically, with very little similarity except for a loose homology over the following 20 residues, up to glycine at position 54 (which is conserved in all type IV subunits). There is also some degree of conservation between bacterial species especially at the caboxy-terminal region (Dalrymple and Mattick, 1987). In both of these regions the class II subunits of *D. nodosus* appear to have more in common with those of other bacterial species such as *Pseudomonas* than they do with class I (Mattick et al., 1993).

Class I and II subunits also differ in the distribution and nature of conserved and variable sequence motifs in the remainder of the protein, as well as in the number and position of cysteine residues. Class I subunits contain cysteine residues at positions 56 and 100 that form a presumptive disulfide loop in the central third (region II) of the protein. Class II subunits contain two closely spaced pairs of cysteine residues, at positions 50 and 62 near the end of the conserved amino-terminal region and at positions 138 and 152 near the carboxy-terminus of the protein (Mattick, et al., 1993). Fimbrial sequences of class I share 60-80% amino acid sequence homology between themselves and within class II it is about 70%, but sequences between the 2 classes share less than 40% similarity (Elleman, 1988; Mattick et al., 1991).

Point mutations seem to be the cause of antigenic variation with strains of the same serotype, and accumulation of sufficient point mutations lead to minor changes in

epitope configuration which perhaps is responsible for differences in serotype. Similarly a process of accumulation of point mutations leading to alteration of a major epitope might be responsible for differences between serogroups. Antigenic switching to a different serogroup might only be possible when many changes are accumulated within a short time (Cox, 1992). Genetic drift alone might not be able to create sufficient changes in a limited time so genetic rearrangements like DNA inversion or recombination with either foreign DNA or with DNA within the same chromosome would be required to change an isolate from one serogroup to another. The occurrence of recombination in or around the *fimA* locus has been suggested (Cox, 1992) but so far there is no direct evidence of such events altering serogroup specific epitopes in *D. nodosus* fimbriae.

Despite the major differences between the 2 classes of fimbriae, transcription initiation and termination is under a similar control system. Two promoters function in fimbriae of both the classes, initiating transcription at position -63 and -45/-46 in response to fimbrial initiation codon (Hobbs et al., 1991). Downstream of the fimbrial subunit coding region, 16-32 base pairs from the stop codon, lies a region of hyphenated dyad symmetry (inverted repeats) followed by a run of thymidine residues (Elleman, 1988; Mattick, et al., 1991), where a majority of *fimA* transcripts terminate (Hobbs, et al., 1991).

1.10.4 Features of Class I fimbrial subunits

There is close relationship in the sequence and organisation of the class I fimbrial subunits of *D. nodosus*. Following the highly conserved amino-terminal region up to residue 30, all class I subunits show a degree of homology through to region 1c. After this region in class I fimbrial subunits 3 hypervariable clusters (HVC) are identified which perhaps represent the region of the protein not under structural constraints, (Dalrymple and Mattick, 1987; Elleman, 1988; Mattick et al., 1991). The first 2 HVC are located on either side of the presumptive disulfide loop. The first is between aligned positions (AP) 57-65 inclusive, the second between AP 85-99 inclusive, and the region between AP 137 – 147 represents the third HVC. The second HVC represents the area of profound differences between the isolates of different serogroups, which suggests that this region may be a substantial contributor to

serological profile (Mattick et al., 1991). No specific pattern or positions for variation were identified within isolates of a serogroup. Sequence variations within isolates of the same serogroup were recorded both within and outside the HVC (Cox, 1992).

On the basis of sequence comparisons, subunits of this class are divided into 2 subclasses: (a) comprising of serogroups A, B, E, F, and I, and (b) serogroups G and C. The subunits G and C show clear relationship and are distinct from others in both conserved and variable regions of proteins. Distinction among A, E, F, B and I subunits is much less marked, and the subdivision of this larger group is mainly based on the differences in the relatively conserved region between residues 37 and 54, wherein B and I subunits show close similarity to C and G (Mattick et al., 1991).

In other conserved regions of the protein there is no clear subgrouping of A, E, F, B and I, and the variable regions exhibit different relationships. The fimbrial subunit of A is more similar to B and I than E or F in the first and to a lesser extent, the third HVC, whereas in the case of the second HVC the only close similarity is between the B and F subunits (Mattick et al., 1991). Serogroups of B and F have previously been observed to cross-react in agglutination tests (Claxton, 1981). The nucleotide and the deduced amino acid sequences of the fimbrial subunits of serogroup M were found to be more similar to that of serogroup F than any other (Ghimire et al., 1998).

Sequence data have been obtained from almost all of the known serotypes of *D. nodosus* and it has been observed that there are 35 to 50 amino acid changes between serogroups, 8 to 15 changes between serotypes within a serogroup and only 0 to 5 changes within a serotype (Mattick et al., 1993). Although most changes between serogroups occur within the HVC, these motifs are relatively conserved within a serogroup, and would appear to be the major serogroup specific epitopes (Mattick et al., 1993). However, there may be some strains that are structurally intermediate, like serotype H2 (Hoyne et al., 1989, Mattick et al., 1993) and cross-reacting strains reported by Claxton (1981, 1986), Day et al. (1986), and Chetwin et al. (1991) may represent such exceptions. Another example is that the serotypes B2, B3 and B4 but not B1 show cross-reaction with serogroup I (Claxton, 1986). This is caused by the asparagine-proline motif in HVC I, because this is the only area in which B2, B3 B4 and I share residues independently of B1 (Mattick et al., 1991). Similarly, serogroups

B and F are known to be closely related antigenically (Claxton, 1981) and they share general similarities in HVC I and III and not so much in HVC II (Mattick et al., 1993).

The changes within a serogroup are not as localised. Single or occasionally double, differences occur throughout the carboxy-terminal two thirds of the protein, both within and outside the hypervariant regions (Mattick et al., 1993). The available data suggest that, within a serogroup there is a more apparent continuum, with the number and nature of changes determining the degree of antigenic relationship and classification. This is said to be consistent with protein and immunological studies that indicate a more continuous spectrum of minor variation within a serogroup and less discrete divisions between serotypes (Mattick et al., 1993).

1.10.5 Features of Class II subunits

The fimbrial subunits of class II serogroups D and H are almost identical until the end of region 1c, the only difference being a conservative valine-isoleucine substitution at position 51 within the first putative disulfide loop. The structure of the protein is distinctly different from that of class I and is more closely related to those of *Moraxella bovis* and *Pseudomonas aeruginosa*, especially in the semiconserved region, 1c and at the end of region II, where there is a disulfide bridge that is found in all type IV subunits analysed to date except *D. nodosus* class I (Dalrymple and Mattick, 1987; Elleman, 1988; Mattick et al., 1991). This feature suggests that class II subunits may have evolved in a different environment and been acquired by genetic exchange (Mattick et al., 1993).

Differences between the prototype strains of serogroup D and of serotypes H1 and H2 are distributed throughout the carboxy-terminal two-thirds of the protein, with only five short segments (4 – 5 amino acids) of sequence conservation between them (Mattick et al., 1993). The fimbrial subunits of D and H exhibit antigenic relationships in western transfer analyses (Anderson et al., 1987). Cross-reaction in the case of serogroup H is limited to the amino-terminal half of the protein and the sequences at this region show very few differences between the subunits (Mattick et al., 1993). The common epitope shared between D and H seems to be located within region 1c since

1b is highly conserved among all subunits, and region 1c is exposed to some degree of antibody recognition *in vivo* (Mattick et al., 1993).

Although the subunit of H2 has more in common with H1 than with D, it is sufficiently different from both in primary sequence to suggest it should constitute a separate serogroup (Hoyne et al., 1989).

1.10.6 Distribution and usage of codon

Analysis of codon usage in the *D. nodosus* fimbrial subunit gene by Mattick et al. (1991) has shown a pattern consistent with that of highly expressed genes in *E. coli* (Grantham et al., 1981; Grosjean and Fiers, 1982; Sharp and Li, 1986), exhibiting third base preferences related to optimization of codon - anticodon pairings for efficient translation with reasonable fidelity (Grosjean and Fiers, 1982). This is supported by the fact that the fimbrial subunit is probably the most abundant protein produced by the *D. nodosus* cell (Mattick et al., 1984) and has similar G + C content to *E. coli* (Holdeman et al., 1984). However, some exceptions include, relative scarcity of codons GTC and TCC from the fimbrial subunit of *D. nodosus* which are common in the other species (Maruyama et al., 1986), and virtual absence of CTG codon, which is predominantly a leucine codon in both procaryotes and eucaryotes. However, this particular codon is relatively common in adjacent genes on the *D. nodosus* genome (Hobbs et al., 1991; Mattick et al., 1993).

The unusual aspect of the *D. nodosus* fimbrial subunit genes is the obvious imbalance in the silent base changes in codons specifying conserved amino acids in different regions of the protein (Mattick et al., 1991). The areas upstream the *fimA* gene are highly conserved with no changes until –30. This nucleotide conservation extends into the 5′ coding region of the gene in all class I and II sequences and is virtually identical up to + 71 (Mattick et al., 1991). There are also very few base substitutions in codons specifying invariant amino acids in the rest of 1b and 1c regions. In contrast, a large number of silent base changes occur in codons in the rest of the protein. Particularly this is evident in class I genes where approximately 25% of such codons have silent base changes (Mattick et al., 1991). This seems to be significantly higher than that

found in adjacent areas of the genome, which suggests that the 3′ sequences of the fimbrial genes may represent a hypermutable region. Almost absolute conservation at the 5′ coding sequences suggests that these sequences have some important secondary function, possibly in the regulation of gene at transcriptional or translational region or in facilitating exchange by recombination (Mattick et al., 1993).

1.10.7 Organisation and expression of the fimbrial gene region

The genomic organisation immediately down-stream from the fimbrial subunit gene, *fimA*, is different in the 2 classes of *D. nodosus* strains and this difference appears only to be a local alteration because the rest of the genome appears essentially similar (Mattick et al., 1993).

The two classes are quite different downstream from *fimA*, but the homology is abruptly restored about 0.9-kb in class I and 2.5-kb in class II downstream at a bidirectional transcription terminal signal, after which lies a large gene *clpB* which also lies in opposite orientation (Figure 1.2). *ClpB* encodes the regulatory subunit of an ATP-dependent protease and is highly conserved both in procaryotes and eucaryotes (Hobbs et al., 1991).

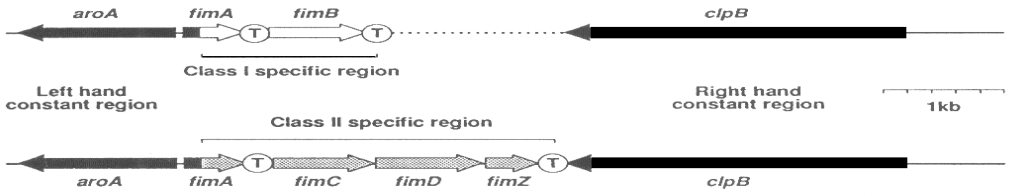
In both classes, upstream from *fimA* is the gene *aroA* (Figure 1.2) which encodes the aromatic aminoacid biosynthetic enzyme 5 – enoypyruvyl shikimate-3- phosphate synthase and lies in opposite transcriptional orientation to *fimA* (Hobbs et al., 1991).
The sequences 5′ to *fimA* in both classes are highly conserved and contain the promoter region (Hobbs et al., 1991). The sequence conservation extends into the *fimA* coding sequences but then diverges within region Ib. Even with this divergence the *fimA* gene in both classes is closely followed by transcription stop signal, yielding a major transcript 570 to 590 nucleotides long (Hobbs et al., 1991). The promoters of the *D. nodosus fimA* as well as other type IV fimbriate bacteria, are recognised by an alternative form of RNA polymerase whose initiation factor is the *rpoN* gene product (Hobbs et al., 1991).

In class I strains there is only one other open reading frame, *fimB* of 257 codons downstream from *fimA*, (Figure 1.2) and its initiation codon in some cases is only 18bp from the end of transcription stop signal of *fimA*. The translation product of *fimB* is possibly an inner membrane protein involved in fimbrial assembly (Hobbs et al., 1991). This gene does not have a separate promoter and is co-transcribed with *fimA* at a level attenuated by the strength of the transcription termination signal in the intergenic region (Mattick et al., 1993).

FimA in class II strains is followed by a more extended region of three genes which appear to have transcriptional arrangement similar to class I operon (Hobbs et al., 1991; Mattick et al., 1993). The first of these extra genes in class II is *fimC* which encodes a highly hydrophobic protein with no known similarity to other genes in these operons. *FimD* is the second gene which encodes a protein of 395 amino acids and may represent an analog of *fimB* of class I even though the sequences do not show close homology. At the 3′ end of the class II specific region, after *fimD* is the third gene *fimZ*, (Figure 1.2) a variant fimbrial subunit gene, and is virtually identical in serogroups D and H. It appears to represent a duplicate, possibly redundant, gene closely related to *fimA* gene found in these strains (Hobbs et al., 1991).

Figure 1.2 Schematic representation of the genetic organisation of the fimbrial gene region of Class I and Class II strains of *D. nodosus*

Source: Hobbs *et al.* (1991). Arrows represent genes (see text for description) and shows the direction of transcription. Darkly shaded area represents regions which are homologous in all strains examined. 'T' represents transcription termination signals.



1.10.8 Identification of the epitopes in *D. nodosus* fimbriae

It was demonstrated by Claxton (1981) that *D. nodosus* whole cells possessed two distinct K-agglutination serotyping epitopes. Beesley, et al.(1984) were able to show multiple epitopes on fimbriae with immuno-gold labelling. Day et al. (1986) postulated four different epitopes for the fimbrial molecule. In a series of monoclonal antibody (MoAb) studies Young et al. (1989) found evidence that both continuous and discontinuous serological epitopes are important on the *D. nodosus* fimbriae. They showed that there were at least 4 separate epitopes in the fimbriae of serotype A1 (1001). Three of these were non-linear, one which agglutinated whole bacteria of A1 and not A2 thus shown to be serotype specific, the other two were serogroup specific epitopes. Agglutinating epitopes were always found to be conformational and serotype-specific. It is suggested by Cox (1992) that in relating the antigenic reactivity of *D. nodosus* strains of subunit sequences, simple comparisons of stretches of sequence should be useful for identifying likely serogroup or serotype determinative regions. However, these comparisons are not adequate for determining which segments form different components of epitopes.

1.10.9 Possible serogroup-specific epitopes

Cox (1992) has proposed AP 31 to 34 of amino acid sequence of the fimbrial subunit either as a part of a serogroup-specific or even class specific agglutinating epitope, if such a segment existed independently. From AP 34 to the first HVC at AP 57 sequences are well conserved with some variation across groups, but there is almost absolute conservation within serogroups. This region possibly contributes to a serogroup-specific agglutinating epitope (Cox, 1992).

1.10.10 Possible serotype-specific epitopes

Based on amino acid sequence comparisons and predicted protein regions, Dalrymple and Mattick (1987) have predicted serotyping epitopes in class I strains of *D. nodosus* to lie in region II and III of the subunit. The sequence comparison made by Cox (1992) has identified seven sections of the fimbrial subunit, in addition to the proposed class-specific and serogroup specific segments, which vary in line with the

serological hierarchy. Billington and Rood (1991) made a sequence comparison of five of the serogroup B isolates and have suggested aminoacids AP 126 and 137 to be the likely serotype specific area.

1.11 Conclusions

1. Serogroup identification of *D. nodosus* is the prerequisite to formulate any footrot control/eradication program, more so to achieve this by vaccination. Blanket vaccination with multivalent vaccines covering all of the serogroups would not make it necessary to identify serogroups present in the affected flocks. This would be the ideal situation for the control of any disease. However, antigenic competition is being seen as the major constraint in such blanket vaccinations with multivalent footrot vaccines (Schwartzkoff et al., 1993; Raadsma et al., 1994; Hunt et al., 1994, 1995). Monovalent or bivalent vaccines are highly efficient with both curative and protective effects compared to the multivalent vaccines (Raadsma et al., 1994).

Specific/target vaccination with autogenous vaccine has proven to be very efficient in controlling footrot (Egerton et al., 1996; Ghimire, 1997). For target vaccination with autogenous vaccine it is essential to identify the serogroup present in the affected flock. It is also important to monitor the disease status in a vaccination program with a quick test to check the efficacy of the specific vaccine.

There is no single test for specifically identifying and grouping *D. nodosus* so that only the desired serogroup(s) are incorporated in an autogenous vaccine. The currently available procedure of isolation of *D. nodosus* from lesion samples by culture techniques and identification by serogrouping/serotyping using slide agglutination/tube agglutination takes 4 to 6 weeks. These tests are also complicated by occasional cross reactions between different serogroups (Claxton 1986; Stewart et al., 1991 and Gradin et al., 1991). It is essential to monitor the specific vaccination program with regular checks on footrot status and even more important to know the serotypes of the new isolates in cases of breakdowns or non-response to the vaccine. The 4 to 6 weeks taken to identify the isolates can be too long to effectively implement the control/eradication program. Rapid determination of serogroups

infecting a flock during an outbreak or in case of endemic infection could also allow more accurate formulation of vaccines.

PCR technology has provided a faster diagnosis of disease directly from lesion samples. PCR amplification of the fimbrial gene of *D. nodosus* (Cox, 1992) gives the opportunity to make rapid diagnosis. It is possible to apply a diagnostic PCR on footrot lesion samples but the sensitivity and specificity of the test is compromised by the extraneous materials present in the lesion samples (Rood and Egerton, personal communications). However, with the advances made in the PCR technology modified methods have been developed for diagnosis from such samples (Millar, et al., 1995; Widjojoatmodjo, et al., 1992). It now seems feasible to use these techniques with acceptable sensitivity and specificity on footrot lesion samples.

Various studies on fimbrial subunit gene sequences as reviewed above have repeatedly indicated some serogroup and serotype specific regions in the carboxy-terminal of the subunit. Cox (1992) has suggested the possibilities of developing serogroup/serotype specific PCR for fimbrial subunit. Study of John et al., (1999) showed the possibility of serogroup specific PCR but their evaluation was not extensive. Zhou et al., (2001) described a PCR/oligotyping method using PCR amplification and hybridisation to immobilised oligonucleotides to type the strains of *D. nodosus*. Though this test may be more accurate test it is more elaborate. These two studies have not been very extensive and also have not looked into the application of the tests on direct footrot samples. The need for an extensively studied rapid PCR serogrouping test with the possible application on direct samples is very much lacking. If such a test can be developed a PCR based serogrouping directly from lesion samples would be an ideal test for diagnosis, control and monitoring of footrot in the field. It would also be the ideal test to monitor disease status in autogenous vaccine trials.

2. Conventionally, diagnosis and differentiation of the forms of footrot is achieved by clinical examination of affected sheep and *in vitro* characterisation of the strains of *D. nodosus* isolated from those animals. However, these methods have limitations, particularly in differentiating flocks free of VFR from those previously affected but recovered from infection. Sheep apparently recovered from VFR can be carriers for

up to 2 years without showing clinical signs of the disease (Egerton and Raadsma, 1993; Depiazzi et al., 1998). Serological tests can therefore provide valuable information for the diagnosis of footrot by indicating recent VFR status (Whittington et al., 1990; Whittington, 1996).

Previous studies have shown that in VFR the sheep's immune system produces antibodies directed against many antigens of *D. nodosus* (Whittington et al., 1990; Whittington et al., 1997) and that diagnosis using serological tests could be quite sensitive (Whittington and Egerton, 1994). ELISA using a potassium thiocyanate (KSCN) extract of outer membrane protein (OMP) antigen was extensively evaluated for diagnostic purposes particularly in relation to the severity of foot lesions (Whittington and Egerton, 1994; Whittington and Nicholls, 1995a).

Immunological memory responses in sheep recovered from footrot can be aroused by natural recurrent infection or by subcutaneous injection of OMP antigens of *D. nodosus* (Whittington and Marshall, 1990; Whittington and Nicholls, 1995b; Whittington, 1996). The magnitude of this anamnestic immunological response is an indicator of the primary immune response of the animal, and is directly correlated with the highest antibody response attained during infection which itself is determined by the severity of foot lesions. The memory response lasts for at least a year after recovery from footrot. However, a low proportion of healthy but older animals show non-specific responses to OMP antigens both before and after anamnestic challenge (Whittington and Nicholls, 1995b; Whittington, 1996). For this reason, several other antigens of *D. nodosus*, both native and recombinant, either independently or in combinations, were evaluated (Whittington et al., 1997). That study indicated that pilus was suitable for use in diagnostic ELISA in individual sheep, and had high specificity, but anamnestic responses were not evaluated.

There is a need for evaluation of the specificity of pilus of *D. nodosus* for use as a diagnostic antigen in ELISA both during natural infection and after anamnestic challenge of animals with VFR, more so in places like Nepal as described above. Also the use of multivalent pilus combinations for anamnestic testing in flocks with multivalent infection needs to be tested.

3. The extracellular proteases (Kortt et al., 1993) and the polar type IV fimbriae (Elleman, 1988) of *D. nodosus* are considered to be the virulence factors but there is little direct evidence for this. The *vap* and *vrl* genomic regions have also been shown to be associated with isolates of *D. nodosus* from severe disease (Katz et al., 1992; Cheetham et al., 1995; Rood et al., 1996; Billington et al., 1999). Study of the role of these proposed virulence factors has not been possible due to the absence of a genetic transformation system in *D. nodosus*. This difficulty was overcome by the successful transformation of different strains of *D. nodosus* by Kennan et al; in 1998. In their experiment a tetracycline resistance gene, *tet (M)*, which was presented on a suicide plasmid, was inserted into the chromosome by reciprocal cross over events. These studies provided the tools required to investigate the role of the putative virulence factors of *D. nodosus*. So these studies have opened a new era to understand the role of fimbriae and other virulence factors in the pathogenesis of footrot.

This thesis aims at investigating the above highlighted issues – 1. development and application of the retrospective diagnosis of virulent footrot by anamnestic test with pilus antigens, 2. study the role of fimbrial gene (*fimA*) in the pathogenesis of footrot, 3. development and application of *D. nodosus* serogroup specific PCR diagnostic test.

Figure 1.3 (Source Cox, 1992) Comparative nucleotide sequences of the fimbrial subunit gene of prototype strains of *D. nodosus*

Nucleotide sequences are aligned from AP –110 (AP 1 is the A of the first translated codon of the fimbrial subunit which forms the first residue of the leader sequence). The large boxed areas show firstly, the highly conserved 5' region of the subunit, and thereafter, the three hypervariable domains of class I subunit genes. Upstream of the gene, boxes indicate variation from the consensus sequence at a position. Within the highly conserved 5' region of the gene any nucleotide variations at a position are indicated by a thick, round-edged box. Within the remainder of the coding sequences boxes designate variations from the serogroup prototype sequence. Downstream of the gene in class I strains boxes indicate single base pair variations in characteristic sequence motifs. The region of homology to the *rpo*N-dependent promoter consensus sequence is indicated between P –91 and P –75. The proposed ribosome binding site upstream of *fimA* is shown as **rbs**. Site where nucleotides are undetermined are indicated by (.). The aligned stop codons are indicated by **S.** Gaps (-) have been inserted in order to optimise the alignment.

CHAPTER 2

General materials and methods

Contents

2.1 Commonly used solutions, buffers and reagents

2.1.1 Phosphate buffered saline (PBS)

Phosphate buffered saline consisted of 0.85% (w/v) sodium chloride, 0.205% (w/v) disodium hydrogen phosphate dodecahydrate (Na_2HPO_4, $12H_2O$) and 0.0156% (w/v) sodium dihydrogen phosphate dihydrate (NaH $PO_4.2H_2O$) in deionised water. The reagents were dissolved and pH adjusted to 7.4 by the addition of sodium hydroxide solution, and autoclaved. Usually 20 x concentrate solution was made, which was diluted 1:20 in deionised water, pH adjusted to 7.4 and autoclaved before use.

2.1.2 Formal phosphate buffered saline (FPBS)

Formal phosphate buffered saline was used for the harvest of antigens from solid media and dilution of antigens. It was made by adding 1 part of formalin in 80 parts (12.5ml in 1 l) of PBS (Claxton, 1981).

2.1.3 Normal saline

Normal saline consisted of 0.85% (w/v) sodium chloride in deionised water. The solution was sterilised by autoclaving.

2.1.4 Tris - EDTA (TE)

Tris-EDTA consisted of 10mM Tris HCL pH 8.0 and 1mM ethylene diamine tetraacetic acid (EDTA) pH 8.0 (Sambrook et al., 1989). Sterile TE solution was used to dissolve or to dilute DNA samples.

2.1.5 Lysis buffer

Bacterial cell lysis buffer consisted of 20mM Tris HCL pH 8.9, 2mM EDTA and 1% triton X 100. This was used for the heat lysis of bacterial cells for polymerase chain reaction (PCR) templates.

2.1.6 DNA loading buffer (6x)

DNA loading buffer was made by dissolving 40% (w/v) sucrose and 0.25% (w/v) bromophenol blue in sterile distilled water (Sambrook et al., 1989).

2.1.7 Protein loading buffer (6x)

Protein loading buffer consisted of 10.28% (w/v) sodium dodecyl sulphate (SDS), 0.35M Tris HCL (pH 6.8), 0.6M dithiothreitol, 36% (w/v) glycerol and 0.12% (w/v) bromophenol blue in sterile distilled water (Asubel et al., 1992).

2.1.8 Tris – acetate (TAE) buffer

TAE buffer consisted of 40 mM Tris – acetate and 1 mM EDTA. Commonly 50 X concentrate solution was made by dissolving 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) in deionised water to a final volume of 1 l (Sambrook et al., 1989). The buffer was sterilised by autoclaving, and diluted 1:50 in deionised water before use. This was used for the electrophoresis of DNA in agarose gels.

2.1.9 Tris – borate (TBE) buffer

This buffer consisted of 90 mM Tris – borate and 2 mM EDTA. Usually 5 X concentrate solution was prepared by dissolving 54 g of Tris base, 27.5 g boric acid and 20 ml of 0.5 M EDTA (pH 8.0) to a final volume of 1 l in deionised water (Sambrook et al., 1989). A 0.5 X solution was used for the electrophoresis of DNA in agarose gels and 1 X solution used in polyacrylamide gels.

2.1.10 Tris – glycine buffer

This buffer consisted of 25 mM Tris, 250 mM glycine (pH 8.3) and 0.1% SDS (Asubel et al., 1992). This buffer was used for the electrophoresis of proteins in SDS – polyacrylamide gels (SDS – PAGE).

2.1.11 Reagents for ELISA

1. Borate coating buffer

Boric Acid	6.18 g
Disodium tetraborate ($Na_2B_4O_7.10.H_2O$)	9.54 g
NaCl	4.38 g
Distilled water	to 1 L

Autoclave and stored at 4^0 C

2. 10 x Phosphate buffered saline (PBS)

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	11.5 g
KH_2PO_4	3.10 g
Distilled water	to 1 L

For working strength diluted 1:10 and adjusted pH to 7.2

3. Diluent for serum and conjugate

10 x PBS	10 ml
Tween 20	50 µl
Ovalbumin or gelatin	100 mg
Distilled water	to 100 ml

4. ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] solution

Citrate phosphate buffer

Citric acid	21 g
Na_2HPO_4	14 g

Distilled water to 900 ml ; adjust pH to 4.2; make up to 1 L

ABTS	0.55 g
Citrate phosphate buffer	1 L

Dispense in 22 ml aliquots and store at -20^0 C

Immediately prior to use add 10 µl of H_2O_2 per 22 ml aliquot

5. ABTS stop solution (0.1% w/v NaN_3 in 0.1 M citric acid)

Citric acid	10.5 g
Distilled water	to 500 ml

Add 50 mg or 1 ml of 5% w/v solution of sodium azide.

2.1.12 Collection and handling of serum

Blood samples were collected from the jugular vein and allowed to clot at room temperature. Serum was separated by centrifugation at 3000 rpm for 10 min and kept at - 20^0 C until required for tests.

2.1.13 ELISA

Serum samples were examined for anti-*D. nodosus* antibodies by ELISA. Antibodies against OMP antigens as well as pilus antigens were measured both for primary and anamnestic responses to *D. nodosus* infection. The OMP antigen ELISA was performed by the method of Whittington and Egerton (1994) and pilus ELISA by the method of Whittington et al. (1997). Optical density (OD) is referred to as the "antibody level" of a serum.

2.2 Bacteriology

2.2.1 Bacteria

Prototype *Dichelobacter nodosus* strains (Claxton et al., 1983) from the culture collection of the Faculty of Veterinary Science, University of Sydney as listed in Table 2.1 were used for studies presented in this thesis. Rabbit antisera prepared against these prototype strains were used for serogrouping and serotyping (Claxton et al., 1983) of other isolates used in these studies.

Table 2.1 Prototype *D. nodosus* strains used from the collection of the Faculty of Veterinary Science, university of Sydney.

Sl. No.	Prototype/Strain No.	Serogroup/ Serotype
1	VCS 1001	Serogroup A, Serotype A1
2	VCS 1251	Serotype A2
3	VCS 1006	Serogroup B, Serotype B1
4	VCS 1208	Serotype B2
5	VCS 1190	Serotype B3
6	VCS 1125	Serotype B4
7	VCS 1008	Serogroup C, Serotype C1
8	VCS 1630	Serotype C2
9	VCS 1172	Serogroup D
10	VCS 1137	Serogroup E, E1
11	VCS 1114	Serotype E2
12	VCS 1017	Serogroup F, Serotype F1
13	VCS 1244	Serotype F2
14	VCS 1220	Serogroup G, Serotype G1
15	VCS 1004	Serotype G2
16	VCS 1687	Serogroup H, Serotype H1
17	VCS 1057	Serotype H2
18	VCS 1623	Serogroup I

2.2.2 Culture media for *D. nodosus*

Four percent (Thorley, 1976) and 2% (Thomas, 1958) hoof agar (HA) was used for the culture of *D. nodosus*. 4% HA was used for the primary isolation and purification cultures and 2% HA for subculturing of pure cultures. Trypticase arginine serine (TAS) broth (Skerman, 1975) was the liquid media used for large scale growth and antigen purifications.

2.2.3 Laboratory culture of *D. nodosus*

The standard procedure for the isolation and identification of *D. nodosus* was followed (Stewart and Claxton, 1993). Briefly, samples for bacteriology were taken from an active part the footrot lesions, using a cotton swab or an applicator stick, either from the interdigital skin or the advancing edge of the underrun hoof, and streaked onto 4% HA plates. These plates were incubated at 37^0C anaerobically in gas jars using hydrogen and carbon dioxide gas generating kits (Gas Pak, BBL, Becton Dickinson and Company, Cockeysville, USA). Plates were examined for *D. nodosus* growth after 3 to 4 days and up to 12 colonies of different size and morphology were selected for subculture or other procedures. Cultures without visible *D. nodosus* colonies were re-incubated anaerobically for another 3 to 4 days before being discarded. Each colony picked and subcultured was treated as individual isolate. Once these isolates had been purified by a series of subcultures on 4 % HA plates they were subcultured onto 2% HA. Isolates selected for preservation were suspended in 0.25 M sucrose solution and lyophilised following the instructions of the manufacturer (Model alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, am Harz, Germany).

Preserved cultures of *D. nodosus* were grown on 4 % HA plates after reconstitution in sterile distilled water. Culturing and incubation procedures were similar to that described above.

2.2.4 Antigen preparation

Antigens for serogrouping and agglutination tests were prepared from pure cultures of *D.nodosus* grown on 2 % HA plates. For serogrouping a thick suspension of bacterial cells was prepared by scraping a pure culture of *D. nodosus* grown on a 2 % HA plate in 10 – 15 drops of FPBS.

The concentration of the suspension was standardised to contain 5 x 10^8 cells per ml, which corresponded to the optical density of 0.5 (absorbence) of the suspension at a wave length of 420 nm in a spectrophotometer (model PU 8620 series, Philips Scientific and Analytical equipment, Cambridge, England). Usually, 10 X

concentrated stock solution of the antigen was stored at 4^0 C, which was diluted in FPBS for use.

2.2.5 Serogrouping

Serogrouping was done by the slide agglutination test as described by Claxton et al. (1983). Briefly, the test was performed by placing a large drop of the test antigen onto a clean glass slide and mixing with a loop full of the rabbit antiserum prepared against the prototype strains of *D. nodosus*. A test was considered positive (+) if coarse floccular agglutinating particles (K- agglutination) were visible immediately (within 10 secs) after mixing of the antigen and the antiserum prepared against the prototype strains, and negative (–) if the mixture remained clear or fine granular particles appeared after a long time. Some of the antigens cross reacted with more than one antiserum and were recorded as cross reacting (±). The serogroups of such antigens were confirmed by the microtitre agglutination test (as described in section 2.2.6). All antigens were tested against all prototype antisera by the slide agglutination test.

2.2.6 Serotying

Serotyping of *D. nodosus* isolates was done by the tube agglutination test (Egerton, 1973) and modified by Raadsma et al (1995) for microtitre plates which is now known as the microtitre plate agglutination test. Antigens to be serotyped were standardised to contain 5 x 10^8 cells per ml. Wells with a typical floccular agglutination were regarded as positive. The reciprocal of the highest serum dilution that produced a visible K–agglutination was regarded as the titre of the antiserum against that isolate. As a positive control the titre of the antiserum against the homologous strain was included on the same plate.

2.3 In-vitro virulence test

In-vitro virulence testing of the isolates was done by the elastase test (Stewart, 1979) and/or the gelatin gel protease thermostability test (Palmer, 1993).

2.3.1 Elastase test

A loopful of pure culture of *D. ndosus* was streaked onto a quarter of an elastin agar plate (Stewart, 1979) as a linear streak. On each plate isolates with known elastase activity, positive at 4-7 days and negative at 28 days, were streaked onto other quarters as controls. These plates were then incubated anaerobically using gas generating kits as mentioned in section 2.2.3. They were examined at 4, 7, 11, 14, 18, 21, 24 and 28 days after incubation. After each examination they were re-incubated anaerobically. Test isolates were regarded as positive at a particular day at which the elastin particles around the inoculum were digested and a clear zone was visible, and were regarded as negative if they failed to clear the elastin particles even after 28 days of inoculation. If the results of the control samples did not match with the recorded clearing patterns the test was repeated.

2.3.2 Gelatin gel test

The principle behind gelatin gel test developed by Palmer (1993) is that extracellular proteases produced by virulent strains of *D. nodosus* are more heat stable than those produced by benign strains.

Procedure: A pure culture of *D. nodosus* was grown in TAS broth for 2 – 4 days to achieve a concentration of 10^8 cells/ml, measured by spectrophotometric reading. This broth culture (0.5ml) was diluted with 2 ml of Hepes test buffer (Palmer, 1993), mixed well and an aliquot of 20 µl was placed into the top well of the gel. The test dilution was placed in a water bath at 68^0 C. After 8 mins of incubation at 68^0 C another 20 µl of the sample was placed into the middle well. The sample was further incubated for 8 mins and 20 µl of it placed into the lower well. Known gelatin gel positive and negative samples were always included as controls. The gels were incubated overnight (18 hrs) in a moist chamber at 37^0 C, after which undigested gelatin was precipitated by flooding with hot (60^0 –70^0 C) saturated ammonium sulphate solution. The zone of proteolysis indicated by clearing around the wells was measured by callipers. A ratio of more than 50% of the zone of clearance of the preheated compared to 16 min heated sample was taken as positive.

2.4 Preparation of antigens

2.4.1 OMP antigen

OMP antigens were prepared by the KSCN extraction method (Emery et al., 1984; Whitttington et al., 1990). Briefly, depilated cells of *D. nodosus* were washed three times in PBS pH: 7.0, resuspended in 0.8 M KSCN, 80mM NaCl and agitated for 17 h at 37^0 C. After centrifugation the supernatant was dialysed in PBS at 4^0 C overnight then subjected to ultra centrifugation. The supernatant was adjusted to a protein concentration of 1.2 mg/ml by dilution in PBS and frozen at - 20^0 C until required for ELISA or inoculation of sheep. For use, thawed antigen was diluted in sterile water to the required concentration.

2.4.2 Pilus antigen

Pilus was purified from suspensions of *D. nodosus* by the method of Mattick et al. (1984). In addition, a series of precipitations with HCl and 0.1 M $MgCl_2$ was required to achieve a high degree of purity (Whittington et al., 1997).

2.5 DNA extraction

Whenever necessary DNA from *D. nodosus* was purified using a Wizard Genomic Purification Kit (Promega Corporation, Madison, USA) as per the manufacturers instructions in the technical manual for the isolation of genomic DNA from gram negative bacteria.

2.5.1 DNA quantitation

The estimation of the quantity of genomic DNA in solution was made by measuring the absorbence of the solution at a wavelength of 260 nm, whereas ethidium bromide fluorescent quantitation was used to estimate the quantity of small fragments of DNA (PCR amplicons) (Sambrook et al., 1989). For the latter method, a sample with unknown quantity of DNA was compared with standards with known quantity in a 1% or 2% agarose gel containing 0.5 μg /ml of ethidium bromide. The quantity of DNA

sample was estimated by comparing the intensity of fluorescence of the unknown sample with that of the known standard.

2.6 Polymerase chain reaction (PCR)

The nucleotide sequence information of the target DNA was derived from the GeneBank data base (LosAlamos, USA). The primers used for the PCR were either from the published literature, or were designed using primer design program (Primer Premier) or were selected arbitrarily, as described in specific sections of this thesis. The primers were custom prepared either by GIBCO BRL, Gaithersburg, USA or Sigma-Aldrich, Sydney, Australia.

PCR amplifications were performed in a 20 μl volume either in a capillary tube (Corbett Research, Australia) or in 0.2 ml thin walled centrifuge tubes. The PCR mixture contained a final concentration of 20 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$ and 200 μM each of the four nucleotides. The concentration of each of the primers was 0.25 to 0.5 μM. One unit of Taq polymerase (GIBCO – BRL) was added to the reaction mix. Approximately 50 – 100 ng of DNA or 1 μl of cell lysate was added as template. The amplification cycle in the capillary thermocycler (Corbett Research, Australia) consisted of initial denaturation at 94^0 C for 2 mins, followed by 94^0 C for 5 sec, 60^0 C for 5 sec and 72^0 C for 30 sec for 5 cycles, 94^0 C for 5 sec, 58^0 C for 5 sec and 72^0 C for 30 sec for 25 cycles, and final extension at 72^0 C for 2 min. The amplification cycles in microcentrifuge tubes in Peltier thermal cycler – PTC 200 (M J Research, Inc. Massachusetts, USA) consisted of 94^0 C for 4 min, followed by 94^0 C for 30 sec, 60^0 C for 30 sec and 72^0 C for 30 sec for 5 cycles, 94^0 C for 30 sec, 58^0 C for 30 sec and 72^0 C for 30 sec for 25 cycles, and final extension at 72^0 C for 4 min. The PCR products were electrophoresed in 0.8% - 3% agarose gels, stained with ethidium bromide and visualised under ultraviolet (UV) illumination.

2.7 Electrophoresis of DNA

DNA was usually electrophoresed in agarose gels using horizontal electrophoresis apparatus (Owl Scientific Plastics Inc., Cambridge, UK). Molecular grade agarose

(Progen Industries, Ltd, Australia) or Top Vision agarose (MBI Fermentas, USA was used. Depending on the DNA fragment sizes, the concentration of agarose in the gel ranged from 0.8% to 3% in TAE or TBE buffer. Ethidium bromide (0.5 μg per ml final concentration) was added into the melted agarose which was poured into a mould and allowed to set. When the gel was set, 1 – 10 μl of the DNA sample mixed with DNA loading buffer was loaded into each well. DNA molecular weight markers were loaded in each gel along side of the samples. Electrophoresis was conducted at a constant voltage until the bromophenol blue dye in the loading buffer reached the bottom of the gel. The gel was examined in a short wavelength UV illuminator and photographed with a Polaroid DS 34 camera.

2.8 Electrophoresis of proteins

Protein samples were electrophoresed in precast SDS polyacrylamide mini gels (Gradipore, USA) using the discontinuous buffer system of Laemmli (1970). Protein samples were mixed with an appropriate volume of protein loading buffer and boiled for 10 min before loading into the gels. Known protein molecular weight standards were also loaded in each gel. The gels were run in Tris–Glycine buffer at a constant voltage of 200 volts in a Mini-Protean II cell (Bio-Rad, USA). Once the bromophenol blue dye in the loading buffer reached the bottom of the gel, the gel was removed from the apparatus, fixed and stained with Coomassie Brilliant Blue stain (Sambrook et al., 1989).

2.9 Scoring of footrot lesions

The method of Egerton and Roberts (1971) was adopted for scoring footrot lesions in sheep. This is based on the degree of progression of the lesions from the interdigital skin to the sensitive laminae underlying the horn of the hoof.

In brief scores were given as follows:

Score 0: Normal feet.

Score 1: Mild interdigital dermatitis.

Score 2: Severe interdigital dermatitis characteristic of footrot with necrosis, exudation and ulceration of skin.

Score 3: Underrunning of the soft horn of the sole and heel with or without interdigital dermatitis.

Score 4: As in score 3, but the underrunning extending beneath the hard horn of the axial and abaxial wall of the hoof and the toe.

Routinely feet were examined in the following order; left front, right front, left hind right hind. An animal with a score of 2 or more in one or more feet was regarded as "affected" irrespective of bacteriological findings. An animal was regarded as "infected" if the presence of *D. nodosus* was demonstrated in one or more feet irrespective of the score.

The total weighted foot score (TWFS), which is the sum of the scores of the 4 feet after raising scores of 3 and 4 by the power of 2 was used in anamnestic trials (Whittington and Nicholls, 1995a).

CHAPTER 3

Pilus ELISA and an anamnestic test for the diagnosis of virulent ovine footrot and its application in a disease control program in Nepal

Contents

3.1 Introduction:

Virulent footrot (VFR) is a highly infectious disease associated with virulent strains of *Dichelobacter nodosus* and is responsible for significant animal suffering and economic loss in affected flocks of sheep (Egerton and Raadsma, 1993). It is present in most sheep rearing countries. Because of its economic impact and animal welfare considerations, VFR is a notifiable disease in some countries. As a result, quarantine and expensive control/eradication programs are instituted for its management either on a property or on a regional basis so as to reduce the impact of the disease. In contrast, a distinguishable milder form of the disease in which benign strains of *D. nodosus* are present, economically does not justify eradication (Egerton,1986; Raadsma et al., 1991). Hence, accurate diagnosis of the form of footrot present in a flock is a prerequisite to any control program. There are many serogroups of *D. nodosus*, defined by fimbrial antigens, but virulence is independent of serogroup (Egerton and Raadsma, 1993).

Conventionally, diagnosis and differentiation of the forms of footrot is achieved by clinical examination of affected sheep and *in vitro* characterisation of the strains of *D. nodosus* isolated from those animals. However, these methods have limitations, particularly in differentiating flocks free of VFR from those previously affected but recovered from infection. Sheep apparently recovered from VFR can be carriers for up to 2 years without showing clinical signs of the disease (Egerton and Raadsma, 1993; Depiazzi et al., 1998). Serological tests can therefore provide valuable information for the diagnosis of footrot by indicating recent VFR status (Whittington et al., 1990; Whittington, 1996).

Previous studies have shown that in VFR the sheep's immune system produces antibodies directed against many antigens of *D. nodosus* (Whittington et al., 1990; Whittington et al. 1997) and that diagnosis using serological tests could be quite sensitive (Whittington and Egerton, 1994). ELISA using a potassium thiocyanate (KSCN) extract of outer membrane protein (OMP) antigen was extensively evaluated for diagnostic purposes particularly in relation to the severity of foot lesions (Whittington and Egerton, 1994; Whittington and Nicholls, 1995a).

Immunological memory responses in sheep recovered from footrot can be aroused by natural recurrent infection or by subcutaneous injection of OMP antigens of *D. nodosus* (Whittington and Marshall, 1990; Whittington and Nicholls, 1995b; Whittington, 1996). The magnitude of this anamnestic immunological response is an indicator of the primary immune response of the animal, and is directly correlated with the highest antibody response attained during infection which itself is determined by the severity of foot lesions. The memory response lasts for at least a year after recovery from footrot. However, a low proportion of healthy but older animals show non-specific responses to OMP antigens both before and after anamnestic challenge (Whittington and Nicholls, 1995b; Whittington, 1996). For this reason, several other antigens of *D. nodosus*, both native and recombinant, either independently or in combinations, were evaluated (Whittington et al., 1997). That study indicated that pilus was suitable for use in diagnostic ELISA in individual sheep, and had high specificity, but anamnestic responses were not evaluated.

In Nepal, VFR was first diagnosed clinically in 1971, was confined to several districts and was suspected to have been introduced with imported rams. Later, an extensive bacteriological survey of affected flocks showed that only 2 serogroups, E and B were involved in VFR in this country (Ghimire et al., 1996). The flocks are maintained under a transhumance husbandry system, migrating from relatively low altitude villages to high altitude alpine pastures during the summer months (May to August) and spending about 6 months away from human habitation. This migration period, which is during the warm and moist season, is the critical period for transmission of footrot within and between flocks (Ghimire and Egerton, 1996). Conventional methods of footrot control are not feasible during this period of migration so an experimental program of specific vaccination against the 2 local virulent strains was implemented in March 1993. Extensive surveillance for the disease, which involved clinical examination of individual feet of all sheep and goats has been carried out two to four times a year since 1992 and after August 1993 only mild lesions caused by benign isolates of *D. nodosus* have been seen (Ghimire 1997 and unpublished data). The reduction in prevalence of severe lesions and inability to isolate virulent strains appears due to elimination of these strains by specific vaccination. Vaccination was

withdrawn from all the flocks in 1997 to allow expression of disease, but none occurred, which also suggests that virulent strains have actually been eliminated.

Inspections of all these flocks while they graze in alpine pastures is not possible for logistical reasons and a high proportion of the lesions in affected animals heal naturally before arriving back in the villages after the downward migration in winter. Bacteriological examination of feet without lesions is unrewarding. Thus a test independent of clinical signs which does not require bacteriological examinations to identify episodes of VFR is highly desirable where clinical diagnosis of VFR is not feasible. Lambs which have not been vaccinated and which have gone on a complete migration would be the useful serological indicators of possible persistence of VFR in the flocks of Nepal.

The aim of this study was to evaluate the specificity of pilus of *D. nodosus* for use as a diagnostic antigen in ELISA both during natural infection and after anamnestic challenge of animals with VFR. The test was then applied in the field in Nepal to test objectively whether young sheep had experienced VFR.

3.2 Materials and methods

3.2.1 Strains of D. nodosus

In trial 1 which was conducted at Camden, Australian *D. nodosus* strain VCS 1208, serogroup B was used, while in trial 2 in Nepal, pilus of a Nepalese serogroup E strain, N 101, which was the most prevalent virulent strain in Nepal was used. Both these strains were characterised as virulent using elastase (Stewart, 1979) and gelatin gel tests (Palmer, 1993).

3.2.2 Isolation and characterisation of D. nodosus

Lesion samples were collected from clinical cases of footrot, inoculated onto 4% hoof agar plates and incubated anaerobically (Thomas, 1958). Individual colonies were subcultured and pure cultures were tested for virulence by elastase test and gelatin gel test.

3.2.3 Preparation of antigens

3.2.3.1 Outer membrane protein (OMP) antigen

OMP antigens were prepared by the potassium thiocyanate (KSCN) extraction method (Emery et al., 1984a; Whittington et al., 1990). Briefly, depilated cells of *D. nodosus* were washed 3 times in phosphate buffered saline (PBS) pH 7.0, resuspended in 0.8M KSCN, 80mM NaCl and agitated for 17 hrs at 37^{o}C. After centrifugation the supernatant was dialysed in PBS at 4^{o} C overnight then subjected to ultra centrifugation. The supernatant was adjusted to a protein concentration of 1.2 mg/ml by dilution in PBS and frozen at -20^{0}C until required for ELISA or inoculation of sheep. For use, thawed antigen was diluted in sterile water to the required concentration.

3.2.3.2. Pilus

Pilus was purified from suspensions of *D. nodosus* by the method of Mattick et al. (1984). In addition, a series of precipitations with HCl and 0.1M $MgCl_2$ was required to achieve a high degree of purity (Whittington et al. 1997)

3.2.3.4 Estimation of protein concentration and purity

Concentrations of the antigens were estimated by using a Bicinchoninic acid (BCA) assay (Sigma Chemical Co., USA), which is a colorimetric assay based on the principle described by Lowry et al. (1951). Concentrations and purity were also tested by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with appropriate molecular weight standards.

3.2.4 Collection and handling of serum

Blood samples were collected from the jugular vein and allowed to clot at room temperature. Serum was separated by centrifugation at 3,000 rpm for 10 mins and kept at -20^{0}C until required for tests. In the field in Nepal blood samples were allowed to clot at ambient temperatures. The tubes were placed vertically in racks and the serum was poured off into vials the following day. Vials were kept cool by placing

them in cold river water, transported to the laboratory in insulated containers within 5 days and were then kept at -20^{0}C until required for tests.

3.2.5 Enzyme linked immunosorbent assay (ELISA)

Serum samples were examined for anti-*D. nodosus* antibodies by ELISA. Antibodies against OMP antigens as well as pilus antigens were measured both for primary and anamnestic responses. The OMP antigen ELISA was performed by the method of Whittington and Egerton (1994) and the pilus ELISA by the method of Whittington et al. (1997). Optical density (OD) is referred to as the "antibody level" of a serum sample.

3.2.6. Anamnestic challenge

For anamnestic challenge, sheep were injected subcutaneously with 0.1 ml OMP or pilus in the hairless inguinal region (Whittington and Marshall, 1990). Doses of 10 μg of OMP, and of 10 μg and 50 μg of pilus in 0.1ml injection volumes were used in trial 1. In trial 2, 10 μg of pilus was used in 0.1ml volumes. For control sheep, 0.1ml of saline was injected similarly. A blood sample was collected before challenge and again 7 days later (7-9 days later in trial 2). A ratio greater than 1.5 between the ELISA OD of day-7 and day-0 serum samples was defined as a positive response.

3.2.7 Clinical examination and lesion scoring

All the animals were examined and their feet scored for footrot lesions at the start of each trial, then weekly or at monthly intervals depending on the stage of the trial. The scoring method of Egerton and Roberts (1971) was used. In brief, scores of 1 and 2 (mild) were given to lesions confined to interdigital skin and scores 3 and 4 to severe lesions with underrunning of the soft and/or hard horn of the digit. The total weighted footscore (TWFS), which is the sum of the scores of the four feet after raising scores of 3 and 4 by the power of 2 was used to rank the sheep for the anamnestic challenge in trial 1 (Whittington and Nicholls, 1995).

3.2.8. Trial 1 - Australia

Four-month-old Merino sheep (n = 86) which were free of footrot were used. Each sheep was identified by a numbered ear tag. Sheep were kept in an experimental animal house on wire-meshed floors and fed lucerne hay and oaten chaff. A group of 70 sheep was randomly selected and each sheep was artificially challenged with a known virulent strain of *D. nodosus* (B2, VCS 1208) by the method of Egerton et al (1969). Briefly, the feet of the animals were predisposed to infection by keeping them on wet mats for 4 days before challenge to facilitate maceration of the interdigital skin. They were then challenged by applying 4 day old pure cultures of *D. nodosus* on plugs of 2% hoof agar to the interdigital skin and holding them there with bandages for 4 days. The remaining 16 sheep were kept as non-exposed controls in similar conditions as the challenged group but were not in contact with the challenged animals.

Three weeks after challenge the sheep were moved out to paddocks with lush green irrigated pasture comprising mainly kikuyu grass (*Pennisetum clandestinum*) to allow natural transmission within the infected group. The controls were kept isolated on a similar but separate paddock.

After 3 weeks at pasture (i.e. 6 weeks after challenge), sheep were moved back to the pens in the animal house for treatment with 2500 mg procaine penicillin, 2500mg dihydrostreptomycin as a sulphate and 200mg procaine hydrochloride (Streptopenicillin, Ilium Penstrep, Troy Laboratories, Australia) by intramuscular injection and a walkthrough footbath in 5% v/v formalin solution twice weekly for 2 weeks.

Three weeks following treatment, when all the animals were apparently free of footrot, they were moved to Elizabeth Macarthur Agricultural Institute, Menangle and grazed on an unimproved pasture. Their diet was supplemented with lucerne hay and oats.

Table 3.1. Allocation of control and footrot affected sheep to different anamnestic challenge groups for trial 1

Group	Controls not exposed to *D. nodosus*	Exposed to *D. nodosus*		Total
		Without severe lesions (n)	With severe lesions* (n)	
Group 1 Saline	4	-	6	10
Group 2 OMP	4	5	6	15
Group 3 Pilus 50μg	4	4	24	32
Group 4 Pilus 10μg	4	-	22	26
Total	16	9	58	83

* Severe lesions are foot scores of 3 and 4.

Four months after recovery from footrot all 83 surviving animals were tested with anamnestic challenge. Sheep were allocated to one of four anamnestic challenge treatment groups: 1, saline control; 2, OMP; 3, pilus 50 μg, or; 4, pilus 10 μg (Table 3.1). The control sheep that had not been exposed to *D. nodosus* were stratified by ear tag number and allocated sequentially to each treatment group. Sheep that had been exposed to *D. nodosus* were stratified in ascending order of the total of TWFS between weeks 1 and 6 (their foot scores), and those with underrun lesions highlighted. Of the subset with underrun lesions, 2 each with low, moderate and high foot scores were allocated to treatment groups 1 and 2. Of the subset without underrun lesions, sheep were allocated sequentially to groups 2 and 3. The remaining sheep were allocated sequentially to groups 3 and 4. This method of allocation ensured that

sheep with different degrees of lesion severity were represented in treatment groups according to the perceived importance of comparisons between treatments.

The proportions of sheep in each pilus treatment group responding to anamnestic challenge were compared using a Chi-square test. The ELISA ratios of sheep in different treatment groups were compared by analysis of variance.

3.2.9 Trial 2 - Nepal

To examine the application of the anamnestic test as a supplementary test for VFR in the flocks of Nepal, experiments were conducted in two geographic areas with a history of high and fluctuating footrot prevalence. Young animals which had not been vaccinated and which had returned from a single migration to the alpine pastures were selected.

In separate studies (unpublished) we have shown that the serological response of goats to VFR is lower than that of sheep. For this reason sheep were used as the indicator species in mixed flocks of sheep and goats.

As part of the footrot surveillance program clinical examination of every foot of the animals in every flock was undertaken before the anamnestic trials.

Based on flock history and detailed clinical examinations of animals over the period of the footrot control program in Nepal (1975 –1992), endemic footrot areas were classified as 'high prevalence' or as 'fluctuating prevalence' with respect to VFR. The high prevalence flocks had >8% of sheep with underrun lesions upon their return from the alpine pastures in November. In fluctuating prevalence areas some flocks had >8% of sheep with underrun lesions in November while other flocks had <8% prevalence in November. All flocks in both areas had <8% prevalence each year after 1993 (Ghimire, 1997). Based on detailed field observations (Ghimire and Egerton 1996) it is known that the prevalence of severe footrot lesions is markedly lower in November when animals have descended from alpine pastures than in the preceding period of upward migration. The prevalence of severe lesions in affected flocks during the transmission period (i.e. May – August) ranged from 40 - 90%.

Due to the particular patterns of migration and grazing on alpine pastures, the flocks of high prevalence areas overlap in distribution and mix irregularly with one another. Similarly, flocks from the fluctuating prevalence areas mix irregularly with one another. However, there is essentially no contact between flocks of high and fluctuating prevalence areas because of differences in migration routes and natural geographical barriers such as high mountains ridges and deep river valleys (Ghimire and Egerton 1996). Therefore for sampling purposes the flocks of these 2 areas were assumed to represent separate populations. Because of the high rate of seasonal transmission of VFR during the migratory period it can be assumed that the disease prevalence in any one flock is representative of the prevalence in the population within the respective prevalence areas. Flocks from Siklis area were chosen to represent the high prevalence population while flocks from Ghanpokhara area were chosen to represent the fluctuating prevalence area (Figure 3.1).

For the purposes of sampling to detect infection, a within flock VFR prevalence of 6% was assumed, with the knowledge that this was likely to be a very conservative estimate if VFR was in fact still present in either the high or fluctuating prevalence areas given that prevalences of >8% had been recorded in November1990 (Ghimire, 1997) and much higher prevalences occurred during transmission periods. Sampling was directed at young unvaccinated sheep (<18 months of age) as these were the most likely to have succumbed to footrot if it was present, with the knowledge that the prevalence of severe lesions in lambs and older sheep had previously been similar (Ghimire, et al., 1992; Ghimire and Egerton, 1996) This approach would also maximise the specificity of the responses to anamnestic challenge (Whittington and Egerton, 1994). The population size of unvaccinated sheep < 18 months old (lambs) that had participated in a complete migration cycle in 9 Ghanpokhara and 7 Siklis flocks was estimated to be 300 each based on the census carried out the previous year and after allowing for mortality. Based on a confidence level of 95%, assumed prevalence of 6% and test sensitivity of 75%, the number of animals to be sampled in each area to detect at least 1 case was estimated to be 61 (Cannon and Roe, 1982). In the field, lambs were selected at random from each flock; a target number of lambs was defined for each flock in proportion to the number of lambs in each flock, which was known from the previous years' flock records. Lambs were manually caught on

dry terraces where they were camped for the night. Every alternate lamb was subjected to anamnestic challenge and sampling ceased when the required number of lambs for that flock had been sampled. Sampling and anamnestic challenge was undertaken in March/ April 1998 (for lambs born between September 1996 - April 1997, and that participated in the 1997 migration), and was repeated (only in high prevalence flocks) in November/ December 1998 (for lambs born between September 1997 - April 1998, and that participated in the1998 migration). The actual number of animals in the trial from each area is given in Table 3.3.

Figure 3.1 : Schematic diagram of routes of migration of flocks from high prevalence Siklis area villages (1, 2, 3) to alpine pastures at A, B, D, and fluctuating prevalence Ghanpokhara area villages (4, 5) to alpine pastures at C, E, F in Nepal.

3.3 Results

3.3.1. Trial 1 - Australia

3.3.1.1. Clinical findings

Of the 70 animals exposed to *D. nodosus* experimentally, 69 (98.57%) developed footrot lesions of which 9 (13.0 %) had mild lesions while 60 (87.0%) had severe lesions in one or more feet. The total weighted foot score (TWFS) at week 2 after the artificial infection was 10.22 ± 1.23 (mean ± s.e) and this gradually increased to 31.45 ± 2.3 at week 6. When all the animals were transferred to dry pens and treated with antibiotics and formalin footbaths the TWFS regressed within a week to 4.39 ± 0.76 at week 8 (Figure 2.2). None of the animals in the control group had any footrot lesions. Anamnestic injection sites were examined for adverse reaction. There were no detectable local reactions to the antigens in any of the animals tested.

3.3.1.2. Serological responses

3.3.1.2.1. Overall pattern of humoral responses

The antibody levels against pilus prior to challenge with *D. nodosus* were 0.16 ± 0.003 and 0.12 ± 0.006 (mean ± s.e.) in challenge and control groups, respectively and these levels remained low until week 4 after infection. At week 4 the mean ELISA OD in the challenge group increased to 0.51 ± 0.05 and rose further to 0.85 ± 0.06 at week 8. The antibody level declined after footrot was cured by the treatments applied at week 6. The level returned to approach the pre-infection level by week 15 and remained there until week 25 when sheep were injected with pilus. After a week the antibody level rose dramatically in the challenge groups. In the control group the levels remained low (between 0.11 ± 0.005 and 0.14 ± 0.005) throughout the whole period of the trial (Figure 3.2).

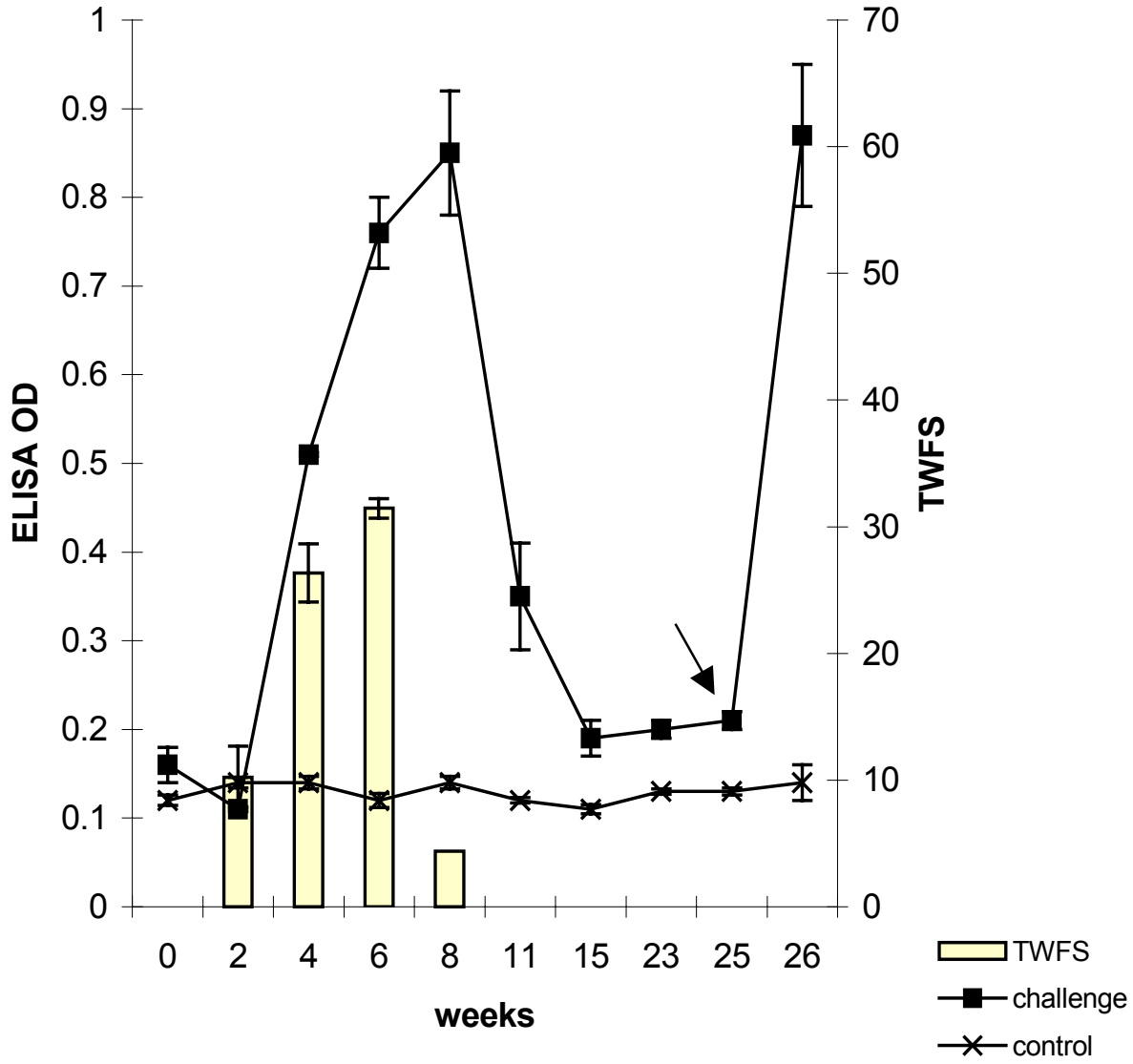


Figure 3.2 : Mean total weighted foot scores (TWFS) (bars) in the challenge group and mean antibody responses to pilus antigen (lines) in sheep in trial 1 with standard error bars. Anamnestic challenge at week 25 is indicated by the arrow. Data for the 50μg and 10μg pilus groups is pooled for week 26.

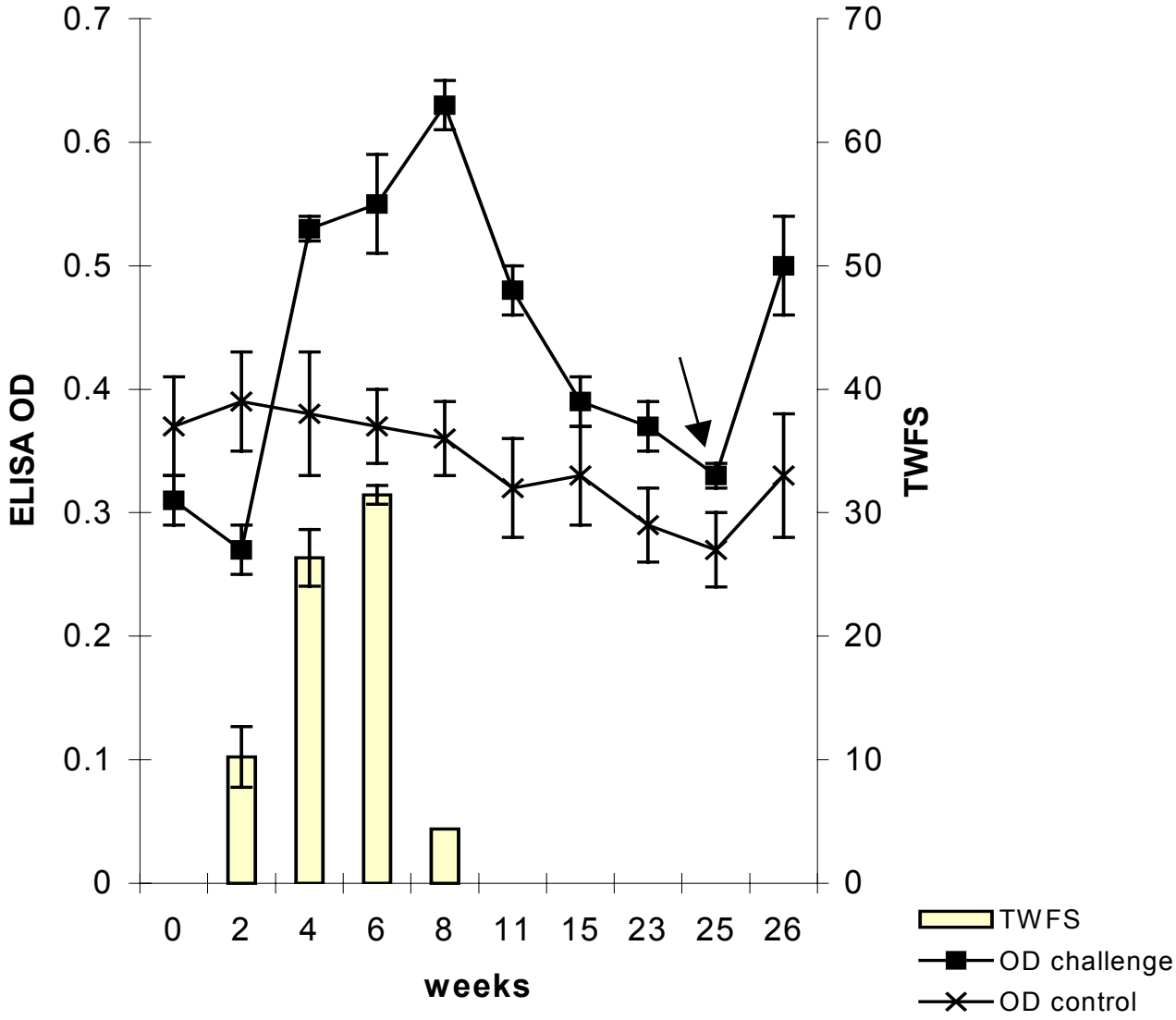


Figure 3.3: Mean total weighted foot scores (TWFS) (bars) in the challenge group and mean antibody responses (lines) to OMP antigen in sheep in trial 1 with standard error bars. Anamnestic challenge at week 25 is indicated by the arrow.

The trend in primary response to OMP antigen in the challenge group was similar to that for pilus. Minimum mean OMP ELISA OD prior to infection was 0.27 ± 0.01 and the maximum after infection was 0.69 ± 0.02. At week 26, a week after the injection with OMP antigen the level increased to 0.50 ± 0.03 whereas in the control group the level remained between 0.27 ± 0.03 and 0.40 ± 0.04 for the whole period of the trial (Figure 3.3).

3.3.1.2.2. Anamnestic response to test antigens

None of the control or affected sheep challenged with saline responded in either the OMP or the pilus ELISA (Table 3.2). The saline control group was included to test for time trends in OD response - saline did not evoke a response detected by either ELISA. Thus the increases in OD seen in other groups were due to the injection of OMP or pilus.

The anamnestic challenge with OMP antigen produced non-specific responses detected by OMP ELISA in each of the control sheep that had not been exposed to *D. nodosus*. Eight of the 11 sheep exposed to *D. nodosus* also responded in this test (Table 3.2).

In contrast to OMP antigen, the responses to anamnestic challenge with pilus were specific; none of the control sheep responded. The sensitivity of detection of sheep exposed to virulent *D. nodosus* was 79 and 86% for the 50 μg and 10 μg dose groups, respectively (Table 3.2). However, there was no effect attributable to the dose of pilus used for anamnestic challenge. Neither the proportion of sheep responding to each dose (Chi-square 0.51, P = 0.5) nor the magnitude of the ELISA ratios (P = 0.9) differed significantly between dose groups. On the basis of these results a dose of 10 μg of pilus and a conservative sensitivity estimate of 75% were chosen for the field application of anamnestic challenge in Nepal in trial 2.

Table 3.2. Trial 1: Comparison of OMP and pilus antigens in eliciting ELISA responses to anamnestic homologous challenge.

Treatment Group	Footrot Status	N	Day7/day0[+] ELISA ratio	Sheep with ELISA ratio >1.5	Sensitivity (specificity)	95% confidence limits of point estimates
1. Saline	Control	4	1.06 ± 0.16*	0		
			0.96 ± 0.05**	0		
	Affected	6	1.00 ± 0.10*	0		
			0.98 ± 0.20**	0		
2.OMP	Control	4	2.39 ± 1.34	4	(0)	0-60
	Affected	11	2.20 ± 0.90	8	73	40 - 94
3. Pilus 50 μg	Control	4	0.87 ± 0.13	0	(100)	40 – 100
	Affected	28	4.52 ± 3.25	22	79	59 – 92
4. Pilus 10 μg	Control	4	1.00 ± 0.18	0	(100)	40 – 100
	Affected	22	4.40 ± 2.98	19	86	65 – 97

*OMP ELISA, ** Pilus ELISA

[+] Mean ± S.D.

One of 4 sheep with mild footrot lesions reacted to anamnestic challenge with 50 μg of pilus and developed an ELISA ratio of 14.6 while the remaining 3 sheep with mild lesions that were challenged with this dose had ratios of 1.0 - 1.1. The reactor had a total TWFS of only 8, the worst lesions seen being transient interdigital skin lesions in 3 feet. Conversely three sheep with severe (under-running) lesions failed to respond to anamnestic challenge with 50 μg of pilus while 3 sheep failed to respond to challenge with 10 μg of pilus even though each of these sheep had had underrun lesions in more than 1 foot.

3.3.2. Trial 2 - Nepal

The feet of all the animals in the flocks selected for the trial were scored before and after the anamnestic challenge and none had any clinical signs of VFR at either of these inspections. However a few animals in some of these flocks had lesions of benign footrot. This diagnosis was confirmed by the laboratory virulence testing (Stewart, 1979; Palmer, 1993) on 4 isolates obtained from samples collected from 15 suspected footrot lesions. A total of 198 animals from Siklis and Ghanpokhara flocks were tested for an anamnestic response with pilus antigen. All the animals tested had day 7/day 0 ratios of less than 1.5 and the percentage of responders to the pilus antigen was therefore nil (Table 3.3). The 0 day and 7 day ELISA OD ranges were 0.09 to 0.35 and 0.11 to 0.32 respectively.

Table 3.3. Trial 2: Anamnestic ELISA responses of young sheep in two footrot prevalence areas of Nepal.

Prevalence area	Time	No. Animals (flocks) tested	Day 7/day 0 Mean ELISA ratio ± s. d.	Sheep with day 7 /day 0 ELISA ratio >1.5
High (Siklis)	Post 1997 migration	72 (7)	1.10 ± 0.15	Nil
	Post 1998 migration	62 (5)	1.04 ± 0.12	Nil
Fluctuating (Ghanpokhara)	Post 1997 migration	64 (9)	1.08 ± 0.12	Nil

3.4 Discussion

It has been shown that VFR stimulates the sheep's immune system to produce antibodies against *D.nodosus* pilus which can be detected by ELISA (Whittington et al., 1997). ELISA using OMP- antigen had been thoroughly evaluated for diagnosis of footrot by Whittington and Egerton (1994). In this study, an evaluation of pilus for diagnosis of footrot in sheep was undertaken and the results indicate that pilus antibodies can be detected over time in a manner similar to OMP antibodies.

The results of this study reinforced and extended the earlier findings with OMP antigen (Whittington and Marshall, 1990; Whittington and Nicholls, 1995; Whittington 1996) and confirmed that a retrospective assessment of VFR status can also be made by an anamnestic test with pilus. As sheep of increasing age have

increasing nonspecific OMP antibodies detected by ELISA (Whittington and Egerton, 1994; Whittington and Nicholls, 1995; Whittington, 1996) it was decided to use only young animals in this study. Young animals were also the most appropriate indicators for new infections in the flocks of Nepal. In Nepal most of the older animals in the footrot endemic area have been vaccinated with pilus vaccine and/or have had a history of VFR.

The anamnestic response to pilus was similar in character to OMP antigen but unlike OMP was highly specific. In Trial 1 there was a false positive ELISA response in the serum of control animals free of footrot challenged with OMP. There was no response in similar animals challenged with pilus. This indicates that the nonspecific antibody response to OMP antigen can be avoided by the use of pilus in this test. However, serogroup specificity is a factor to consider in the use of pilus for diagnosis, something which does not apply to the use of OMP which is cross-reactive between serogroups. Serogroup restriction with pilus could probably be overcome by multiple antigen injections and a series of ELISA tests for detection of the different serogroups likely to be present. The presence of only two virulent serogroups in the flocks in Nepal made this testing easier, particularly as only one serogroup (E) was known to be dominant in the study area (Ghimire, 1997).

The response of sheep in trial 1 to anamnestic challenge with pilus antigen was determined mostly by the severity of the lesions they had expressed, with severe lesions triggering the greatest responses. However, there was variation between individuals, with some (6 of 46 with severe lesions) failing to respond. Conversely, one of 4 sheep with mild lesions had a dramatic response to anamnestic challenge with 50 μg of pilus. This variation is probably mediated genetically and also has been observed in the response of sheep to vaccination with pilus antigen (Raadsma et al., 1995)

The comparison between high dose (50 μg) and low dose (10 μg) of pilus in trial 1 did not show any significant difference in response. Based on these results a 10 μg dose was used in the field. This is relatively small amount of pilus and is quite economical to produce.

VFR was once a recurrent problem in Nepal but apparently has been completely controlled by specific vaccination (Ghimire, 1997). This assessment was based on clinical findings that were largely restricted to observations made during late autumn, winter and spring under conditions not favourable for expression of footrot. Thus some doubt remained as to whether virulent footrot really had been eradicated. However, animals with the highest risk of having had episodes of footrot in recent transmission seasons were tested with pilus anamnestic challenge and none of them reacted. These results supported the clinical findings suggesting that virulent strains of *D. nodosus* had been eliminated from these flocks. Although presence of serogroups other than E could also explain negative ELISA results due to serogroup restriction in anamnestic challenge responses, bacteriological findings indicated that serogroup E was the dominant serogroup, accounting for 84.3% of virulent isolates in both Siklis and Ghanpokahara areas when virulent footrot occurred there prior to the vaccination program (Ghimire, 1997). Weather/seasonal patterns in the hill regions of Nepal are relatively unchanging from year to year and emergence of genetic resistance amongst the livestock is improbable so that elimination of VFR is most likely to be due to the control program. Benign footrot is endemic in these flocks and isolates of *D. nodosus* with benign phenotype have been obtained from these affected flocks. The observations made by Whittington (unpublished) that animals with benign footrot in Australia remained negative to anamnestic tests with OMP were confirmed by the findings of this study in Nepal with pilus. The reaction seen in trial 1 in one sheep with mild lesions due to a virulent strain of *D. nodosus* did not occur in sheep in Nepal where only benign strains appeared to be present. The pilus used was of the same serogroup as some of the benign strains endemic in parts of Nepal. The other advantages of this test are that a very low dose is required to elicit a response, no adjuvants are required and there are no observable reactions at the site of injection.

This retrospective pilus anamnestic test was a valuable complementary diagnostic tool for confirming the absence of outbreaks of VFR in the migratory flocks of Nepal. Clinical diagnosis was difficult to apply, more so during the most critical phases of the disease transmission when the animals were not accessible. Even though this anamnestic test was evaluated in the Baruwal breed of sheep in Nepal there is no reason to believe that the response there would be different from the trial conducted in

Australia in Merino sheep. Egerton, et al., (1996) and Ghimire (1997) have shown that the serological responses to footrot and footrot pilus vaccines are similar in Baruwal and other breeds so it is expected that the anamnestic response to the same antigens would also be similar.

CHAPTER 4

An assessment of multi-valent pilus anamnestic ELISA in the diagnosis of footrot

Contents

4.1 Introduction

Immunological memory responses in sheep recovered from virulent footrot can be reactivated by natural recurrent infection or by subcutaneous injection of OMP and pilus of *D. nodosus* (Whittington and Marshall, 1990; Whittington and Nicholls, 1995b; Whittington, 1996; Dhungyel et al., 2001). The magnitude of this anamnestic immunological response is an indicator of the primary immune response of the animal, and is directly correlated with the highest antibody response attained during infection. This is determined by the severity and duration of foot lesions. Antibody responses are weak in animals with lesions confined to interdigital skin, as in most cases of intermediate and benign footrot. The memory response lasts for at least one year after recovery from footrot. Memory response to OMP antigen is not very specific (Whittington, 1996) but for pilus it is specific and this test has been tested for the retrospective diagnosis of virulent footrot in Nepal (Dhungyel et al., 2001). This response is detectable in blood samples using ELISA and peaks a few weeks after lesions are fully developed and declines over about 4 months from the time of resolution of lesions. With anamnestic challenge using pilus antigen there is need to know the serogroup (s) of the strain (s) causing the infection in the flock. It is possible that this could be overcome by using multivalent pilus antigen. However, this hypothesis has not been tested.

This anamnestic ELISA test can be used even when the animals appear free of lesions at least one year after an episode of virulent footrot thus making it possible to assess objectively the severity of past footrot lesions in a flock. It can be tested on blood samples before and after a subcutaneous injection of *D. nodosus* antigens on two farm visits making it much easier than clinical examinations with frequent visits.

ELISA test results for flocks infected with most isolates would be expected to correlate with the gelatin gel or other protease test results (Whittington 1995a). However, strains with positive gelatin gel test results have been identified in flocks with no clinical evidence of virulent disease (Hall et al., 2001).

Based on the history of low prevalence of footrot in the area, Armidale Rural Lands Protection Board (RLPB) was declared as a ‘Footrot Protected Area’ in 1969, and

with the introduction of the NSW Footrot Strategic Plan (1988) this Protected Area was extended to cover most of the New England Tablelands (Hall et al., 2001). The NSW Footrot Strategic Plan has the objective of eradicating virulent but not benign footrot. Virulent footrot is diagnosed clinically supplemented by *in vitro* virulence assessment of isolates using the gelatin gel test (Palmer, 1993).

A survey in the New England region identified an unexpectedly high number of flocks with virulent footrot (Walker, 1997) and quarantine restrictions were enforced in these and other flocks. Some of flocks were quarantined because gelatin gel positive *D. nodosus* were isolated from infections which were clinically benign (Hall et al., 2001). Subsequently trials with sheep were conducted by a farmer`s group involved in this problem. Data from those trials, isolates of *D. nodosus* from them, some serum samples and sheep used in the trials were made available for the work described in this chapter.

4.2 Materials and methods

4.2.1 Cicerone Project Trial- Phase 1 Uralla

The aim of the Cicerone Project was to compare the clinical expression of less virulent forms of footrot under similar conditions. *D. nodosus* had been isolated from these different outbreaks. The trial was conducted for 20 weeks on a farm near Uralla, in the New England region. (Gaden, 2001; Hall et al., 2001).

A paddock previously used for a pasture trial was slashed, fertilised and divided into 14 one hectare plots. The plots were assessed for clover content and were then paired to contain one with a higher and one with a lower clover content.

Merino sheep were selected from six farms in the New England area based on the form of footrot present and the results of virulence tests on *D. nodosus* isolated from those outbreaks. The categories were: virulent footrot associated with *D. nodosus* positive in the gelatin gel test (VFR/GG+); benign footrot associated with the isolation of organisms which were gelatin gel positive (BFR/GG+) and benign footrot associated with gelatin gel negative isolates BFR/GG-).

In October 1999 each of the six infected groups was divided into 2 groups of four or five sheep and one group each was put into one plot of high clover and its paired low clover pasture along with 5 uninfected sheep. Each plot had a total of 9 or 10 sheep (Hall et al., 2001).

Each group of sheep was inspected for footrot and 10 samples were collected for the isolation and characterization of the *D. nodosus* involved. Foot scoring of all the animals in each group was done every week by the method of Stewart, et al.(1982) in the period between October and March. Stringent measures were taken to avoid cross contamination between the plots during inspections. Scoring was not done for 5 months (March – August 2000). After 20 weeks, samples were again taken for bacteriology. Blood samples were collected from each of the animals in March and October 2000.

4.2.2 Cicerone trial- phase 2 Molong

Five of the above groups of sheep (1x VFR/GG+; 2 BF/GG+ and 2x BFR/GG-) were transferred to Molong, an area thought to be more favourable for footrot, in August 2000. The objective was "to ascertain whether the different diseases behaved differently when taken into an environment more favourable for the expression of the disease" (Hall et al., 2001). Two groups of Molong animals (Groups 6 and 7 Table 4.1) were added to the trial and all groups were managed in separate paddocks. Foot scoring was done at weekly intervals for 14 weeks.

In general the results from the Cicerone project suggested that at both Uralla and Molong there were no changes in the clinical expression of the diseases observed on their properties of origin. It was noted that whereas in the VFR/GG+ groups transmission to unaffected sheep occurred this did not happen with either the BFR/GG+ or BFR/GG- groups.

At the conclusion of these observations 98 animals became available to this project. They presented an opportunity to test a group of sheep, with a recorded history of footrot, for antibodies to the ELISA test described in Chapter 3. Subsequently it would also be possible to test their anamnestic responses to pilus antigens thus

enabling an objective assessment to be made of the severity of disease which had been present in them. Thus one week prior to their departure from Molong for Camden remaining infected animals were treated with 8 – 10 ml of 200 mg/ml long acting oxytetracycline (Terramycin LA, Pfizer). They were walked through a 5% formalin footbath when loading to the truck.

The aims of the work described in this chapter were:

1. To compare clinical observations and ELISA antibody levels in the assessment of disease severity.
2. To test the application of multivalent pilus for anamnestic test.
3. Thus to evaluate both tests as an alternative to *in vitro* testing of *D. nodosus* as an aid to flock diagnosis.

4.2.3 Anamnestic trial, Camden

On arrival at EMAI all the animals were again treated with 8 – 10 ml of 200 mg/ml long acting oxytetracycline (Terramycin LA, Pfizer) and walked through a 5% formalin footbath. They were managed as one group in a quarantine area with native pasture which had been kept free of sheep for a prolonged period. They were periodically inspected for footrot by the author.

Animals (6) not responding to the treatments were separated from the others and managed in a separate paddock. These measures were designed to prevent the development of footrot during the anamnestic trial.

Table 4.1: Trial groups with their clinical and bacteriological status

Group	Area of origin	Status
1.	New England	VFR/GG+
2.	New England	BFR/GG+
3.	New England	BFR/GG+
4.	New England	BFR/GG-
5.	New England	FR-
6.	Molong	FR-
7.	Molong	VFR/GG+

VFR/GG+ - virulent footrot with +ve gelatin gel results; BFR/GG+ - benign footrot with +ve gelatin gel results; BFR/GG- - benign footrot with –ve gelatin gel results; FR - - unaffected controls.

4.2.4 Bacteriology

4.2.4.1 Collection of samples and culture of *D. nodosus*

Forty five isolates of *D. nodosus* cultured during Phase 1 of the Cicerone trial were made available by OIC RVL, NSW Agriculture, Orange. During the investigations conducted at Camden an additional 14 isolates were obtained from the affected feet of some of the trial sheep. They were cultured, isolated and characterized as described elsewhere in this thesis. Altogether 59 isolates were available to the study.

4.2.4.2 Serogrouping

Serogrouping of the purified antigen of the culture samples was done by slide agglutination test as described by Claxton et al., (1983).

4.2.4.3 *In-vitro* virulence tests

In-vitro virulence testing of the isolates was done by the elastase test (Stewart, 1979) and the gelatin gel protease thermostability test (Palmer, 1993).

4.2.5 Preparation of antigens

Serogroup specific pilus was purified from suspensions of *D. nodosus* of all the serogroups (A – I) by the method of Mattick et al., (1984). In addition, a series of precipitations with HCl and 0.1M $MgCl_2$ was required to achieve a high degree of purity (Whittington et al., 1997)

4.2.5.1 Estimation of protein concentration and purity

Concentrations of the antigens were estimated by using a bicinchoninic acid (BCA) assay (Sigma Chemical Co., USA), which is a colorimetric assay based on the principle described by Lowry et al., (1951). Concentrations and purity were also tested by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with appropriate molecular weight standards and known standards of the earlier purified pilus samples.

4.2.6 Collection and handling of serum

Blood samples were collected from the jugular vein and allowed to clot at room temperature. Serum was separated by centrifugation at 3,000 rpm for 10 mins and kept at -20^0C until required for tests.

4.2.7 Enzyme linked immunosorbent assay (ELISA)

Serum samples available from the Cicerone trial and were examined for anti-*D. nodosus* antibodies by ELISA. Antibodies against pilus were measured both for primary and anamnestic responses. The OMP antigen ELISA was performed by the method of Whittington and Egerton (1994) and the pilus ELISA by the method of Whittington et al. (1997). Optical density (OD) is referred to as the “antibody level” of a serum sample.

4.2.8 Anamnestic challenge

For anamnestic challenge, 92 sheep apparently free of footrot were injected subcutaneously with 0.1ml of multivalent pilus (serogroups A – I) antigen on the right side in the hairless inguinal region (Whittington and Marshall, 1990). Doses of 10 μg of each serogroup pilus were combined and injected in a total volume of 0.1ml. A blood sample was collected before challenge and again 7 days later. A ratio greater than 1.5 between the ELISA OD of day-7 and day-0 serum samples was defined as a positive response (Whittington and Marshall, 1990).

4.2.9 Statistical analysis

Statistical analysis, summary statistics, correlations and analysis of variance of the data was done using Minitab Statistical Software (MINITAB INC, USA).

4.3 Results

4.3.1 Clinical findings

The mean total weighted foot score (TWFS) for all groups in the period November 19 99 to May 2001 was low except for that of group 7 (VFR/GG+) (Table 4.2). Similarly the range of maximum TWFS is much greater in this group than the others which is clearly reflected in Figure 4.1. The results indicate that underrunning lesions (> 2) occurred in each of the groups at least on some occasions. The disease was least severe in one of BFR/GG- groups and most severe in the VFR/GG+ group.

Table 4.2 Range of maximum TWFS recorded for different groups for the trial period (November 1999 to May 2001).

Group	Footrot status	Range of weekly group maximum TWFS
1	VFR/GG+	0 – 18
2	BFR/GG+	0 – 36
3	BFR/GG+	0 – 32
4	BFR/GG-	0 – 29
5	FR-	0 – 11
6	FR-	0 – 16
7	VFR/GG+	12 – 64

4.3.2 Bacteriology

Samples for *D. nodosus* culture were collected only a few times before the animals were brought to Camden, where it was done more frequently. The *in vitro* virulence tests (elastase and gelatin gel tests) and serogrouping were conducted on these samples. The results of the culture samples tested from different groups are presented in Table 4.3

Table: 4.3 Distribution and characterisation of *D. nodosus* isolates among different clinical groups

Group	N=	Footrot status	Bacteriological status		Serogroup	No.of isolates
			Elastase	Gelatin gel		
1	16	VFR/GG+	7-10 days +ve	Stable	A, B, E, H	23 (RVL)
2	19	BFR/GG+	7-10 days +ve	Stable	A, B, F, H	12 (RVL)
3	19	BFR/GG+	7-10 days +ve	Stable	D	3 (RVL)
4	10	BFR/GG-	28 days –ve	Unstable	B, E, H	7 (RVL)
5	9	FR-	NA	NA	NA	NA
6	9	FR-	NA	NA	NA	NA
7	10	VFR/GG+	7-10 days +ve	Stable	A, B, E, H	14(Camden)

4.3.3 Serological responses

4.3.3.1 Pre-anamnestic challenge humoral responses

The mean antibody levels against pilus antigens known to be present in the flock in March 2000 and October 2000 are presented in Table 4.4. As indicated by the results the response was variable between groups and between different pilus antigens. The trends were that the responses to all pilus antigens were relatively low in all groups except for the VFR/GG+group in which sheep had relatively high responses to all antigens other than F antigen. However, responses to F pilus in March 2000 were relatively high in Group 1(VFR/GG+) and group 2 (BFR/GG+). Responses to H pilus in March 2000 were lower in group 6 (FR-) than other groups. Samples for the VFR/GG+ group were not available for March 2000 because they were included in trial at Molong only from August 2000.

4.3.3.2 Anamnestic response to test antigens

The results of antibody responses to anamnestic challenge with different antigens for all the groups are presented as mean 7 day/ 0 day ELISA ratios in Table 4.5 and Table 4.6.

Positive anamnestic responses (OD ratio > 1.5) to serogroup A pilus antigen were seen in only four animals, 2 from group 1 (VFR/GG+), and one each from groups 2 and 7. All these animals had a history of underrun lesions and serogroup A was isolated from all these three groups.

Positive anamnestic responses to B pilus antigen occurred in all groups except group 6 which had been free of footrot. Seven responder animals, one in group1 (VFR/GG+), 4 in Group 3 (BFR/GG+) and 2 in group 5 (BFR/GG-) were not observed with severe lesions at any of the inspections.

Serogroup D pilus antigen had positive anamnestic responses in group 3 except for one reactor each in groups 2, 4 and 7. Serogroup D isolates were identified only from group 3. Four of the positive responders from group 3 and one from the group 2 had no observed underrun lesions.

The majority of the positive responders to serogroup E pilus antigen were from the groups 2 and 3 but 3 were from group 7, and one each from the groups 1 and 4. Serogroup E isolates were identified only from groups 4 and 7 during our trial. Four of the positive responders to E antigen from group 3 and one from group 2 had no underrun lesions.

The only 4 animals were positive responders to F pilus antigen; 3 from group 2 and one from group 3 but serogroup F was isolated only from group 2. All the positive responders to this group had underrun lesions.

Responders to H pilus were from all groups except group 6. Serogroup H was isolated from all responding groups except groups 3 and 5. Five of the positive responding animals, one of group 1, one of group 2, 2 of group 3 and one of group 5 did not have underrunning lesions during our trial period.

None of the animals in group 6 considered free of footrot had any positive responders to any of the antigens tested. This indicates that the test is very specific. Although 2 animals were recorded as affected on two consecutive inspections no transmission occurred and no isolations of *D. nodosus* were made. It seems likely then that these records were inaccurate.

Of the total of 92 sheep in all groups, 7 of the 53 animals (13.2%) with underrun lesions (Scores >2 or TWFS > 8) did not respond to any of the antigens tested.

Correlations between the responses to different pilus antigens and the maximum TWFS are presented in Table 4.7. Statistically significant correlations were observed between the day 7/day 0 ELISA ratios for antibodies against several serogroups. For example responses against serogroup B were correlated with those against serogroup A. As there is believed to be little antigenic cross reaction between serogroups the results suggest that antibodies to more than one serogroup were present in the serum of some sheep. This is consistent with the recovery of more than one serogroup of *D. nodosus* from footrot lesions in most of the groups in the trial.

Figure 4.1 Maximum TWFS of the animals of different groups in the trial for the whole period of the trial (Legend: B - Group1, P - Group 2, PI - Group 3, RE - Group 4, O- Group 5, Y- Group 6, LB - Group 7).

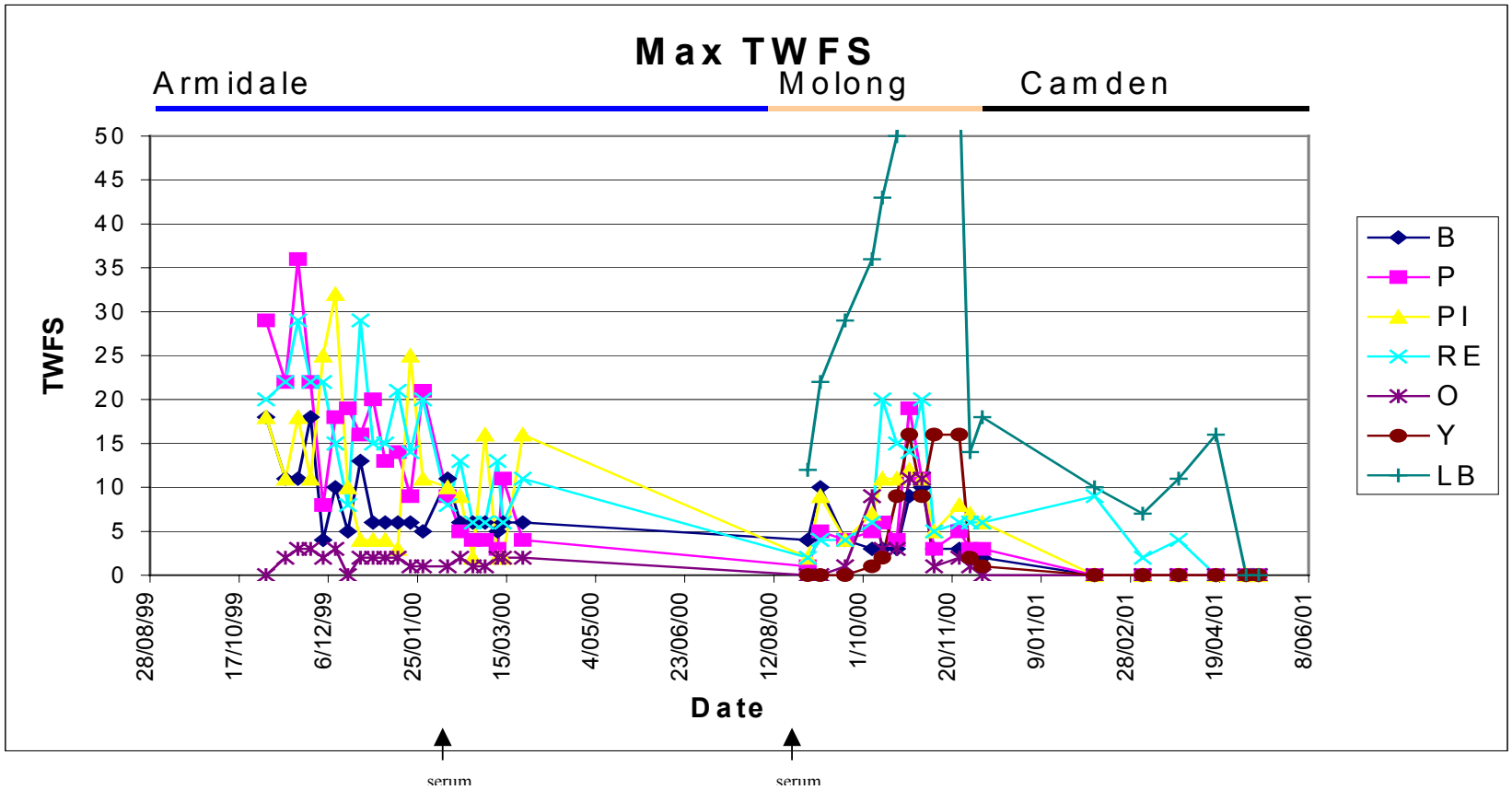


Table 4.4 Mean antibody responses (standard deviation) to pilus antigens in different clinical groups (pre Camden samples).

Group	A pilus		B pilus		D pilus		E pilus		F pilus		H pilus	
	March	October	March	October	March	October	March	October	March	October	March	October
1	0.15(0.04)	0.12(0.02)	0.13(0.04)	0.11(0.02)	0.21(0.10)	0.14(0.05)	0.16(0.08)	0.14(0.05)	0.29(0.48)	0.12(0.02)	0.37(0.35)	0.17(0.08)
2	0.10(0.06)	0.13(0.02)	0.14(0.10)	0.14(0.05)	0.13(0.08)	0.20(0.11)	0.10(0.06)	0.21(0.20)	0.24(0.56)	0.15(0.09)	0.21(0.16)	0.19(0.08)
3	0.15(0.08)	0.14(0.04)	0.22(0.18)	0.22(0.12)	0.22(0.17)	0.29(0.20)	0.23(0.14)	0.26(0.16)	0.15(0.11)	0.16(0.07)	0.26(0.20)	0.26(0.16)
4	0.18(0.06)	0.12(0.02)	0.32(0.21)	0.16(0.08)	0.23(0.13)	0.21(0.08)	0.18(0.07)	0.12(0.01)	0.18(0.10)	0.12(0.01)	0.42(0.23)	0.23(0.04)
5	0.15(0.06)	0.16(0.07)	0.33(0.21)	0.21(0.09)	0.18(0.08)	0.20(0.03)	0.12(0.04)	0.19(0.06)	0.14(0.04)	0.14(0.02)	0.27(0.17)	0.26(0.10)
6	0.10(0.02)	0.16(0.06)	0.17(0.05)	0.19(0.06)	0.15(0.04)	0.18(0.03)	0.14(0.05)	0.16(0.03)	0.14(0.06)	0.15(0.02)	0.18(0.06)	0.27(0.04)
7	-	0.27(0.10)	-	0.40(0.21)	-	0.34(0.14)	-	0.35(0.14)	-	0.17(0.04)	-	0.50 (0.15)

Table 4.5: Mean 7 day/ 0 day ELISA ratio of different pilus antigens tested (± S.D) for the multivalent pilus anamnestic challenge.

Group (serogroup found)	N	Footrot status	Affected **	Mean 7 day/ 0 day ELISA ratio for different pilus antigens tested (± S.D)						Sheep with footscore > 2 with ELISA ratio > 1.5	Sensitivity	Sheep with footscore ≤ 2 with ELISA ratio > 1.5
				A pilus	B pilus	D pilus	E pilus	F pilus	H pilus			
1 (A B E H)	16	VFR/GG+	8	1.2 (±0.19)*	1.5 (± 0.39)*	1.03 (± 0.19)	1.07 (± 0.21)*	1.21 (± 0.14)	1.43 (± 0.34)*	7	87.5	1
2 (A B F H)	19	BFR/GG+	11	1.13 (± 0.23)*	1.34 (± 0.64)*	1.14 (± 0.17)*	1.31 (± 0.44)*	1.19 (± 0.28)*	1.39 (± 0.47)*	10	91	2
3 (D)	19	BFR/GG+	10	1.1 (± 0.14)	1.34 (± 0.44)*	1.59 (± 0.68)*	1.32 (± 0.44)*	1.19 (± 0.16)*	1.55 (± 0.87)*	10	100	4
4 (B E H)	10	BFR/GG-	10	1.18 (± 0.12)	1.94 (± 0.75)*	1.18 (± 0.20)*	1.21 (± 0.22)*	1.2 (± 0.18)	1.42 (± 0.21)*	10	100	0
5 -	9	BFR/GG-	3	1.04 (± 0.21)	1.4 (± 0.35)*	1.1 (± 0.12)	1.16 (± 0.15)	1.05 (± 0.08)	1.17 (± 0.35)*	3	100	3
6 -	9	FR-	2	1.0 (± 0.12)	1.16 (± 0.12)	1.04 (± 0.09)	1.09 (± 0.17)	1.05 (± 0.12)	1.04 (± 0.25)	0	-	0
7 (A B E H)	10	VFR/GG+	9	1.22 (± 0.18)*	1.54 (± 0.55)*	1.23 (± 0.18)*	1.37 (± 0.33)*	1.15 (± 0.12)	1.6 (± 0.37)*	7	78	0

** with underrunning lesions (score > 2) in one or more foot. * Responders to anamnestic test in these groups.

Table 4. 6 No of reactors (ELISA ratio ≥ 1.5) in each of the maximum foot score category (number of animals tested = 92).

Pilus serogroup ELISA	No. of reactors (ELISA ratio ≥ 1.5) in each of the maximum foot score category							
	0 n = 29	1 n = 8	2 n = 7	3a n = 21	3b n = 10	3c n = 3	4 n = 4	5 n = 10
A	0	0	0	2	1	0	1 (1)	2 (1)
B	3	2	4 (1)	14 (5)	6 (3)	2 (1)	1 (1)	3 (1)
D	2	1	3 (1)	1	3 (1)	0	0	2
E	2 (1)	0	2 (1)	3 (1)	3 (1)	1	3 (1)	2
F	0	0	0	3	1	0	0	0
H	4 (2)	1	2 (1)	7 (2)	5 (1)	2	0	0

( ) No. of reactors when cut off ratio increased to ≥ 2 .

Table 4.7 Correlations between responses to different pilus antigens and the maximum TWFS.

Correlations between 7 Day/ 0 Day ELISA responses to different pilus antigens (P value)						
	A ratio	B ratio	D ratio	E ratio	F ratio	H ratio
B ratio	0.42(0.00)					
D ratio	0.11(0.27)	0.27(0.01)				
E ratio	0.29(0.01)	0.24(0.02)	0.37(0.00)			
F ratio	0.56(0.00)	0.40(0.00)	0.22(0.04)	0.16(0.13)		
H ratio	0.18(0.09)	0.40(0.00)	0.33(0.00)	0.43(0.00)	0.22(0.04)	
MaxTWFS	0.27(0.01)	0.21(0.05)	0.09(0.41)	0.24(0.02)	0.08(0.45)	0.14(0.18)

4.4 Discussion

The origin of the Cicerone trial was the identification of flocks which had apparently mild footrot but from which gelatin gel positive *D. nodosus* cultures had been isolated i.e. the *in vitro* results were at variance with the field observations. The Cicerone trial confirmed the mild nature of the disease in the flocks of origin in two different environments.

In this investigation I elected to use the clinical data from the Cicerone trial to generate TWFS (Whittington and Nichols, 1995) for all the groups for the duration of

both phases of the Cicerone trial and at Camden. As indicated by the results in Figure 4.1, the clinical footrot status was severe only in group 7. However, the plot of maximum TWFS over time indicates the occurrence of scores of 3 and 4 in most groups. This seemed to be in conflict with the original clinical findings in some groups. TWFS is the sum of the scores of four feet after raising scores of 3 and 4 by power of 2 and was used to illustrate the results. The clinical diagnoses made in the Cicerone trial were based on the diagnostic guidelines of the NSW Strategic plan (NSW Footrot Eradication Manual) and not on TWFS which has not been evaluated in the field.

The primary humoral response to *D. nodosus* antigens is only an indication of the immediate history of virulent footrot. The results of the humoral responses prior to the anamnestic challenge were variable between different groups and to different pilus antigens. There are also very wide variations in response within the groups as reflected by the large SD values. Samples collected at single time points would not be expected to give uniform responses to all antigens. The higher responses seen in some animals would have coincided with the immediate history of footrot caused by those serogroups in those responding animals. The response to H serogroup was relatively high in some groups which could be because this infection was more recent. These results from only 2 samplings are expected only to give an indication of the history of footrot and could not be used for diagnostic purposes.

All the pilus antigens tested in anamnestic challenge except serogroup A had some apparently non-specific positive responses i.e. the serogroup had not been isolated from that group of sheep and also positive responses were obtained from animals with no recorded underrunning infections. Relatively few samples were collected for culture so it is likely that some serogroups may have been present but not identified. Seven animals with scores of 3a or higher did not respond to the test with any of the pilus antigens used. This also suggests the presence of undetected serogroups.

The number of positive reactors among sheep in each of the maximum foot score categories is presented in Table 4.6. Over the trial period there were many reactors in sheep where the maximum recorded score was 3a. Whereas lesions of this severity are obviously sufficient to provoke an immune response their presence in some sheep in a

flock (in the absence of more severe lesions) would not result in a clinical diagnosis of VFR. Changing the cut off for the positive ELISA OD ratio from ≥ 1.5 to ≥ 2 still yielded positive reactors in sheep from flocks which were clinically benign. These scores used in my investigation were collected for the trial period only and the complete prior history of footrot in these animals is not known. Possible explanations for these apparently non-specific positive responses could be: 1) that some animals had VFR before being included in the Cicerone trial. 2) the scores recorded in some cases may have been inaccurate. 3) the test is too sensitive. 4) more severe lesions occurred but were not observed. No observations were made for 5 winter months (March – August 2000) during which advancement of pre-existing infections was considered unlikely. Nevertheless Abbott (2000) has reported advancement of lesions of intermediate footrot in apparently unfavourable environments. Also, while scoring of footrot lesions is relatively simple, inaccuracy and variability between scorers can occur.

The fact that none of the animals in group 6 responded to any of the antigens either in primary or anamnestic tests provides evidence that this test is specific so the positive responses seen in other groups may not all be non-specific.

The work described in chapter 3 demonstrated that is possible in some circumstances to distinguish VFR from BFR but the results from the investigations with the Cicerone sheep did not allow that distinction to be made. It may be that, if the test were applied to a representative random sample of the flocks from which the Cicerone groups were derived, a more accurate assessment of its value could be made. That assessment may need to be based on the prevalence of positive reactors in a flock

There were significant positive correlations between the mean of the ELISA ratios of most serogroups. This could be an indication of the multiple infection with different serogroups. There were only “moderately significant” positive correlations between the means of the ELISA ratios and the maximum foot scores in three of the serogroups.

Nine pilus antigens were included in the anamnestic challenge in a single injection in a single site. Anamnestic responses were elicited against multiple serogroups

suggesting that antigenic competition was not a major issue. However, further studies in sheep with footrot defined through controlled experimental challenge with known serogroups of *D. nodosus* would be required to confirm this. There is some evidence of antigenic competition when pilus antigens are administered in adjuvants to elicit protective immunity (Schwartzkoff, et al. 1993; Raadsma, et al. 1994; Hunt, et al., 1994, 1995).

The results of this study have provided:

1) based on the foot scores available there was disagreement between the original clinical diagnosis and the results of the anamnestic ELISA test
2) evidence that multivalent pilus can be used in one injection for anamnestic challenge and this avoids the need to know the identity of serogroups present in any flock to be tested, and
3) this test may be used as an indirect test to check the presence of serogroups in a flock.

The accuracy of this test in distinguishing between BFR and VFR needs to be further evaluated in flocks in which clinical histories have been recorded accurately over a long period of time.

CHAPTER 5

The role of fimbrial gene (*fimA*) of *D. nodosus* in the pathogenesis of footrot

Contents

5.1 Introduction

Knowledge of the role of *D. nodosus* in the pathogenesis of ovine footrot is limited. The extracellular proteases (Kortt et al., 1993) and the polar type IV fimbriae (Elleman, 1988) of *D. nodosus* are considered to be the primary virulence factors but there is little direct evidence for this. The *vap* and *vrl* genomic regions have also been shown to be associated with isolates of *D. nodosus* from severe disease (Katz et al., 1992; Cheetham et al., 1995; Rood et al., 1996; Billington et al., 1999). Study of the role of these proposed virulence factors has not been possible due to the absence of a genetic transformation system in *D. nodosus*. This difficulty was overcome by the successful transformation of different strains of *D. nodosus* by Kennan et al., (1998). In their experiment a tetracycline resistance gene, *tet (M)*, which was presented on a suicide plasmid, was inserted into the chromosome by reciprocal cross over events. These studies provided the tools required to investigate the role of the putative virulence factors of *D. nodosus*.

In a variety of bacteria, fimbriae are associated with virulence as they are the organelles for attachment to epithelial surfaces (Johns and Isaacson, 1983; Klemm, 1985; Moore and Rutter, 1989). In *D. nodosus* the role of fimbriae in attachment and colonisation of skin has not been demonstrated but the degree of fimbriation is correlated with virulence of an isolate (Stewart, 1973; Thorley, 1976; Stewart and Egerton, 1979; Skerman et al., 1981; Every and Skerman, 1983). However, this correlation is not definite as a few strains known to be benign were shown to have as many fimbriae as virulent strains (Depiazzi and Richards, 1985; Stewart et al., 1986). Fimbriation was determined by electron microscopy in most of the above studies, rather than by quantification of purified fimbrial protein from a known number of cells. It is known that *D. nodosus* fimbriae can easily detach from cells in the process of preparing for microscopy (Mattick et al., 1984). So it is not known whether the results of above studies are accurate.

D. nodosus fimbriae are classified as type IV because of their polar location, association with twitching motility and the presence of an N-methylphenylalanine residue as the N-terminal amino acid (Strom and Lory, 1993). Based on the agglutination reactions of the fimbrial antigens *D. nodosus* isolates have been

classified into 9 major serogroups, A to I (Claxton, 1989). *D. nodosus* fimbriae are highly immunogenic and vaccination of sheep with whole cell *D. nodosus*, or purified fimbrial proteins gives protection against the disease, although this protection is serogroup specific (Egerton, 1974; Every and Skerman, 1982; Elleman, 1988). Recombinant fimbrial vaccines have been prepared by expression of the fimbrial subunit gene, *fimA* in *Pseudomonas aeruginosa* (Egerton et al., 1987). Multivalent vaccines have been tested but antigenic competition with consequent reduced host immune response has limited the use of these vaccines (Hunt et al., 1994; Hunt et al., 1995; Raadsma et al., 1994).

The *fimA* gene encodes for the major fimbrial subunit of all the nine serogroups. Based on the nucleotide sequences and the genetic organisation of the fimbrial gene region *D. nodosus* serogroups have been classified into two major classes (Mattick et al., 1991; Hobbs et al., 1991). In class I, which includes serogroups A, B, C, E, F, G and I, the *fimA* is followed by *fimB*. The latter gene encodes a potential 29.5 Kda membrane protein of unknown function but was postulated to play a role in the export of fimbrial subunits to the cell surface (Hobbs et al., 1991). *FimB* does not have a separate promoter and is co-transcribed with *fimA* (Mattick et al., 1991). Serogroup D and H belong to class II and have three additional genes downstream of *fimA*, namely *fimC*, *fimD* and *fimZ* (Hobbs et al., 1991). The *fimD* gene is thought to be a functional homologue of *fimB* (Hobbs et al., 1991), *fimC* has a similar sequence to *trax* from F plasmid (Firth and Skurray, 1995) and *fimZ* is thought to represent a redundant fimbrial subunit (Hobbs et al., 1991). At the protein level there are differences between class I and class II fimbriae, namely in class I cysteine residues are at positions 56 and 100 which form a presumptive disulphide loop in the central third of the protein whereas in class II cysteine residues are at positions 50 and 60, near the end of conserved amino-terminal region and at positions 134 and 148, near the carboxy terminal region of the protein (Mattick et al., 1991). The first pair of cysteine residues are unique in *D. nodosus* whereas the second pair occur in a similar position and environment in the fimbrial subunits of other type IV fimbriate species of bacteria (Dalrymple and Mattick, 1987).

As part of a larger study to determine the role of fimbriae in the pathogenesis of footrot using allelic exchange to disrupt the *fimA* and *fimB* genes of a class I strain

(serogroup G) this study contributed a detailed characterisation of the resultant mutant and the wild strain, engineered at Monash University (Kennan et al., 2001) and testing of these strains for virulence in sheep.

5.2 Materials and methods

5.2.1 Strains of D. nodosus

The strains used were:

1. VCS 1703 (K strain) – Wild strain.
2. JIR 3727 (J strain) – *fimA* mutant 1.
3. JIR 3728 (L strain) – *fimA* mutant 2.
4. JIR 3737 (M strain) – *fimB* mutant.

Cultures of these strains were obtained from Dr. Kennan at Monash University and VCS 1703 came from the collection of the Faculty of Veterinary Science, University of Sydney. These strains were initially grown on 4% hoof agar and subcultured on 2% hoof agar (Thomas, 1958).

5.2.2 Characterisation of trial strains

5.2.2.1 Microscopy

Smears were made from cultures and stained by the Gram method. These stained smears were examined microscopically to confirm that cultures were free of contaminants and that cells were morphologically normal.

5.2.2.2 **Serogrouping**

The serogroup of each strain was confirmed using the microtitre plate agglutination test (Raadsma et al., 1995).

5.2.2.3 Polymerase chain reaction (PCR) tests

a. D. nodosus specific PCR:

All the strains were subjected to a PCR test using species specific primers (La Fontaine et al., 1993). These primers amplify a region of DNA encoding for the 16S ribosomal RNA of *D. nodosus*.

b. Fimbrial gene PCR

The strains were tested by Class 1 *fimA* specific primers (PTC 830 and PTC 5) (Cox, 1992).

c. Serogroup specific PCR

A *D. nodosus* serogroup specific PCR (see Chapter 6) was used to further confirm the serogroups of the strains used.

d. **omp-gene PCR**

Copies of the *omp 1* gene which encodes for major outer membrane proteins of *D. nodosus* were amplified from all the test strains by primers A and C (Moses, 1993). The details of all the primers used in the characterisation study are presented in Table 5.1.

Amplification in PCR tests was done in 20 µl volumes in capillary tubes (Corbett Research, Mortlake, Australia). The reaction mixture contained a final concentration of 20mM Tris-HCL, 50mM KCL, 3mM $MgCl_2$ and 200 µM each of the four nucleotides. The concentration of each primer was 0.5 µM. One unit of Taq polymerase (Gibco-BRL, Gaithersburg, MD, USA), and approximately 50 – 100 µg of DNA was added in the form of cell lysates. The products were electrophoresed in 1% and 2% agarose in tris EDTA (TAE) buffer, stained with ethidium bromide and visualised under ultra violate (UV) illumination.

Table 5.1 Details of the primers used in the characterisation study.

Name	Primer sequence	Source
1. Ac	5' CGG GGT TAT GTA GCT TGC 3'	La Fontaine et al., 1993
2. Cc	5' TCG GTA CCG AGT ATT TCT ACC CAA CAC CT 3'	La Fontaine et al., 1993
3.PTC830	5' GGC CGG AAA GCG CTG AGA CCG G 3'	Cox, 1992
4. PTC5	5' AGC TAA CAG GGC AGC AAC GGT 3'	Cox, 1992
5. A	5' AAT CAA GGA ACT GAA GAA 3'	Moses, 1993
6. C	5' AAT GCC GTA CAT TAA AGC A 3'	Moses, 1993
7. FP	5' CCTTAATCGAACTCATGATTG 3'	Dhungyel et al., 2001
8. RG	5' CTTAGGGGTAAGTCCTGCAAG 3'	Dhungyel et al., 2001

Note: Primers Ac and Cc are forward and reverse for the amplification of *D. nodosus* species specific 16S rRNA gene. Similarly primers PTC830 and PTC5 are for the fimbrial gene region, primers A and C for *omp* gene, and FP and RG are serogroup G specific.

5.2.2.4 Restriction fragment length (RFLP) of *omp* gene PCR products

15 µl of the clean *omp* gene PCR products were used directly for endonuclease digestion. Three units of the restriction enzyme *HpaII* (Gibco-BRL, Gaithersburg, MD, USA) and the buffer provided by the supplier was added to the product and incubated overnight at 37 ^{0}C. The digested products were electrophoresed in a 4-20% gradient polyacrylamide minigel (Novex, San Diego, CA, USA) in tris borate EDTA (TBE) buffer than stained with ethidium bromide and visualised under UV illumination. The band sizes were estimated by visual comparison with standard molecular weight markers. Each distinct pattern was assigned a PCR-RFLP number.

5.2.2.5 In-vitro virulence tests

All the test strains were subjected to elastase (Stewart, 1979) and gelatin gel tests (Palmer, 1993).

5.2.2.6 Pilot in-vivo virulence test

Ten young sheep were challenged with wild type (VCS 1703) by the method of Egerton et al. (1969) (see below) to confirm the virulence of this strain in a pilot trial before conducting the comparative trial with mutant strains.

5.2.2.7 Trial animals and allocation to trial groups

Six month old Merino sheep (n = 48), free of footrot were obtained from a farm at Marulan in the southern highlands of NSW. Each sheep was identified by a numbered ear tag. They were randomly divided into 5 groups, 10 in 4 groups and 8 in one group as presented below in Table 5.2.

Table 5.2. Allocation of animals to challenge groups

Group	N	Treatment (challenge strain)
1	10	Wild (K) strain
2	10	Plain agar (negative control)
3	10	J strain (*fimA* mutant 1)
4	10	K strain (*fimA* mutant 2)
5	8	M strain(*fimB* mutant)

5.2.2.8 Management of animals

Sheep were kept in an experimental animal house on concrete floors. Each group was in a separate temperature controlled room at 22^0 C and fed lucerne hay and oats. Water was provided ad-libitum.

5.2.2.9 Viable cell count

Pure lawn cultures of *D. nodosus* on 2 % hoof agar plates were harvested in 1 ml of sterile TAS broth. Cells in these suspensions were counted in a Thoma counting chamber. Briefly, 4 µl of the 1:100 dilution of *D. nodosus* cells were placed on a clean counting chamber and covered with a cover slip. Cells from 4 groups of 16 small squares (total 64 squares) were counted. Duplicate samples were counted. Calculation of the count per ml of the original suspension was based on the mean of the duplicate counts by using the formula below.

CPM = (Average count for 64 squares/64) x ($2x10^7$) x dilution factor.
$2x10^7$ is the reciprocal of the volume of one square .

Once the total cell counts were known 10-fold serial dilutions were made in TAS broth. 25 µl each of the final dilutions (X x 10^2 dilution) were inoculated into 3 quarters of 4 % HA plates, the fourth quarter inoculated with 25 µl of the undiluted cell suspension as a control, and incubated anaerobically at 37^0 C for 4 days. Colonies were counted from these 3 quarters and the mean of these was used to calculate the number of colony forming units (CFU) in the sample. This counting procedure was done on duplicate plate cultures.

5.2.2.10 Challenge procedure

Sheep were artificially challenged with the strains (Table 5.2) by the method of Egerton et al. (1969). Briefly, the feet of the animals were predisposed to infection by keeping them on wet foam plastic mats for four days before challenge to facilitate hydration of the stratum corneum of the skin. The interdigital skin of foot of all the animals were sampled and cultured for *D. nodosus* infection prior to challenge. They were then challenged by the application of four day pure cultures of the relevant strains. A full plate of culture was scraped on to plugs of 2% hoof agar which was placed on to the interdigital skin and held with bandages for 4 days. Animals in group 2 were challenged with sterile agar plugs. Mats were removed from the floors 2 weeks after the challenge. The interdigital skin of all the animals in each group were

sampled for *D. nodosus* culture two weeks after the challenge. All the isolates recovered were again characterised as above.

5.2.2.11 Scoring of feet

All the animals were examined and their feet scored for severity of lesions at the start of the trial, then at weekly intervals. The lesion scoring method of Whittington and Nicholls (1995), a modification of the method of Egerton and Roberts (1971) was used. The total weighted foot score (TWFS), which is the sum of the scores of the four feet after raising scores of 3 and 4 by the power of 2 was used to get the overall score for the animal. Scoring of feet was done by an experienced research team member who was unaware of the identity of the strains used on each group of sheep.

5.2.2.12 Preparation of antigens for ELISA

a. Outer membrane protein (OMP) antigen

Outer membrane protein antigen of *D. nodosus* was prepared by the potassium thiocyanate (KSCN) extraction method (Emery et al., 1984; Whittington et al., 1990). A concentration of 1.2 mg/ml was made in phosphate buffered saline (PBS) and frozen until required for ELISA. Prior to use, thawed antigen was diluted in PBS to the required concentration.

b. Pilus antigen

Pilus was purified from broth culture suspensions of native (K) strain by the method of Mattick et al. (1984). In addition a series of precipitations with HCL and 0.1M $MgCl_2$ was required to achieve a high degree of purity (Whittington et al., 1997).

c. Estimation of antigen purity and concentration

Concentrations and purity of both the antigens were tested by SDS - PAGE with appropriate molecular weight standards. Concentrations were also estimated by using

a Bicinchoninic acid (BCA) assay (Sigma Chemical Co., USA), which is a colorimetric assay based on the principle described by Lowry et al. (1951).

d. Collection and handling of serum

Blood samples were collected from the jugular vein and allowed to clot at room temperature. Serum was separated by centrifugation at 3,000 rpm for 10 mins and kept at -20^0 C until required for tests.

e. Enzyme linked immunoabsorbent assay (ELISA)

Serum samples were tested for anti – *D. nodosus* antibodies by ELISA. Antibodies against OMP as well as pilus antigens were measured for primary response of the sheep to footrot. The OMP antigen ELISA was performed by the method of Whittington and Egerton (1994) and the pilus ELISA by the method of Whittington et al. (1997). Optical density is referred to as the "antibody level" of the serum sample.

5.3 Results

5.3.1 Characterisation

All 4 strains used in the trial were found to be Gram negative, bipolar rods. They were all positive to *D. nodosus* species specific 16S rRNA PCR (Figure 5.1). PCR with primers based on the fimbrial gene (*fim A*) sequence of these strains were able to amplify PCR product only from the wild (K) and *fim B* mutant (M) strains. Serogrouping, both by slide agglutination and serogroup specific PCR confirmed the serogroups of the wild (K) and *fim B* mutant (M) strains to be serogroup G (Figure 5.1). The *OMP* gene PCR/RFLP of all the challenge strains, both before the challenge and recovered from lesions in sheep during the trial was the same (Figure 5.2).

In elastase and gelatin gel tests only the wild (K) and *fimB* mutant (M) strains were positive and both *fimA* mutant strains (J and L) were negative. All the results of the characterisation tests are summarised in Table 5.3.

Table 5.3: Characterisation tests of the trial strains.

Strain	Gram staining	16S rRNA PCR	Sero-group	FimA PCR	OMP/RFLP pattern	Elastase test	Gelatin gel test
K	- ve bipolar rods	+ ve	G	+ ve	1	7 days +ve	+ ve
J	- ve bipolar rods	+ ve	-	-	1	28 days - ve	- ve
L	- ve bipolar rods	+ ve	-	-	1	28 days –ve	- ve
M	- ve bipolar rods	+ ve	G	+ ve	1	7 days +ve	+ ve

5.3.2 Viable cell counts of the challenge cultures

The viable cell counts of the challenge cultures of the strains K, J, L and M were 8.58 x 10^5 colony forming units (CFU), 9.53 x 10^5 CFU, 8.43 x 10^5 CFU and 10.82 x 10^5 CFU, respectively.

Figure 5.1 Results of *D. nodosus* specific and serogroup specific PCR. Lanes M- DNA marker Lanes 1-4 D.nodosus specific products (1-strain K, 2-strain J, 3-strain L, 4-strain M), Lanes5- 8 Serogroup specific products (5-K, 6-J, 7-L, 8-M), Lane 9 negative control.

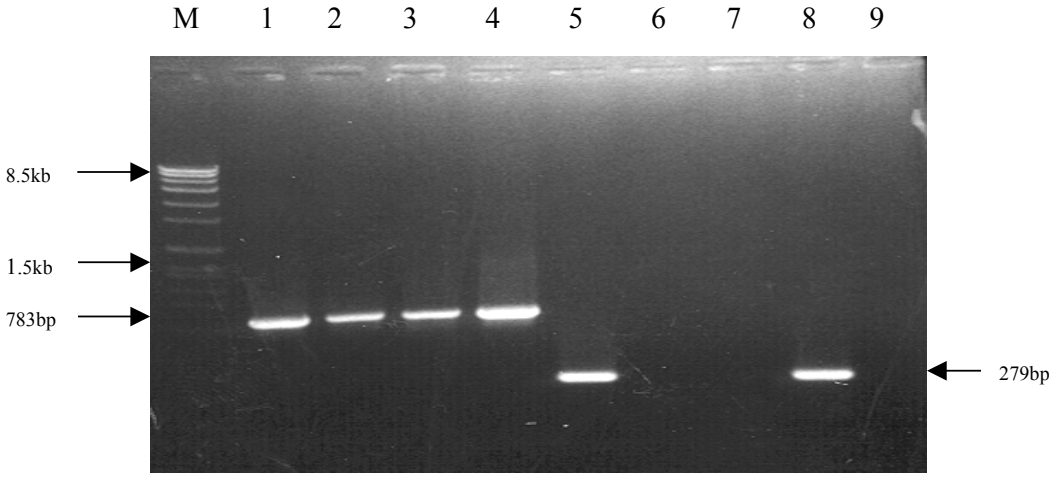
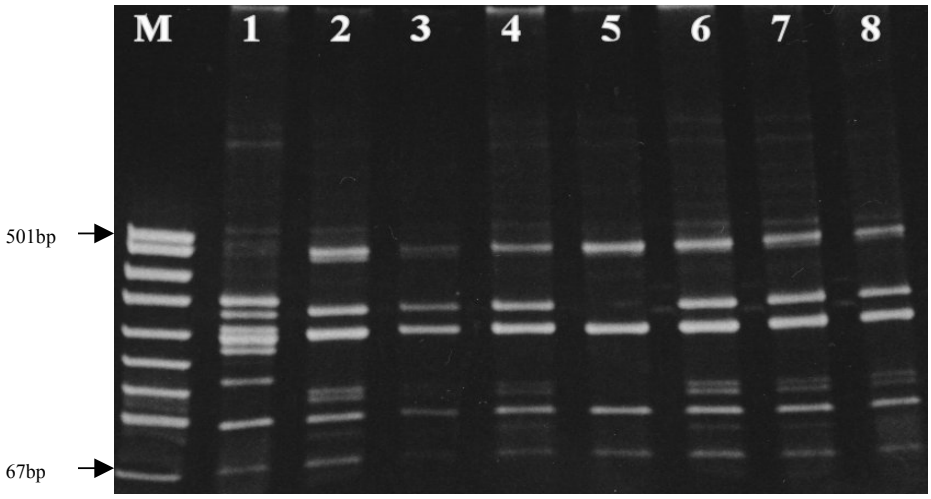


Figure 5.2 Comparison of the PCR/ RFLP patterns of OMP genes of the trial strains (M- Puc19DNA marker, Lane 1- I strain, 2- Wild K strain, 3 & 5- other G strains, 4 – Wild K strain recovered from infected feet, 6 - fimA mutant J, 7- fimA mutant L, 8- fimB mutant M).



5.3.3 Clinical findings

Clinical footrot lesions were observed only in group 1 (wild K strain) and group 5 (*fimB* mutant). The mean total weighted foot scores (TWFS) in all the groups on week 0 were nil. At week 1 mean TWFS were 2.7, 52.1 and 12.1 in the pilot trial group, groups 4 & 5, respectively. On week 2 these increased to 30.2, 78.2 and 12, and on week 3 they were 64.3, 61.1 and 9.8 respectively. Whereas footrot was not seen in the other groups, group 2 (negative control), groups 3 and 4 (both *fimA* mutant strains). The mean TWFS of the foot lesions of different groups are presented in Table 5.4 and Figure 5.3A.

Table 5.4: Mean TWFS of different treatment groups

Group	Treatment (challenge)	N	Mean TWFS			
			Week 0	Week 1	Week 2	Week 3
1	Wild strain (K)	10	0	52.1	78.2	61.1
2	Plain agar	10	0	0	0	0
3	*FimA* mutant 1	10	0	0	0	0
4	*FimA* mutant 2	10	0	0	0	0
5	*FimB* mutant	8	0	12.1	12	9.8

5.3.4 Bacteriology

All the animals prior to challenge were negative when cultured for *D. nodosus*. *D. nodosus* was recovered only from the animals in groups that developed footrot as indicated by results in Table 5.5. The characteristics of the isolates re-isolated were same as the original strains including the *OMP* gene RFLP (Figure 5.2).

Table 5.5: Results of *D. nodosus* culture of foot swabs from different groups

Group	Treatment (challenge)	Day 0 (prior to challenge)	Week 2 after challenge
1	Wild strain (K)	- ve	+ ve
2	Plain agar	- ve	- ve
3	*FimA* mutant 1	- ve	- ve
4	*FimA* mutant 2	- ve	- ve
5	*FimB* mutant	- ve	+ ve

5.3.5 Serological response

The antibody levels (mean ELISA OD) against OMP antigen at week 0 in all the groups were relatively low. At week 2 and 3 it increased only in group 1. There was no change in animals in groups 2, 3 and 5 (Table 5.6). As in the case of OMP antibody, concentration against pilus antigen was low in week 0. Similar to OMP response, there was no change in response to pilus in groups 2, 3, 4 and 5 (Table 5.6 and Figure 5.3B).

Table 5.6: Mean ELISA responses to OMP and pilus antigens

Group	Treatment (challenge)	Mean ELISA OD (responses)					
		Week 0		Week 2		Week 3	
		OMP	Pilus	OMP	Pilus	OMP	Pilus
1	Wild strain (K)	0.34	0.24	0.53	0.26	0.74	0.39
2	Plain agar	0.41	0.27	0.38	0.20	0.37	0.19
3	*FimA* mutant 1	0.38	0.27	0.39	0.31	0.37	0.31
4	*FimA* mutant 2	0.28	0.31	0.25	0.26	0.26	0.25
5	*FimB* mutant	0.24	0.20	0.29	0.22	0.31	0.24

Figure 5.3A Diagrammatic representation of the total weighted footscores (TWFS) in different groups of the experiment (Wild strain, plain agar, fimA mutant 1, fimA mutant2 and fimB mutant).

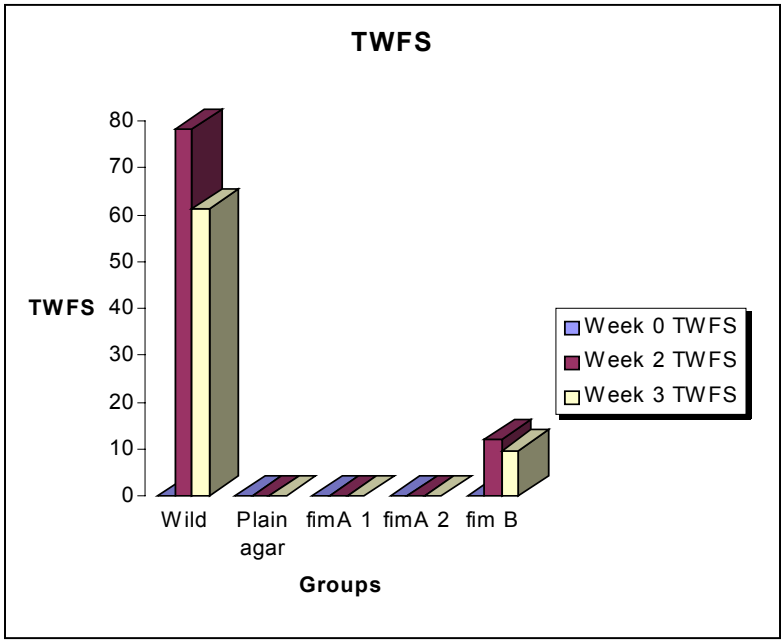
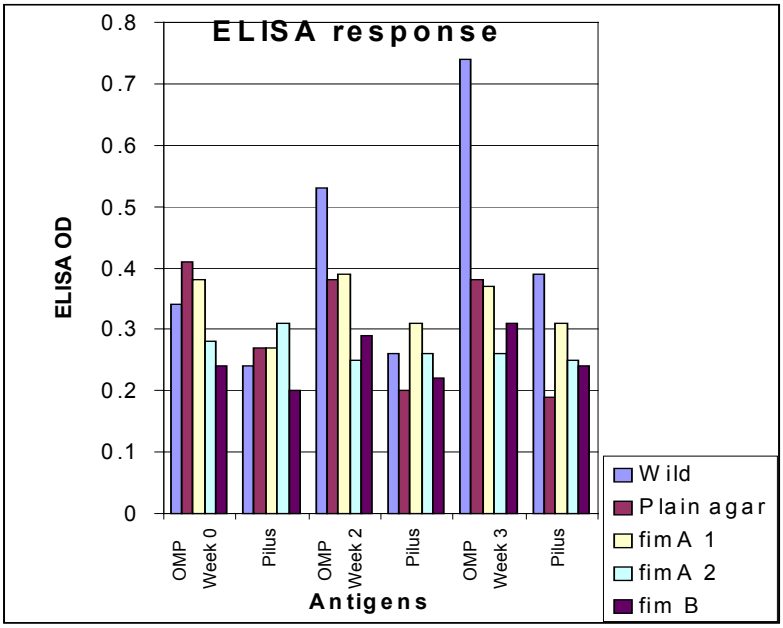


Figure 5.3B Diagrammatic representation of the ELISA response to OMP and Pilus antigen in different groups of the experiment (Wild strain, plain agar, fimA mutant 1, fimA mutant2 and fimB mutant).



5.4 Discussion

A detailed *in-vitro* characterisation study of trial strains of *D. nodosus* was necessary to independently confirm and compare the characters of the wild strain with that of the mutant strains before they were tested in the *in-vivo* trial in sheep. As indicated in the results in Table 5.2 all of the four trial strains had the staining and morphological characteristics of *D. nodosus*, and were positive on species specific 16S rRNA PCR (Figure 5.1). This is in agreement with the initial part of the study by Kennan when the mutant strains were generated, molecular and other characterisation procedures like southern hybridisation experiments using *fimA* and *tet* (M) specific probes, immuno-blotting with serogroup G specific antiserum, electron microscopy and twitching motility were conducted (Kennan et al, 2001).

Initial screening of these trial strains with the characterisation tests revealed that one of the strains was of serogroup I instead of G and had to be exchanged for the correct strain. So the characterisation with various tests described above was essential to confirm that all the strains used for animal trial originated from original wild strain of serogroup G from which the *fimA* and *fimB* mutants were generated. In this process evidence of serogroup conversion was also found (Kennan et al. in preparation).

Serogrouping both by agglutination testing and *D. nodosus* serogroup specific PCR (Dhungyel et al, 2001), and *fimA* PCR (Cox, 1992) are based on the fimbrial gene unit. As reflected by results in Table 5.2 the mutant strains (J and L) were negative to these tests whereas wild strain (K) and *fimB* mutant strain (M) in which only the *fimB* is inactivated react positively to *fimA*. As expected *fimA* mutant strains (J and L) were negative to serogroup specific PCR which is based on *fimA* gene confirming that the mutants were devoid of intact gene (Figure 5.1). The *Omp* gene RFLP profile remain the same in all the strains confirming that this characteristic remains unchanged even with the changes in *fimA* and *fimB* genes. OMP/RFLP is thought to remain constant even in cases where switching of *fimA* was suspected to have occurred to cause a change in serogroup (Ghimire and Egerton, 1999; Abbott, 2000).

Phenotypic *in-vitro* virulence tests, the elastase and the gelatin-gel protease thermo-stability tests, on these 4 strains showed that the wild strain and the *fimB* mutant

strains were positive to both, whereas the two *fimA* mutant strains were negative. Lu et al. (1997) have shown that the mutation of fimbrial subunit gene *pilA* of *P. aeruginosa* results in loss of the ability to secrete extracellular proteins such as elastase. The results of this study were suggestive of the fact that the *fimA* mutants of *D. nodosus* were not able to produce or secrete wild type levels of extracellular proteases. However, RT-PCR experiments on these *fimA* mutant strains confirmed that the synthesis was normal as in the wild strains (Kennan et al, 2001). Hence it was concluded that the extracellular proteases were still synthesised in the *fimA* mutants but they were not secreted as efficiently as in the wild strains.

The clinical results of the *in-vivo* trial (Table 5.3) indicated that sheep challenged with *fimA* mutant strains (groups 3 and 4) failed to develop footrot as in the case of negative control group (2) despite the fact that the viable counts of the cultures used for challenge were same as in other groups (1 and 5). Also the conditions for the experiment were similar except for the strain of culture used for challenge. TWFS values (Table 5.3) which provide an objective summary of the footrot lesions in a sheep (Egerton and Roberts, 1971; Whittington and Nicholls, 1995b) clearly indicated the presence of virulent footrot in the groups 1 and 5. The lower values of TWFS in the *fimB* group (5) could not be explained but there was clear evidence that this strain could cause footrot. However, the highest TWFS value of 12.1 was lower that that observed with the wild strain (78.2) suggesting that the *fimB* mutant may be attenuated. The result obtained from the *fimB* mutant challenge showed the need for further testing of this hypothesis. However, since this strain clearly causes footrot further experiments could not be justified on the grounds of animal ethics.

All of the *D. nodosus* isolates re-isolated from the infected animals of groups 1 and 5 were characterised in detail in same way as the strains used for challenge. They were found to be identical in all the characteristics tested confirming that they were the same strains used for the challenge, and that no other infection or cross infection had taken place.

The serological responses to both OMP and pilus antigens were nil in the groups challenged with *fimA* mutant strains indicating that they had remained uninfected. The responses in the groups challenged with wild strains were typical of those seen in

sheep with virulent footrot three weeks post infection and it was anticipated that they would have increased further if the sheep had not been treated with antibiotics for welfare reasons as the humoral responses are lesion severity and duration dependent (Whittington and Nicholls, 1995). This was thought to be the cause for no significant increase in the responses in *fimB* mutant group.

The results presented in this study provide the first definitive evidence that fimbrial gene (*fim A*) is essential for virulence of *D. nodosus* in sheep. *In vivo* virulence testing of the two *fimA* mutants showed that they were not able to establish any footrot lesions whereas the wild type of the same strain produced virulent footrot in the same trial conducted under similar and blind conditions. These bacteria were not re-isolated from interdigital skin after in vivo challenge. This indicates that *fimA* mutant strains cannot colonise the ovine foot, and the simplest and most likely explanation for these results is that colonisation of the interdigital skin and subsequent penetration of the stratum corneum requires the adhesive activity of type IV fimbriae. However, since these mutants also had altered ability to secrete extracellular proteases, and perhaps other as yet unknown extracellular proteins, the possibility of the involvement of these factors as the major determinants in colonisation or penetration cannot be excluded.

The role that the proteases play in the disease process needs to be examined by isolating mutants in each of the respective protease genes. However, separating the direct effects of mutations on the biological function of the fimbriae from their effects on extracellular secretion will be a much more complex experiment.

CHAPTER 6

Development of rapid PCR test for serogrouping of *Dichelobacter nodosus*

Contents

6.1 Introduction

Footrot is mixed bacterial infection of the feet of ruminants in which *Dichelobacter nodosus* is the essential transmitting agent (Beveridge, 1941; Egerton, et al., 1969). *D. nodosus* is a slow growing and fastidious anaerobe requiring special media and conditions for growth. Antigenic variation among strains of *D. nodosus* was first observed by Beveridge (1941) and ascribed to surface (K) antigens that were detected using a slide agglutination test (Egerton 1973). The K agglutination reaction, attributable to fimbriae (pili) which is the major immunogen (Thorley and Egerton, 1981; Egerton et al., 1987), was used to define a number of serogroups of *D. nodosus* (Egerton, 1973; Schmitz and Gradin, 1980; Claxton et al., 1983, Claxton, 1986). In the Australian classification system there are 9 serogroups (A – I). Serotypes have been described within most groups.

Commercial footrot vaccines contain representatives of the common serogroups and typically are octo- or nona-valent. Whole flock vaccination with multivalent vaccine can be effective but antigenic competition results in suboptimal antibody titres of short duration. This necessitates administration of booster doses after intervals as short as several months during the seasons when footrot is spreading between sheep (Egerton and Burrell, 1970; Schwartzkoff et al., 1993; Raadsma et al., 1994, Hunt et al., 1994, 1995). In contrast to multivalent vaccines, monovalent or bivalent vaccines are more potent, inducing persistent high antibody titres that have both curative and protective effects (Egerton and Morgan, 1972; Raadsma et al., 1994). Furthermore, specific vaccination has been associated with eradicating virulent footrot from mixed flocks of sheep and goats in Nepal (Egerton et al., 1996; Ghimire, 1997; Dhungyel et al, 2001) and from sheep in Bhutan (Egerton and Gurung, unpublished). Before specific vaccination can be considered the first priority is to identify the serogroup(s) present in the affected population. Claxton et al. (1983) have reported the presence of multiple serogroups of *D. nodosus* within affected flocks.

Current methods for the identification of the serogroup of *D. nodosus* present in the bacterial population requires isolation of the organism and after purification by subculture, antigenic analysis. This usually takes at least 4 weeks. So there is a need

for a reliable and rapid typing method that correlates with the current serogrouping system.

Antigenic diversity in the fimbriae of *D. nodosus* is based on variation in the DNA sequence of the fimbrial gene (*fimA*) (Elleman, 1988; Mattick et al., 1991). Variation in the 3' region of fimA facilitates a PCR-based test for rapid identification of *D. nodosus* strains (John et al., 1999; Zhou and Hickford, 2000; Zhou et al., 2001, Dhungyel et al., 2001). In the study of Zhou et al. (2001), genotyping of *D. nodosus* was done by PCR amplification and hybridisation to immobilised oligonucleotides (PCR/Oligotyping) and requires sequence data for all serotypes to be detected. In this chapter I describe the development of a simple and rapid serogroup specific PCR test which distinguishes between the prototypes of each of the major *D. nodosus* serogroups; sequence data are required only for each serogroup, enabling the detection of 'unknown' serotypes.

6.2 Materials and Methods

6.2.1 Bacterial strains

Eighteen cultures of *D. nodosus* covering all the Australian prototypes (i.e., serogroups and serotypes) were obtained from the collection of the Faculty of Veterinary Science, University of Sydney (Table 6.1). These prototypes were used for the establishment of sensitivity and specificity within and between serogroups (Claxton et al., 1983). To confirm specificity within serogroup, 146 lyophilised *D. nodosus* cultures which had previously been serogrouped were examined from the same collection.

Table 6.1 Australian prototype *D. nodosus* strains used in this study.

Sl. No.	Prototype/Strain No.	Serogroup/ Serotype
1	VCS 1001	Serogroup A, Serotype A1
2	VCS 1251	Serogroup A, Serotype A2
3	VCS 1006	Serogroup B, Serotype B1
4	VCS 1208	Serogroup B, Serotype B2
5	VCS 1190	Serogroup B, Serotype B3
6	VCS 1125	Serogroup B, Serotype B4
7	VCS 1008	Serogroup C, Serotype C1
8	VCS 1630	Serogroup C, Serotype C2
9	VCS 1172	Serogroup D
10	VCS 1137	Serogroup E, Serotype E1
11	VCS 1114	Serogroup E, Serotype E2
12	VCS 1017	Serogroup F, Serotype F1
13	VCS 1244	Serogroup F, Serotype F2
14	VCS 1220	Serogroup G, Serotype G1
15	VCS 1004	Serogroup G, Serotype G2
16	VCS 1687	Serogroup H, Serotype H1
17	VCS 1057	Serogroup H, Serotype H2
18	VCS 1623	Serogroup I

A total of 84 other bacteria, most commonly found in the normal flora of sheep or representative of other bacterial families and including *S. indolegenes* and *C. hominis* (Table 6.4) (Whittington, 1994) were used to check the specificity of PCR assay among the bacteria. Strains were grown as described and stored as cell pellets at -20°C (Whittington, 1994).

6.2.2 Design and synthesis of oligonucleotide primers

D. nodosus fimA sequence data were available for all the Australian prototypes (Table 6.1). Sequences were aligned using BLAST (NCBI, USA) and primers were then

selected using Primer Premier (Stanford University, USA). A common forward primer (FP) was designed from the constant amino-terminal region of *fimA*. Reverse primers were designed for each of the 9 serogroups from the variable carboxy-terminal region of the sequence. The criteria for selection were *D. nodosus* specificity and serogroup specificity, and different sizes of the products for all the nine serogroups. The primer sequences, their positions in the fimbrial gene sequence and product sizes when used in a PCR assay with the common forward primer are presented in Table 6.2. Primers were custom synthesised by Gibco-BRL, USA or Sigma Aldrich, USA. A number of reverse primers were examined by trial and error for several serogroups where sensitivity or specificity were problematical, but data are given only for the most useful primers. In addition all of the primers presented in the study of John et al. (1999) were synthesized and tested with the prototype strains.

Table 6.2: Details of the common forward primer and serogroup specific reverse primers used in PCR assays.

Primer name	Nucleotide sequence	Position in *fim A*	Product size	GenBank accession number
FP	5'CCTTAATCGAACTCATGATTG 3'	26-46	-	X52403
RA	5'AGTTTCGCCTTCATTATATTT 3'	421-441	415 bp	X52403
RB	5'CGGATCGCCAGCTTCTGTCTT 3'	286-309	283 bp	X52404
RC	5'AGAAGTGCCTTTGCCGTATTC 3'	331-351	325 bp	X52405
RD	5' TGCAACAATATTTCCCTCATC 3'	325-345	319 bp	X52389
RE	5'CACTTTGGTATCGATCAACTTGG 3'	367-389	363 bp	X52407
RF	5' ACTGATTTCGGCTAGACC 3'	250-267	241 bp	X52408
RG	5' CTTAGGGGTAAGTCCTGCAAG 3'	283-305	279 bp	X52409
RH	5' TGAGCAAGACCAAGTAGC 3'	412-435	409 bp	X52390
RI	5' CGATGGGTCAGCATCTGGACC 3'	194-215	189 bp	X52410

Note: product sizes given are of the amplicons produced with forward primer (FP) and the respective serogroup reverse primers.

6.2.3 Preparation of samples for PCR

6.2.3.1 Chromosomal DNA

Chromosomal DNA of the prototypes of *D. nodosus* was extracted from cell pellets with the Wizard Genomic DNA purification kit, Promega Corporation, USA, using

the protocol for gram negative bacteria. The concentration of the purified DNA was estimated by measurement of absorbance at 260 nm (Sambrook et al., 1989).

6.2.3.2 Whole cell lysates of prototype strains and other bacterial strains

Prototypes of *D. nodosus* (Table 6.1) were grown anaerobically on 4 % (Thorley, 1976) and 2 % (Thomas, 1958) hoof agar (HA) media at 37^0 C for 4 days. Cells were harvested in PBS and the number of organisms counted visually using a Thoma counting chamber after serial dilution to provide a cell density appropriate for enumeration using this method. Cells were harvested in lysis buffer (20 mM Tris HCl pH 8.9, 2 mM EDTA, 1 % Triton X-100). All suspensions were boiled for 5 min and immediately cooled on ice for 10 min. The suspensions were then centrifuged at 16,110 *x g* for 1 min and 1µl of the supernatant was used as template for PCR assays. To make samples containing more than one serogroup, equal volumes of the lysates from suspensions that contained equal concentrations of cells were mixed. Lysates of these suspensions were mixed also at varying concentrations to provide complex DNA target with multiple serogroups present in unequal amounts. Depending on the number of serogroups/strains in the mixture, 1 – 3 µl of the mixed samples were used as templates for each PCR reaction.

Suspensions of the other bacterial strains were similarly made in lysis buffer and a 1µl sample of the supernatant was used as template for each PCR reaction.

6.2.4 PCR experiments

To test the specificity of the PCR assays among bacterial strains each serogroup primer pair was individually tested by PCR on lysates of 84 bacterial strains which were either from the normal flora of sheep or representative of other bacterial families.

To evaluate the sensitivity and target specificity of the serogroup specific primers, purified chromosomal DNA samples and the cell lysates from the prototype strains of *D. nodosus* were serially diluted (10 ng to 0.1 pg for chromosomal DNA and 10^{10} cells to 10^2 cells per ml) and amplified by PCR. To test serogroup specificity, each

primer pair (common forward primer with serogroup specific reverse primer) was tested on all 18 prototype (9 serogroup and 9 serotype) strains individually. Further, each primer pair was tested separately against a mixture of DNA from all 18 prototypes.

6.2.4.1 Multiplex PCR

In order to make it easier to amplify different serogroups from mixed template in a single tube reaction, various combinations of reverse primers (RA – RI) along with the common forward primer (FP) were tested to optimise the multiplex reaction. Groups of 2, 3, 4, 5 and 9 reverse primers with FP were tried.

6.2.5 Immuno-magnetic capture method

6.2.5.1 Preparation of antisera

Antibodies against outer membrane protein (OMP), known as potassium thiocyanate (KSCN) extracted antigen common to all serogroups of *D. nodosus* were prepared in rabbits. Briefly, KSCN antigen was prepared from *D. nodosus*, strain VRS 171 (serogroup F, virulent) as previously described (Whittington and Egerton, 1994). A New Zealand White rabbit was immunised subcutaneously with 250 μg KSCN antigen (in 1 ml sterile saline) which had been emulsified in 1ml Freund's Complete adjuvant (Sigma). Immunisation was repeated 4 weeks later. Blood was collected 3 weeks later, allowed to clot at room temperature and serum was harvested and stored at -20^0 C until required. Immunoglobulins were purified by affinity chromatography over protein A (pharmacia), dialysed against Tris-saline with sodium azide as preservative, concentrated to the original volume of serum by dialysis against carboxymethyl cellulose (Aquacide, Calbiochem) and stored at -20^0 C until required.

6.2.5.2 Immuno-magnetic capture

To coat magnetic beads (M-280, Dynal A S, Norway) with OMP antibodies, 200 μl of tosylactivated beads were mixed thoroughly with 800 μl of 0.05 M borate buffer (BB, 1.54g boric acid in 500 ml of MQ water). The beads were collected by standing the

tubes on a magnet for 2 mins and carefully removing all of the supernatant without disturbing the beads. The beads were washed twice with 1 ml of 0.05 M BB by mixing thoroughly, collecting them with the magnet and suspending them in 1ml of BB. 625 µl of the OMP antibodies were added to 1ml of bead suspension and made up to 50 ml by adding 0.05 M BB. This preparation was incubated overnight at 37^0 C with gentle end to end rotation. The beads were collected on a magnet, resuspended in 8 ml of phosphate buffered saline with 0.05% v/v tween 20 and 0.1% w/v ovalbumin (PBSTO), and washed twice for 10 mins at room temperature with gentle end over end rotation. Washing was repeated, increasing the incubation time to 30 mins and then again with incubation at 4^0 C overnight. Beads were then collected for 2 mins on a magnet and resuspended in 50 ml of PBSTO with 5 µl of 10 % w/v sodium azide and stored at 4^0 C. Beads in 0.5ml – 1ml of this coated bead suspension were collected with the magnet and washed with 1ml of 0.05M borate coating buffer and used to capture *D. nodosus*.

Cultures of *D. nodosus* were used to test the sensitivity and specificity of the immuno-magnetic capture method. 1ml of the 10 fold serial dilutions of the culture cell suspensions at different dilutions were incubated with OMP antibody coated magnetic beads for 1 hr at room temperature. Beads were collected with the magnet and uncaptured cells were discarded. The beads were washed with 1 ml of 0.05 M BCB and were then resuspended in 50 µl of PBS and 4 µl of this bead suspension was taken as a PCR template and PCR performed by the method described below. Similarly multi-serogroup sample capture was tested with a suspension containing a mixture of different serogroups then examined in the multiplex PCR test.

Swabs were collected from the interdigital space and hoof of healthy animals, suspended in 500 µl of PBS and were used to test the application of the immuno-magnetic bead capture method to footrot samples. The suspensions were seeded with 10-fold serial dilutions of pure cultures of *D. nodosus* and the immunomagnetic capture PCR method described above was applied.

6.2.6 PCR amplification

PCR amplifications were performed in 20 µl volumes either in a capillary tubes (Corbett Research, Australia) or in 0.2 ml thin walled PCR tubes (M J Research, Inc. Massachusetts, USA). The PCR mixture contained a final concentration of 20 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$ and 200 µM each of the four nucleotides. The concentration of each of the primers was 0.25 to 0.5 µM. One unit of Taq polymerase (GIBCO – BRL) was added to the reaction mix. Approximately 50 – 100 ng of DNA or 1 - 3 µl of cell lysate was added as template. The amplification cycle in the capillary thermocycler (Corbett Research, Australia) consisted of initial denaturation at 94^0 C for 2 mins, followed by 94^0 C for 5 sec, 60^0 C for 5 sec and 72^0 C for 30 sec for 5 cycles, 94^0 C for 5 sec, 58^0 C for 5 sec and 72^0 C for 30 sec for 25 cycles, and final extension at 72^0 C for 2 min. The amplification cycles in microcentrifuge tubes in a Peltier thermal cycler – PTC 200 (M J Research, Inc. Massachusetts, USA) consisted of 94^0 C for 4 min, followed by 94^0 C for 30 sec, 60^0 C for 30 sec and 72^0 C for 30 sec for 5 cycles, 94^0 C for 30 sec, 58^0 C for 30 sec and 72^0 C for 30 sec for 25 cycles, and final extension at 72^0 C for 4 min. The PCR products were electrophoresed in 0.8% - 3% agarose gels, stained with ethidium bromide and visualised under ultraviolet (UV) illumination.

The amplification conditions for multiplex PCR were similar to that of single PCR described above except for an increased concentration of forward primer (2.5 times) compared to reverse primers.

6.3 Results:

6.3.1 Analytical sensitivity

PCR assays, each using a single serogroup specific reverse primer and the common forward primer had an analytical sensitivity of 1 pg of chromosomal DNA, and 50 to 100 *D. nodosus* cells (Figure 6.1A). The sensitivity was increased on the samples captured with immuno-magnetic beads by at least 10 times, detecting 5 to 10 cells (Figure 6.1B). The results for each serogroup were similar.

Figure 6.1A: Results of the sensitivity test on the lysates of serial dilutions of the *D. nodosus* cells of serogroup A. (M- Puc19 marker, 1-100ng, 2- 10ng, 3- 1ng, 4- 100pg, 5- 10pg, 6- 1pg)

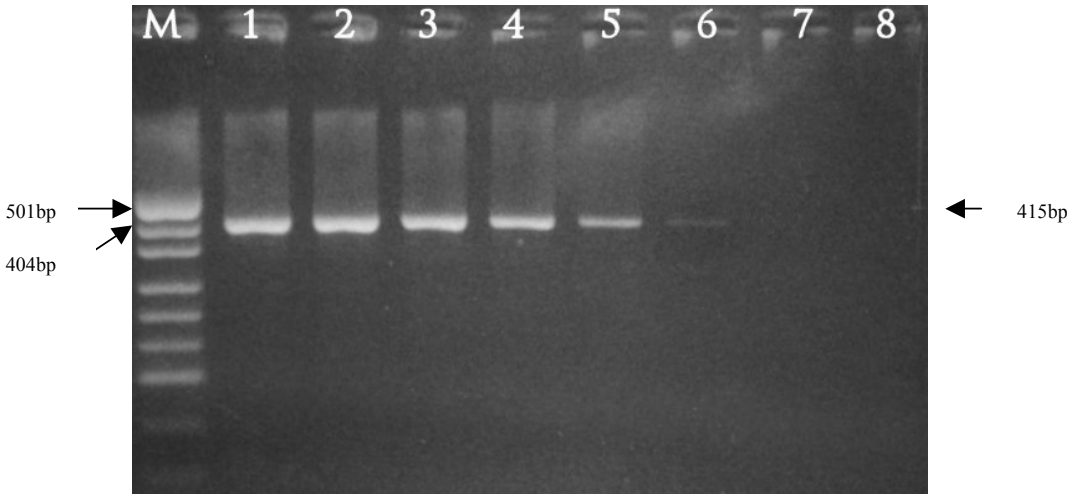
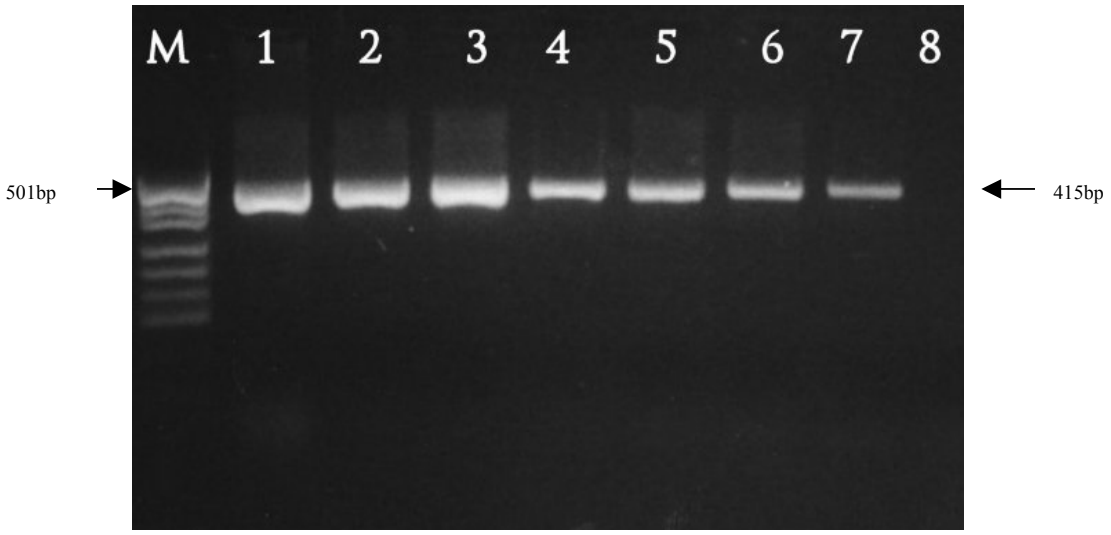


Figure 6.1B: Results of the sensitivity test on the lysates of serial dilutions of *the D. nodosus* cells of serogroup A immuno-captured with magnetic beads. (M- Puc19 marker, 1-100ng, 2- 10ng, 3- 1ng, 4- 100pg, 5- 10pg, 6- 1pg, 7- 0.1pg).



6.3.2 Specificity

The serogroup specificity of PCR assays with each of the individual primer sets within the prototype was tested and assessed by the sizes of the PCR products, which were designed to differ for each serogroup (Table 6.3, Figure 6.2A). Each serogroup specific primer combination was able to amplify all the serotypes within the serogroup eg: serogroup B specific primers were able to amplify all the serotypes (B1, B2, B3 and B4) within this group (Table 6.3). Reactions including the common forward primer and one of the serogroup specific reverse primers on an artificially mixed template of nine serogroups amplified only the appropriate serogroup specific products. Serogroup specificity was further confirmed by a single tube multiplex reaction with the forward primer and all the nine reverse primers on individual serogroup templates (Figure 6.2B) and also on multiple serogroup templates with single serogroup primers (Figure 6.2C) which resulted in amplification only of the appropriate specific products. The specificity of existing published primers (John et al. 1999) was tested and were found to be non specific between serogroups (Table 6.3)**.**

Figure 6.2A: Serogroup specific products. Lane M – Puc19 marker, lanes a - i serogroups A – I in a 3% agarose gel. Individual primers with homologous template.

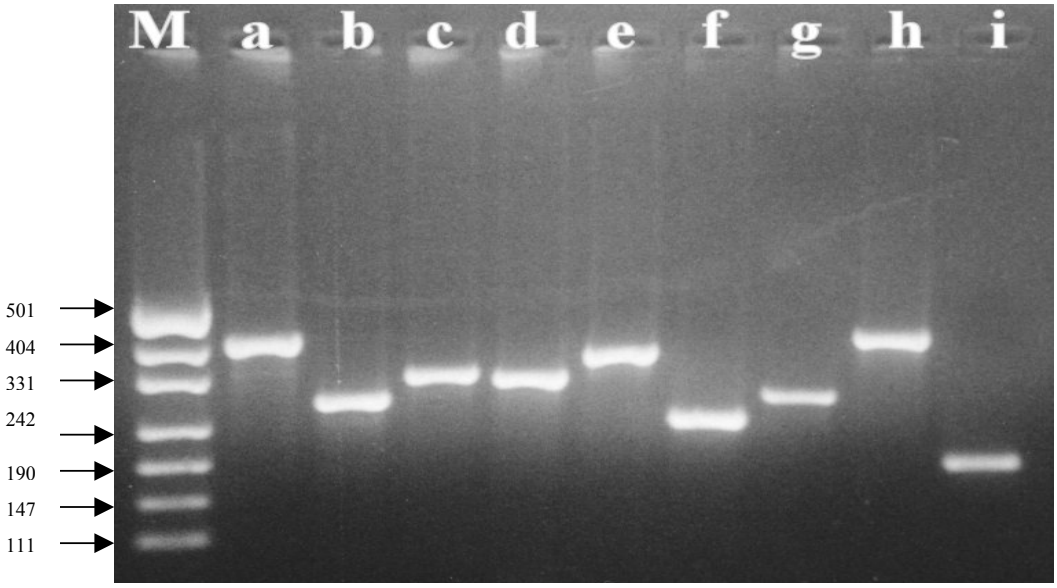


Figure 6.2B: Serogroup specific products (serogroups A – I), multiple serogroup primer mix with single serogroup template.

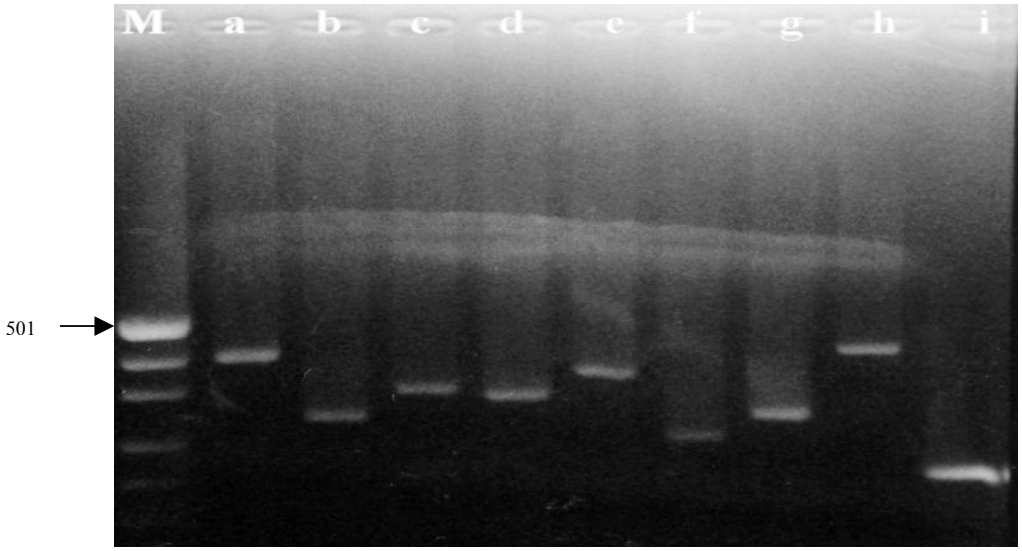


Figure 6.2C: Serogroup specific products (serogroups A – I), single serogroup primers on multiple serogroup template mix.

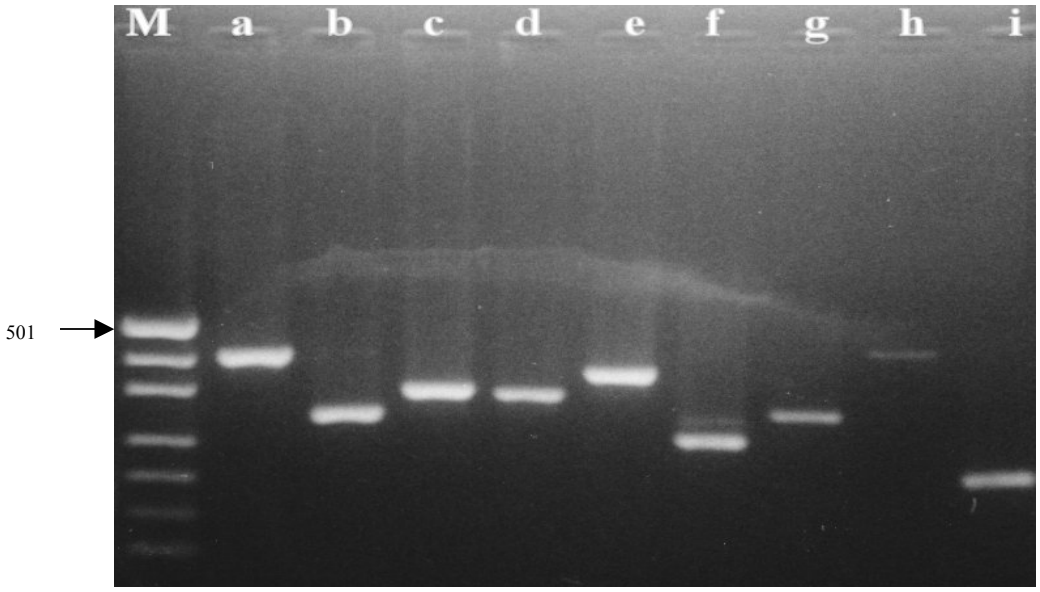


Table 6.3: Results of the serogroup specificity test on all the Australian prototype strains (tested in single primer pair reactions against single template of cells/DNA).

Primer combination	Serogroups/serotypes																	
	A1	A2	B1	B2	B3	B4	C1	C2	D	E1	E2	F1	F2	G1	G2	H1	H2	I
FP + RA	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FP + RB	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
FP + RC	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
FP + RD	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
FP + RE	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
FP + RF	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
FP + RG	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
FP + RH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
FP + RI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
*C1 + A2	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
* C1 + C2	+	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
* C1 + E2	+	-	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-

FP – the common forward primer and the respective (A-I) reverse primers.

*Note: These primers are from the study of John et al., (1999).

None of the 84 other bacterial species tested in PCR with any of the 9 serogroup specific primer sets produced a positive reaction thus confirming the specificity of the assays for *D. nodosus* templates. Of the 3 primer sets of John et al., (1999) for serogroups A, B and C tested with the above 84 bacterial lysates, 2 primer sets (serogroups B and C) were found to give positive results with a number of bacterial species (data not presented).

6.3.3 Multiplex PCR

A multiplex PCR assay that included the common forward primer with all nine reverse primers (RA – RI) on an artificial template mixture of all 9 serogroups in a single tube resulted in amplification of *fimA* from only 6 serogroups. The combination of the common forward primer with groups of 3 reverse primers (eg: ABC, DEF and GHI) led to amplification of each of the relevant serogroups included in each reaction (Figure 6.3). Similarly, the multiplex combination of the common forward primer with groups of 5 or 4 reverse primers (ABCDE & FGHI) was also able to amplify all of the relevant serogroups included in the reactions (Figures 6.4 A and B). To avoid

the limitation of small size differences in the products of some serogroups, the combinations of serogroups ADEGI and BCFH were also tested and were found to work equally well. However, due to size constraints multiplex combinations of groups of 3 primers (ABC, DEF and GHI) may be preferable for identifying PCR products in 3 % agarose gel.

Varying the concentrations of different templates in a mixed template multiplex PCR did affect the sensitivity of the test. If a particular template was present at 100-fold lower concentration than others in a mixture, sensitivity of detection was reduced, and it was not detected when it was present at 1000-fold lower concentration than other components (data not presented).

Figure 6.3A: Amplification of three serogroups in multiplex reactions containing 3 reverse primers (M- DNA Marker followed by products of serogroups A,B,C as singles and ABC in multiplex in 3% agarose gel) .

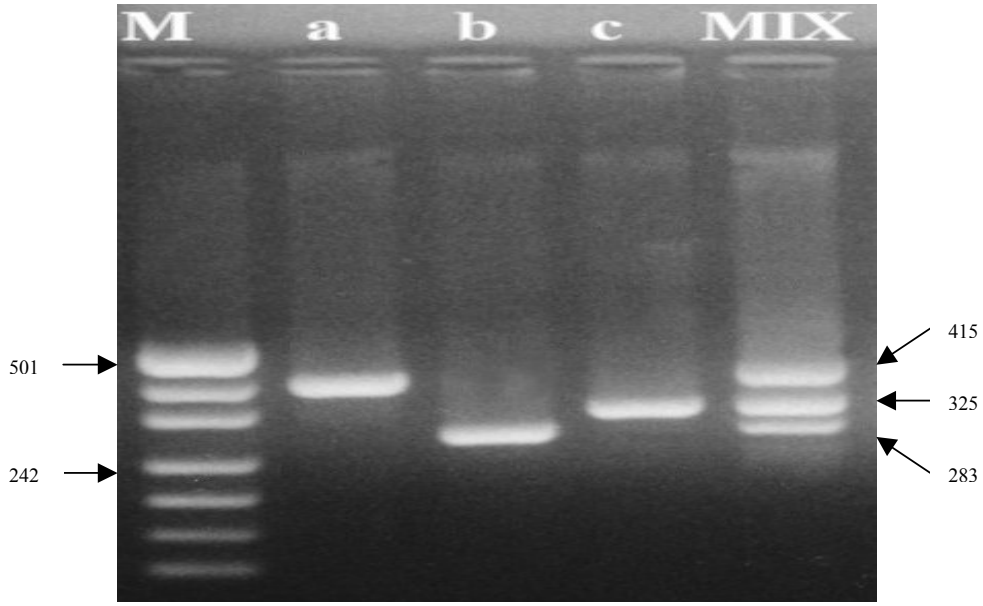


Figure 6.3B: Amplification of three serogroups in multiplex reactions containing 3 reverse primers (M- DNA Marker followed by products of serogroups D, E, F as singles and DEF in multiplex in 3% agarose gel) .

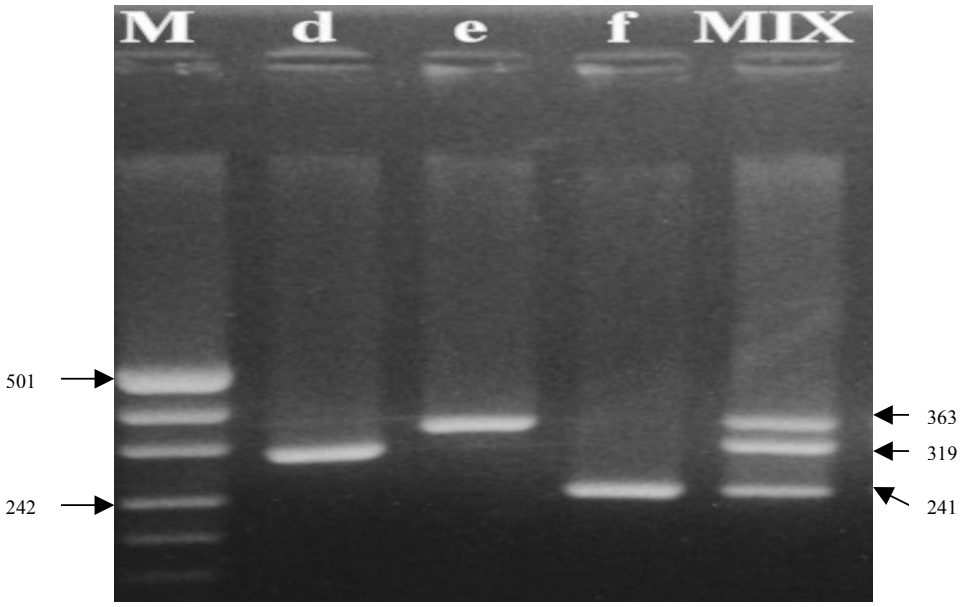


Figure 6.4 A: Amplification of 5 serogroups in group of 5 multiplex reaction (M- DNA Marker followed by products of serogroups A,B,C,D,E as singles and ABCDE in multiplex in 3% agarose gel).

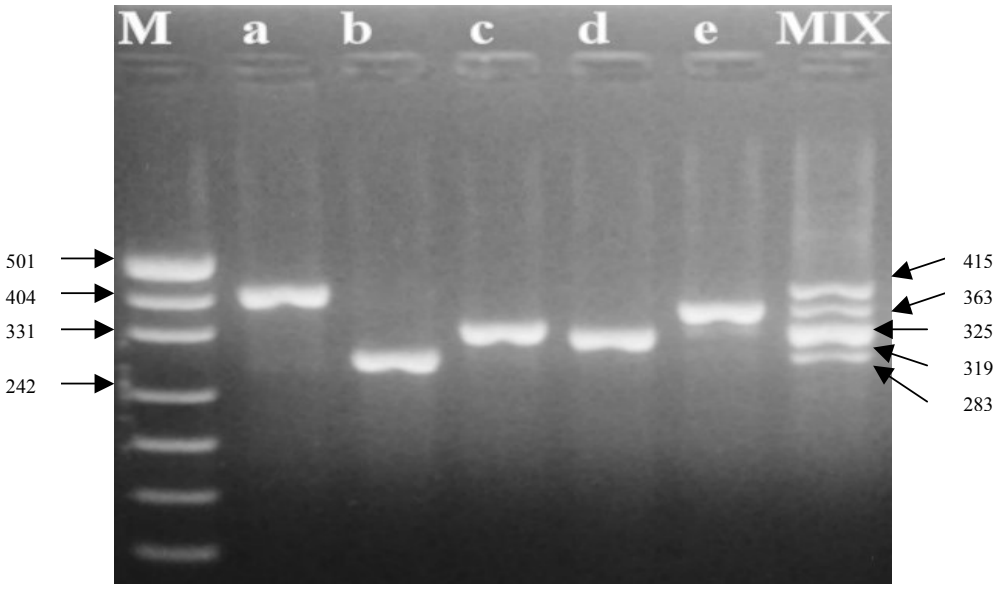


Figure 6.4B: Amplification of 4 serogroups in group of 4 multiplex reaction (M-DNA Marker followed by products of serogroups F,G,H,I as singles and FGHI in multiplex in 3% agarose gel).

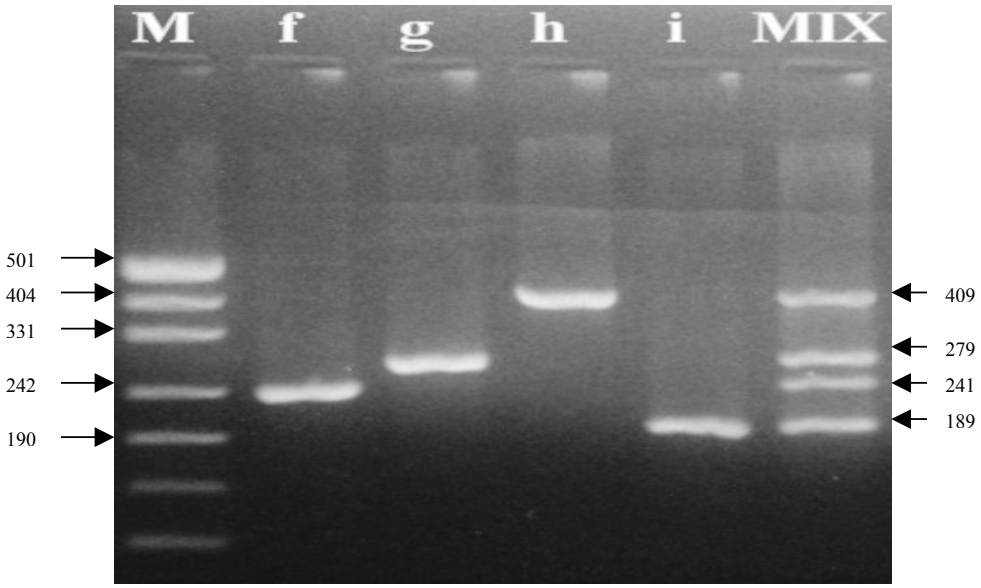
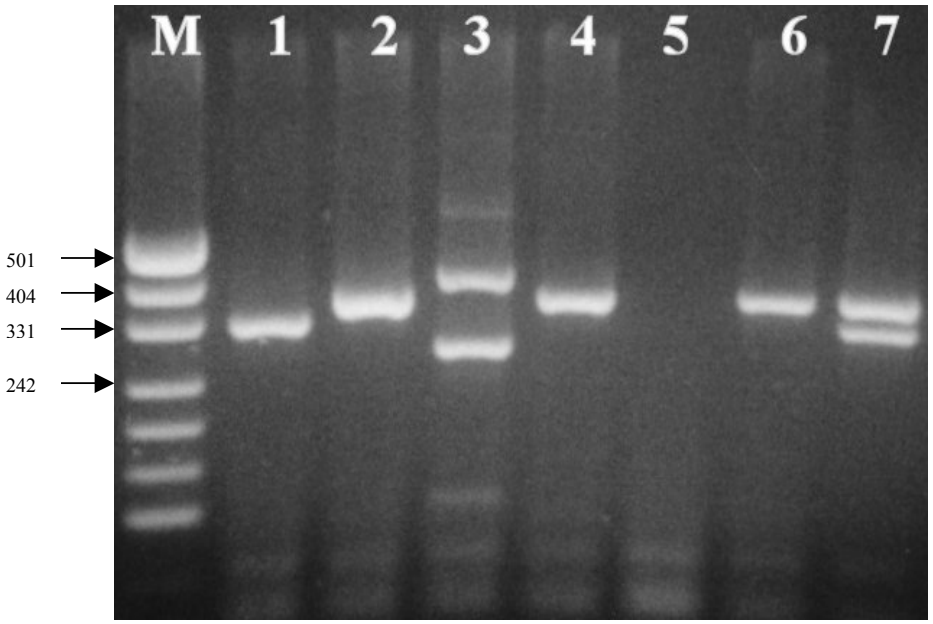


Figure 6.5: Multiplex PCR on lysates of the lyophilised samples in the collection (M-DNA marker, Lanes 1-serogroup G, 2- E, 3- B & H, 4- E, 5- –ve, 6- E and 7- D & E.



One hundred and forty six strains previously serogrouped by agglutination tests, were subjected to the serogroup specific PCR test, both as single reactions and by multiplex reactions in groups of 3 primers (ABC, DEF, GHI). Of these 135 (92.5%) agreed with the initial grouping, 6 (4.1%) were mixture of 2 serogroups, and 5 (3.4%) were different serogroups (Figure 6.5).

The samples which had mixtures of 2 serogroups or were different on PCR test were cultured again on hoof agar and re-tested serologically and by PCR and found to be the same as in first PCR test confirming either the presence of 2 serogroups in the samples and the inaccuracy of the original classification in some cases.

6.4 Discussion

The basis of serogroup variability in *D. nodosus* is localised in a limited region of the carboxy-terminal of the fimbrial gene so the selection of primers from this region was a limiting factor. Despite the attempts to design different serogroup specific primers to amplify sufficiently variable size products to identify on agarose gels, some closely similar size products could not be avoided i.e. Serogroup C and D products vary only by 6 base pairs. However, they can be separated sufficiently to identify them in a 3% agarose gel (Figure 6.2A).

Analytical sensitivity of the serogroup specific PCR on chromosomal DNA was similar to PCR tests in other bacterial species (Kingsford and Raadsma, 1995). Sensitivity of detection from lysates was comparable to but slightly lower than that for *D. nodosus* specific 16S rRNA PCR (La Fontaine et al., 1993). This is probably because the 16S rRNA gene is present in three copies in the genome whereas the fimbrial gene has a single copy. However, the sensitivity of the serogroup specific PCR was greatly increased by immunomagnetic bead capture PCR. This increased the sensitivity by at least 10 fold, detecting 5 to 10 cells in the sample (Figure 1B).

Specificity is equally important for a diagnostic test which is aimed at amplifying only the target organism from the mixtures of bacteria in the footrot lesion. The 84 commonly found bacterial strains or strains related to *D. nodosus* were tested to check the specificity of these *D. nodosus* serogroup specific primers. None of these reacted

in these PCR tests confirming 100% specificity to *D. nodosus*. Such extensive testing of bacterial strains was not reported in contemporary studies (John et al., 1999; Zhou et al., 2001).

These primers were found to be very specific between and within serogroups (Table 3). Specificity of these primers between all 9 serogroups and within the 18 serotypes was the primary objective of this study, so various combinations of primers were tested to get serogroup specific amplifications. In other studies, specificity tests with all serotypes/strains was not undertaken (John et al., 1999; Zhou and Hickford, 2001). Significantly, the primers recommended by John et al. (1999) were found to be cross reactive between serogroups and also not specific for *D. nodosus.* Because immunity is serogroup specific, the ability to identify the serogroups present in a flock accurately is critically important if specific vaccination is being considered.

It is highly likely that more than one serogroup is present in flocks with footrot (Claxton et al., 1983). It is essential therefore to identify all the serogroups present. To overcome the time delay in conducting 9 different PCR amplifications a single tube multiplex PCR was attempted to amplify as many of the serogroups as possible in a reaction. Multiplex reactions with the common forward primer and all of the 9 reverse primers were tried but were able to amplify only 6 of the nine serogroups included in the template presumably due to competition in priming. Multiplex reactions with groups of 3, 4 and 5 reverse primers were successful thus reducing the number of reactions required to 2 (with groups of 4 and 5 primers) or 3 (with groups of 3 primers) to amplify all nine serogroups. Multiplex PCR gives an added advantage to serogroup specific PCR, making it much faster and easier to identify the serogroups in a mixture of multiple templates. For identification of serogroups based on amplicon size using 3% agarose gels, 3 reactions each with 3 primer pairs are recommended (ABC, DEF, and GHI). Using this approach we identified 2 serotypes in each of 6 archival culture isolates and detected earlier serogrouping mistakes in another 5, suggesting that this new technique will be more robust and accurate than culture and agglutination reactions.

Multiplex reactions with different concentrations of the templates were tested and found to influence the amplifications of multiple templates. If a template was 1000

times less concentrated than the others in the mixture it was not amplified. This could be one of the drawbacks of a multiplex reaction whereby in a mixture of different serogroups in a sample only the template with higher concentration may be amplified but the margin for difference is very high.

The immuno-magnetic capture method which has been used in other bacterial capture studies (Widjojoatmodjo et al., 1992; Mason et al., 2000) was tested with *D. nodosus*. On the culture samples it was found to increase the sensitivity of detection by serogroup-specific PCR by 10 – 100 fold. This method also worked on footrot-free swab samples seeded with *D. nodosus*.

This PCR test can only identify at the serogroup level. In some epidemiological investigations it might be desired to identify isolates at the serotype/strain level in which case more elaborate tests like the one developed by Zhou et al., (2001) may be necessary. In the study of Zhou et al., (2001) genotyping of *D. nodosus* was done by PCR amplification and hybridisation of amplicon to immobilised oligonucleotides (PCR/oligotyping) which is more elaborate and expensive than the method described in this study. There is a need to know the *fimA* sequence to design a specific probe for each serotype which may be a drawback to identify the unknown field samples. Also the evaluation of sensitivity and specificity within *D. nodosus* and other bacterial species was not as extensive as described in this study.

A serogroup specific PCR test does not distinguish virulent from the benign strains so a second level test would be required to address this issue. At the present time this test would have to rely on the clinical findings and phenotypic tests like elastase and gelatin gel tests which do not always correlate with the clinical findings. Molecular studies in the area of virulence have identified several virulence associated genetic elements (*vap* and *vrl* regions) in *D. nodosus* genome (Katz et al., 1991; Whittle et al., 1999), and further studies on these may lead to PCR-based tests for identification of virulence.

Serogroup specific PCR is much faster and is more sensitive and accurate than agglutination tests which take 3 to 4 weeks to complete. Multiplex PCR makes it easier to detect different serogroups in a sample with a maximum of 3 PCR tests.

Serogroup specific multiplex PCR will be a useful tool for footrot control based on target vaccination.

TABLE 6.4: Bacterial strains used to test the specificity of the serogroup specific primers (these strains are from the collection of Dr. Richard Whittington, Whittington, 1992).

Sl No.	Culture No.	Organism / strain
1	1	*Yersinia pseudotuberculosis /M492*
2	2	*Dermatophilus congolensis / M272*
3	3	*Staphylococcus aureus / M215*
4	4	*Corynebacterium pseudotuberculosis / M202*
5	5	*Pasteurella multocida / M222*
6	6	*Alcaligenes faecalis / M329*
7	7	*Nocardia asteroides / M385*
8	10	*Pseudomonas aeruginosa / VCS 2412*
9	11	*Yersinia enterocolitica / M92/1587*
10	12	*Moraxella ovis / M515*
11	13	*Clostridium perfringens / M96*
12	15	*Corynebacterium renale / M248*
13	16	*Listeria monocytogenes / M489*
14	17	*Brucella ovis / M426*
15	18	*Histophilus ovis / M175*
16	19	*Staphylococcus aureus / ATCC25923*
17	20	*Shigella sonnei / ATCC11060*
18	21	*Proteus vulgaris / ATCC6380*
19	22	*Streptococcus pyogenes / ATCC10389*
20	23	*Erysipelothrix rhusiopathiae / CM 84/478 685*
21	24	*Staphylococcus epidermis / ATCC14990*
22	25	*Escherichia coli / ATCC14990*
23	26	*Fusobacterium necrophorum / CSIRO*
24	27	*Enterobacter cloacae / ATCC 13047*
25	28	*Pseudomonas aeruginosa / ATCC 9721*
26	29	*Cardiobacterium hominis / NTCC10426 JIR 1048*
27	30	*Suttonella indologenes / ATCC25869 JIR 1040*
28	31	*Bacillus cereus / M841*
29	32	*Salmonella oranienburg / M221*
30	33	*Aeromonas hydrophila / RN 87/81*
31	34	*Histophilus ovis / O 86/1717*
32	36	*Proteus vulgaris / M154*
33	37	*Citrobacter sp. / M 51*
34	38	*Klebsiella pneumoniae/ M 156*
35	39	*Mycobacterium phlei/ CM 1334*
36	40	*Vibrio anguillarum/ 85/3954 – 1*
37	41	*Escherichia coli/ K12 UQK 12F*
38	42	*Haemophilus somnus/ O 207*
39	43	*Haemophilus somnus/ O 84/2319*
40	44	*Actinobacillus seminis/ O 2468/394/2468*
41	45	*Actinobacillus seminis / O 105*

42	46	*Actinobacillus seminis / O 1344/117*
43	47	*Escherichia coli J5 / UQ J5*
44	48	*Haemophilus somnus/ O 107/392*
45	49	*Selenomonas ruminantium / UNE AR53*
46	50	*Bacteroides ruminicola brevis/ UNE AR20*
47	51	*Butyrivibrio fibrisolvens / UNE AR10*
48	52	*Eubacterium ruminantium / UNE AR39*
49	53	*Fibrobacter succinogenes / UNE AR1*
50	54	*Ruminococcus albus / UNE AR67*
51	58	*Fusobacterium sp. / 4705/L*
52	59	*Fusobacterium sp. / 4705/S*
53	60	*Bacteroides sp. / 4713*
54	61	*Fusobacterium sp. / 4449*
55	62	*Fusobacterium sp. / 4365*
56	63	*Fusobacterium necrophorum / 4013*
57	64	*Bacteroides sp./ 4041*
58	65	*Bacteroides tectum / 3302*
59	67	*Unidentified anaerobe / gum A*
60	68	*Unidentified anaerobe / gum B*
61	69	*Unidentified anaerobe / gum C*
62	70	*Bacteroides fragilis / 3902*
63	71	*Bacteroides sp / 4373*
64	72	*Fusobacterium russii / 3327*
65	73	*Porphyromonas sp. / 4849*
66	74	*Bacteroides tectum / 3333*
67	75	*Fusobacterium russii / 3310*
68	76	*Porphyromonas sp. / 4596*
69	77	*Bacteroides tectum /3427*
70	78	*Bacteroides heparinolytica /3324*
71	79	*Veillonella sp. / 4283*
72	80	*Bacteroides fragillis / 3938*
73	81	*Bacteroides sp / 4521*
74	83	*Bacteroides sp / 4169*
75	84	*Bacteroides tectum / 3316*
76	85	*Bacteroides tectum / 156*
77	87	*Bacteroides tectum / 4127*
78	88	*Unidentified anaerobe / 3382*
79	89	*Bacteroides tectum / 3330*
80	90	*Bacteroides sp / 3464*
81	91	*Bacteroides fragillis / 4184*
82	92	*Prevotella zooleoformans / 4010*
83	93	*Bacteroides fragillis / 3411*
84	94	*Bacteroides tectum / 159*

Plain sticks

Figure 6.6 Plate A:

The lesion scoring system used for ovine footrot developed by Egerton and Roberts (1971) and modified by Stewart et al (1982). 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 footrot. Score 2 lesions are restricted to the interdigital space. Score 3 and 4 are characterised by underrunning of the sole of the foot. Score 3 has been subdivided into 3a, 3b and 3c by Stewart et al (1982). Most severe score is also scored as 5 by some workers.

CHAPTER 7

Application of *D. nodosus* serogroup specific PCR diagnosis to clinical samples of footrot

Contents

7.1 Introduction

The serogroup identification of *D. nodosus* is a prerequisite to formulation of a footrot control and eradication program, more so to achieve this by serogroup specific vaccination.

There is no single test for specifically identifying and serogrouping *D. nodosus* from footrot lesion samples. The currently available procedure necessitates that the organism be isolated under strict anaerobic conditions, identified visually in mixed cultures on hoof agar plates, subcultured and enough antigen purified, and subjected to antigenic analysis by subjective tests like slide and microtitre agglutination tests. These procedures generally take at least 4 weeks which becomes a drawback for early intervention in a disease outbreak. Also the cost involved can be quite substantial, mainly because of the time involved.

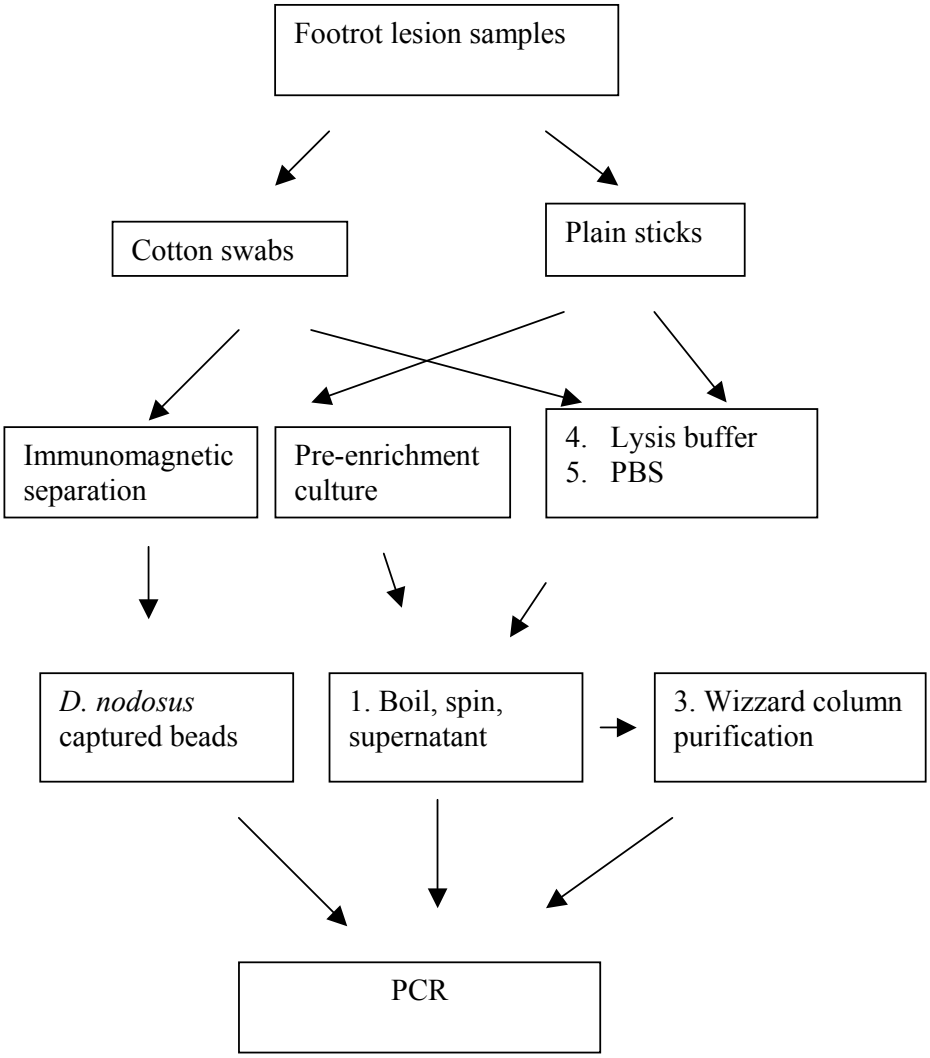
There is need for a method for reliable and rapid typing of *D. nodosus* from footrot lesion samples so that the typing of strains prevalent in a flock can be achieved in a minimum time. In this study we investigated the application of the serogroup specific PCR developed in the preceding study (chapter 6) to clinical samples of footrot by various methods (Fig 7.1) with direct lesion swab suspensions, purification by Promega Wizard columns, pre-enrichment cultures and immunomagnetic capture.

7.2 Materials and methods

7.2.1 Collection of samples from cases of footrot

Exudates were collected from the feet of naturally infected sheep from several farms in south eastern New South Wales. The site sampled was at the apex of the cleft that develops between the horn of the hoof and the sensitive underlying tissues. Samples were collected on cotton swabs and/or sterile plain wooden applicator sticks. The severity scores of the feet from which the samples were collected varied from 2 to 4, scored by the method of Egerton and Roberts (1971). The samples were either processed on return to the laboratory and used for PCR or stored in sterile tubes at –20 ^{0}C until use.

Figure 7.1: Diagrammatic representation of the methods used for processing of footrot samples



7.2.2 Direct lesion suspensions

Suspensions of material present on swabs or sticks were made either in 200 µl of lysis buffer or phosphate buffered saline (PBS) by gently vortexing in 1.5 ml eppendorf tubes. The swab or stick was then removed. For direct PCR these samples were boiled for 5 min, cooled in ice for 10 min and centrifuged at 16,110 *x g* for 1 min, and a 1µl sample of the supernatant was used as template for each PCR reaction.

7.2.3 Purification by Promega Wizard column method

Samples collected as above were also processed to purify DNA using the Promega Wizard® PCR preps DNA purification system (Cat No. A7181). Briefly, fresh samples were collected with sterile swabs and suspended in 600 µl of PBS overnight. After a gentle vortex the swab stick were discarded and the suspensions were incubated at 50 ^{0}C for 30 mins, centrifuged for 30 secs at 16,110 *x g*. The supernatants were mixed with 200 µl of Wizard resin and vortexed gently. These mixtures were passed through the Wizard columns and washed by passing through 2 ml of 80 % isopropanol. After spin drying the columns to remove remaining isopropanol, the DNA bound in the columns was eluted in 50 µl of Tris HCL, pH 8.0. 2 – 5 µl of this purified DNA samples were used as PCR template.

7.2.4 Pre –enrichment culture

Duplicate samples collected from footrot cases were inoculated into 2 ml of trypticase arginine serine (TAS) broth (Skerman, 1975) and incubated anaerobically either overnight or for 4 days at 37 ^{0}C. 1 ml of this culture was centrifuged and the cell pellet was used for preparing cell lysates for PCR tests.

Alternately, swabs were cultured by streaking onto 4 % HA plates and incubated anaerobically for 4 days at 37 ^{0}C. A mixture of *D. nodosus* colonies from these cultures was collected on a sterile loop and suspended in 50 µl of lysis buffer. Cell lysates were prepared as above and 2 µl of these lysates were used for PCR tests.

7.2.5 Immunomagnetic capture

Preparation of antibodies for immuno-magnetic capture and the method for immuno-magnetic capture was done as described in section 6.2.5 of Chapter 6. Samples collected by swabs or sticks from footrot lesions or from cultures were suspended in 500 µl of PBS, vortexed briefly, then coarse material was allowed to settle for 5 mins. 400 µl of supernatant was taken and incubated with the antibody coated beads as described in section 6.2.5 of Chapter 6 for 1 hr. After 1 hr the beads were collected with the magnet and the beads were resuspended in 25 µl of PBS of which 4 µl used as PCR template.

7.2.6 PCR amplification

PCR amplifications were performed in 20 µl volumes in 0.2 ml thin walled PCR tubes (M J Research, Inc. Massachusetts, USA). The PCR mixture contained a final concentration of 20 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$ and 200 µM each of the four nucleotides. The concentration of each of the primers was 0.25 to 0.5 µM. One unit of Taq polymerase (GIBCO – BRL) was added to the reaction mix. 2 - 5 µl of cell lysate or sample prepared as above was added as template. In case of immunocapture method 4 µl of the *D. nodosus* captured bead suspensions was used as template. The amplification cycles in a Peltier thermal cycler – PTC 200 (M J Research, Inc. Massachusetts, USA) consisted of 94^0 C for 4 min, followed by 94^0 C for 30 sec, 60^0 C for 30 sec and 72^0 C for 30 sec for 5 cycles, 94^0 C for 30 sec, 58^0 C for 30 sec and 72^0 C for 30 sec for 25 cycles, and final extension at 72^0 C for 4 min. The PCR products were electrophoresed in 3% agarose gels, stained with ethidium bromide and visualised under ultraviolet (UV) illumination.

7.2.7 Multiplex PCR

As per the results in chapter 6 multiplex PCR with groups of 3 sets of primers was selected to be used for amplification from lesion samples prepared as per the above described procedures. The amplification conditions for multiplex PCR were similar to those of single PCR described above except for an increased concentration of forward primer (2.5 times) compared to reverse primers.

7.3 Results

7.3.1 PCR on direct lesion samples

A total of 27 samples collected from footrot lesions both by plain sticks and cotton swabs were tested by serogroup specific multiplex PCR in groups of 3 primers. Only 2 (7.4%) of the samples collected by plain sticks were positive (both serogroup A) while none of the samples from cotton swabs were positive.

7.3.2 PCR on Wizard column purified samples

When 2 µl of the samples purified by Wizard column method were used as templates for multiplex PCR (groups of 3) none of the 8 samples were positive. When the same templates were increased to 10 µl, 2 (25%) were positive (Table 7.1). Of the 8 samples collected by plain sticks and purified by the columns, in which 5 µl of the samples were used as templates, 5 (62.5 %) were positive on multiplex PCR (Table 7.1).

7.3.3 PCR after enrichment culture

A total of 18 swab samples cultured in TAS broth were tested with multiplex PCR after overnight and 4 days anaerobic incubation at 37^0 C. All these samples were negative in multiplex PCR in groups of 3 primers.

Lysates of mixed colonies from 4 day old lesion cultures (10 plates) on 4 % hoof agar were tested by multiplex PCR in groups of 3 primer sets. All 10 samples were positive (Table 7.1). These colonies were subcultuerd and pure cultures were tested by agglutination test. The serogroup results of slide agglutination test matched those of serogroup specific PCR.

7.3.4 Immuno-magnetic capture method

A total of 44 samples (collected by swabs (12) and sticks (32)) from clinical cases of footrot were tested by immuno-capture method by serogroup specific multiplex PCR in groups of three. Of these only 4 (9%) samples were positive (Table 7.1).

Table 7.1 Comparative results of multiplex PCR with footrot samples processed by different methods

Method		No. of samples (footrot lesions)	% positive on PCR	% positive on culture
1. Direct swabs	a. Cotton swabs	18 (18)	Nil	100
	b. Plain Stick	9 (9)	7.4	100
2. Column Purification		8 (8)	62.5	100
3. Immuno-magnetic capture		44 (44)	9	90.9
4.Enrichment culture	a. TAS broth	18 (18)	Nil	100
	b. 4% HA	10 (10)	100	100

7.4 Discussion

One of the aims of this study was to do sergrouping by PCR of *D. nodosus* directly from lesion samples to make it a rapid diagnostic test for field samples. This was tried with duplicate samples collected both by swabs and plain sticks. The sensitivity of the test on lesion samples was found to be very low. To try and improve the sensitivity an overnight and 4 day old pre-enrichment culture in broth was tested but was found to be no better than direct PCR.

An immuno-magnetic capture method which has been used in other bacterial capture studies (Widjojoatmodjo et al., 1992; Mason et al., 2000) was tested with *D. nodosus*. On the culture samples it was found to increase the sensitivity of detection by serogroup specific PCR by 10 – 100 fold. This method also worked on footrot free

swab samples seeded with *D. nodosus* but sensitivity was too low with footrot lesion samples.

PCR on direct footrot samples was tested in an extensive trial study with 16S rRNA primers for the detection of *D. nodosus* (Egerton and Rood, 1996). That study with lesion samples demonstrated the high specificity of the test but it was not sensitive enough for practical application. As indicated in this study there may be PCR inhibiting factors in the footrot lesion samples which are mostly contaminated with faecal materials. Large amounts of non-target DNA is likely to make PCR less sensitive in samples with less abundant target DNA making it difficult for specific primers to find the target for annealing (Echeverria et al, 1989; Olive, 1989). Miller et al., (1995) have shown that the ability of the DNA polymerase enzyme to amplify specific fragments from target DNA is affected by the proteins and other inhibitory substances present in faecal samples. PCR assay can also be inhibited by haemoglobin breakdown products such as bilirubin and bile salts present in faeces. These could be some of the reasons for less sensitivity of the PCR tests on faecal contaminated samples like footrot lesion swabs.

However, this drawback can be overcome by picking up colonies from 4 days old lesion cultures on hoof agar plates for serogroup specific/multiplex PCR. As indicated in the results this method was found to be very sensitive and can reduce the time taken for serogrouping on slide agglutination test by three weeks. If the colonies are too small/ too few on the first cultures these can be sub-cultured into quarter of 4 % HA plates and then used for the PCR test. Even if subculturing is required the time taken for typing is reduced by a minimum of 2 weeks. The other advantage is that individual colonies do not need to be isolated. A PCR test can be done on pooled colonies just as well and can be used to identify all the serogroups prevalent in the sample (data not presented). The issue of cross-reaction seen in agglutination tests of some samples can be overcome in the serogrouping by PCR tests.

The immuno-magnetic capture method was not used on samples off 4% HA lesion cultures, but it could be tried in future which might increase the chance of detecting rare or low abundant serogroups present in the samples.

Serogroup specific PCR is much faster and is more sensitive and accurate than slide agglutination tests which take 3 to 4 weeks to complete. Multiplex PCR makes it easier to detect different serogroups in a sample with a maximum of 3 PCR tests. Serogroup specific multiplex PCR will be a useful tool for footrot control based on specific vaccination. The difficulty in using the test on direct lesion swabs needs to be further investigated. There may be future advances in the application of PCR tests to clinical samples.

Chapter 8

General discussion and conclusions

The level of humoral response to infection with *D. nodosus* is correlated with the severity and duration of the lesions which develop (Whittington and Nicholls, 1995). These antibodies do not last for long after the recovery from the infection. However, immunological memory responses in sheep recovered from footrot can be aroused by natural recurrent infection or by subcutaneous injection of OMP or pilus antigens of *D. nodosus* (Whittington and Marshall, 1990; Whittington and Nicholls 1995b; Whittington 1996; Dhungyel et al., 2001). These antibodies can be detected with ELISA tests. The magnitude of this anamnestic immunological response is an indicator of the primary immune response of the animal, and is directly correlated with the highest antibody response attained during infection. The memory response to OMP antigen is not very specific (Whittington, 1996) but it is specific for pilus antigen and this anamnestic test can be used for retrospective diagnosis of virulent footrot (Dhungyel et al., 2001). Serogroup specificity is seen to be a deficiency of using pilus for anamnestic test. The use of a single combined multivalent pilus antigen in the anamnestic test was an answer to this problem.

The type IV fimbriae of *D. nodosus* are recognised as a virulence factor (Claxton 1989, Kennan et al., 2001), provide the basis for classification of *D. nodosus*, and are highly immunogenic (Claxton 1989; Egerton 1974). Various studies have associated the severity of infection with the degree of fimbriation of the strain causing the infection. On the other hand some of the highly fimbriated strains have been associated with less severe forms of the disease. Development of a genetic transformation system for *D. nodosus* has opened avenues to study the role of fimbrial genes in virulence of the organism (Kennan et al., 2001). Fimbriae are the major immuno-protective antigens although the protection is serogroup specific (Egerton 1974; Every & Skerman, 1982; Elleman & Stewart, 1988). Serogroup specificity and antigenic competition have been the major technical limitations in the use of multivalent fimbrial vaccines. Monovalent and bivalent serogroup specific vaccines have been used successfully in the control and eradication of virulent footrot (Ghimire, 1997, Egerton et al. 2002, in press).

The sequence variation in the fimbrial subunit protein is the basis of serological diversity seen in *D. nodosus* (Elleman, 1988; Mattick et al., 1991; John et al., 1999; Zhou & Hickford, 2001). In the Australian classification system fimbriae are the basis for the classification of *D. nodosus* into nine major serogroups, A – I. There are 18 serotypes exist within these serogroups (Claxton, 1989). Studies of Cox (1992), John et al, (1999) and Zhou et al. (2001) have shown the possibilities of developing a rapid fimbrial PCR diagnostic test. Such a rapid test would be suited to identify the serogroups present and formulate effective specific vaccination programs against virulent footrot.

The results of the study presented in this thesis on the use of *D. nodosus* antigens reinforced and extended earlier findings that a retrospective diagnosis of footrot can be made by using OMP antigen for an anamnestic test and confirmed that a similar test using pilus antigens can be made more effectively. The pilus antigen was found to be more specific than OMP antigen irrespective of the age of the animals. A pilus based anamnestic test was used as a supplementary test to confirm the eradication of the virulent strains of *D. nodosus* from the flocks of Western Nepal in which footrot was endemic. However, serogroup specificity was thought to be another aspect of this test that needed to be studied.

The pilus antigen anamnestic test can be used as a valuable complementary retrospective diagnostic tool for the diagnosis of virulent footrot or to check the clinical history of the disease. This would be more appropriate in inaccessible flocks like the migratory flocks of Nepal. Other advantages of this test are that only a very low dose (10μg) of the pilus is required to elicite a response, no adjuvants are required and there are no observable reactions at the site of injection.

The possibility of using a combined serogroup (multivalent) pilus anamnestic test was therefore investigated in a study on animals from a pre- existing trial in the New England area of New South Wales. The results of this study indicated that multivalent serogroup pilus antigens can be effectively used to elicit antibody responses in the anamnestic ELISA test. This test was usually positive where there was a record of an underrunning infection (scores ≥ 3) but there was no agreement between the results of the ELISA tests and the original clinical diagnosis in the flocks from which the test sheep came. The antigenic competition seen with the use of multivalent pilus vaccines (Schwartzkoff et al. 1993, Raadsma et al. 1994, Hunt et al. 1994, 1995) does not seem to be a problem with the multivalent pilus anamnestic test.

However, the sensitivity and specificity of this test needs to be further evaluated in flocks with known clinical backgrounds. Sheep in the Cicerone trial were probably not representative of their flocks of origin, i.e. were the worst affected animals. Appropriate cut off ELISA ratios and prevalences of reactors consistent with various categories of footrot (BFR, IFR and VFR) are yet to be defined.

If the anamnestic ELISA test were authenticated it could also be used as an indirect test for checking the presence of different serogroups of *D. nodosus* in a flock. It would also be valuable in certifying sheep offered for sale, export or import.

The role of fimbrial gene (*fimA*) of *D. nodosus* in the pathogenesis of footrot was studied by detailed characterisation of the mutant strains developed at Monash University (Kennan et al., 2001) and testing these strains in sheep in a pen challenge experiment. These mutant strains did not produce any fimbrial proteins and also had altered ability to secrete extracellular proteases. Unlike the wild strains the mutant strains tested were not able to colonise the interdigital skin and cause footrot in sheep indicating the important role of fimbriae in colonisation and penetration of the bacteria into the interdigital skin.

The study on the role of fimbrial gene (*fimA*) in the establishment of footrot was the first of its kind and has shown direct correlation with the ability to secrete extracellular proteases. However, the possibility of the involvement of unknown extracellular proteases as determinants in colonisation or penetration of the interdigital skin cannot be ruled out. This study has opened new avenues for the study of the pathogenesis of footrot at the molecular level.

The role of proteases in the pathogenesis of footrot needs to be studied by creating mutants in each of the respective protease genes. However, studying the direct effects of fimbrial genes on extracellular proteases will be much more complex. This study on the mutant strain also has shown an evidence of transformation mediated serogroup conversion which, could lead to a whole new area of *D nodosus* research. The role of different protease genes in the pathogenesis of footrot is a logical sequence to my work on the fimbrial gene. This is expected to lead to a better understanding of the role of these genes. This is likely to be become an important issue as more and more episodes of non-virulent footrot are seen as the prevalence of VFR declines. This issue was exemplified by the Cicerone trial work described in Chapter 4.

Based on the limited sequence variability in the 3' region of the fimbrial gene of the prototype strains of *D. nodosus*, serogroup specific PCRs were developed and tested by various protocols as discussed in chapter 6. The analytical sensitivity of the serogroup specific PCR on chromosomal DNA was comparable with PCR test in other bacterial species. To increase the sensitivity an immuno-magnetic bead method was tested on pure cultures and seeded collection swabs. Sensitivity was greatly increased (at least 10 fold) by the immuno-magnetic bead capture method. The primers developed in the study were found to be very specific within and between the 9 serogroups. None of the 84 representative bacterial strains tested reacted to these primers confirming 100% specificity to *D. nodosus*. Multiplex PCR with the common forward primer and groups of 3, 4 and 5 reverse primers was developed to reduce the number of reactions required to 2 (with groups of 4 and 5 primers) or 3 (with groups of 3 primers) to amplify all the nine serogroups.

One of the main aims of developing a *D. nodosus* serogroup specific PCR was to apply the test to footrot samples and to develop a rapid diagnostic test. As discussed in chapter 7 various methods were tested to check the field application of this test. The sensitivity of the test on clinical samples was very low irrespective of the method used for processing. This low sensitivity could be due to the presence of known PCR inhibiting contaminants such as the large amount of non target-DNA, proteins and bile salts present in the footrot lesion samples contaminated with faeces. However, this drawback was overcome by performing serogroup/multiplex PCR on colonies from 4 days old lesion cultures on hoof agar plates. Unlike the conventional method, individual colonies did not need to be purified thus reducing the time taken for serotyping by a minimum of 2 weeks. A PCR test can be done on pooled colonies to identify all serogroups prevalent in the sample. The difficulty of applying this test on direct footrot swabs may be overcome by future advances in the application of PCR tests to clinical samples in general.

The serogroup-specific PCR developed is much faster and is more sensitive than the conventional antibody-based serogrouping method. Development of multiplex PCR makes it easier to detect all 9 serogroups if present in a sample with a maximum of 3 PCR tests. This could be useful diagnostic tool for footrot eradication based on target vaccination. Specific/ target vaccination has been shown to be a very useful method of eradication of virulent footrot from Nepal and Bhutan, where only 2 and one virulent strains, respectively, were present

(Ghimire et al., 1996). Such a situation is very unlikely in Australian footrot infections but sequential targetting could be a way to deal with this and it opens new ways of eradication in isolated populations.

Some of the drawbacks of this test are that it can identify isolates at the serogroup level only and may not be ideal for some epidemiological investigations where subtyping could be useful, and also it does not distinguish virulent from benign strains. A second level test would be required. At present virulence assessment generally has to rely on clinical findings of the disease and protease tests. PCR tests to identify virulence characteristics are being developed (Cheetham et al., 2001) and these could become available in future as supplementary tests.

Research to define the virulence factors of *D. nodosus* and the means to detect these is likely to continue in Australia. This must be accompanied by field research to develop better understanding of biological behaviour of strains of low to intermediate virulence. While eradication of virulent footrot can be achieved using accurate clinical and laboratory diagnosis far less is known about the other forms of the disease.

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