Aspects of the aetiopathogenesis and diagnosis of ovine footrot

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Declaration of Authorship

Apart from the assistance stated in the acknowledgements section, this thesis represents the original work of the author. The results of this study have not been presented for any other degree or diploma at any other university.

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BAnVetBioSci (Hons I)

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Summary

Ovine footrot is prevalent in most countries where sheep are reared. The essential causative agent, *Dichelobacter nodosus* (formerly *Bacteroides nodosus*, *Fusiformis nodosus*), is a fastidious, strictly anaerobic bacterium, and an obligate parasite of the ruminant hoof. The clinical disease is the result of a complex interplay between pathogen, environment and host. The severity of these clinical manifestations vary, from a mild interdigital dermatitis through to complete separation of the horny epidermis from the underlying dermal tissues. For descriptive purposes, two clinical forms of the disease are recognised: virulent footrot and benign footrot. The virulent form causes severe lameness, resulting in substantial, ongoing economic losses due to lost productivity, and the cost of controlling the disease. In Australia, virulent footrot is estimated to cost the sheep meat and wool industries approximately $35 million per annum. Consequently, in Australia sheep flocks diagnosed with virulent but not benign footrot may be quarantined and required to undergo a compulsory elimination program, with costs met by the farmer.

Laboratory virulence tests are used to assist diagnosis because clinical differentiation of virulent and benign footrot can be challenging during the early stages of disease or when the disease is not fully expressed due to unfavourable pasture conditions. The most recent published genotypic virulence tests, which were developed in Europe, are based on research conducted in Australia which indicated that the expression of acidic protease isoenzyme 2 (AprV2, encoded by the gene *aprV2*) is the defining phenotypic characteristic of virulent strains of *D. nodosus*. Benign strains express the analogous protease AprB2 (encoded by the *aprB2* allele). Two qPCR tests were developed that target the *aprV2/B2* alleles, both of which were reported to be highly specific. However, validation data were limited and the tests were not validated for use outside of Europe. The aim of the study presented in Chapter 3 was to subject these tests to the validation pathway proposed by the World Organization for Animal Health (OIE), and assess their suitability for use in an Australian context. Using samples collected from foot lesions from 960 sheep from 40 flocks in four different geographic regions of south-eastern Australia, the analytical characteristics of the qPCR tests were evaluated, and one of the tests was compared to phenotypic protease tests (elastase test, gelatin gel test) at the flock- and isolate-levels. There was a low level of agreement between clinical diagnosis and qPCR test outcomes at both the flock-

and sample-levels, and poor agreement between qPCR test outcomes and the results of phenotypic virulence tests, and the diagnostic specificity of the qPCR test was low at both the flock- and individual swab-levels (31.3% and 18.8%, respectively). In contrast, agreement between the elastase test and clinical diagnosis was high at both the flock-level (DSe = 100%, DSp = 78.6%) and isolate-level (DSe =69.5%, DSp = 80.5%), which indicated that the expression of AprV2 is a good indicator of virulence. However, it was apparent that *aprV2*-positive *D. nodosus* strains with benign phenotypes are common in Australian sheep flocks.

Footrot can be controlled with vaccine targeting the fimbriae of virulent strains of *D. nodosus*. However, the bacterium is immunologically heterogeneous, and ten distinct fimbrial serogroups have been identified. Thus in each outbreak the infecting strains must be cultured and serogrouped so that the appropriate serogroup-specific mono- or bi-valent vaccine can be administered, because multivalent vaccines lack efficacy due to antigenic competition. If clinical disease expression is suspected to be incomplete, culture-based virulence tests are also required to confirm the diagnosis, because control of benign footrot is economically unjustifiable. Both diagnosis and vaccination are conducted at flock-level. The aims of the study presented in Chapter 4 were to develop a PCR-based procedure for detecting and serogrouping *D. nodosus* directly from foot swabs, and to determine whether this could be done accurately from the same swab that is cultured. A total of 269 swabs from the active margins of foot lesions of 261 sheep in 12 Merino sheep flocks in south-eastern Australia were evaluated. DNA extracts from putative pure cultures of *D. nodosus* and directly from swabs were evaluated in PCR assays for the 16S rRNA and *fimA* genes of *D. nodosus*. Pure cultures were tested also by the slide agglutination test. Direct PCR from swabs was more sensitive than culture for detecting and serogrouping *D. nodosus*. Using the most sensitive sample collection method of swabs in lysis buffer, *D. nodosus* was more likely to be detected by PCR in active than inactive lesions, and in lesions with low levels of faecal contamination, but lesion score was not a significant factor. PCR conducted on extracts from swabs in Stuart's transport medium that had already been used to inoculate culture plates had lower sensitivity.

Despite the recognition of *D. nodosus* serogroup M in Australia, New Zealand, Nepal, Norway, and the United Kingdom, a serogroup M-specific PCR test has not been published. The aim of the study presented in Chapter 5 was to develop a serogroup M-specific PCR test to accompany

existing multiplex conventional PCRs for the detection of serogroups A to I. A serogroup M PCR test was developed with a sensitivity of 250 *D. nodosus* cells. The test was compared with the slide agglutination test, which was previously the only means of detecting serogroup M, and was shown to be more specific.

The Merino sheep is uniquely susceptible to footrot, but the basis of this susceptibility is contentious. The interdigital skin of foot of the Merino appears to be more susceptible to bacterial invasion than that of British sheep breeds; as such, it may be colonised by a greater number or diversity of opportunistic pathogens following environmental predisposition. In the study presented in Chapter 6, next-generation sequencing and analysis of the bacterial 16S rRNA gene was used to characterise the bacterial communities on the feet of a group of healthy Merino sheep, and two groups of Merino sheep with footrot. The results indicated a qualitative shift in the bacterial community of the Merino foot is triggered by infection with *D. nodosus*. The communities of healthy Merino feet were dominated by Gram-positive, aerobic genera such as *Corynebacterium* and *Staphylococcus*. In contrast, the communities of footrot-affected feet were dominated by Gram-negative, anaerobic genera such as *Porphyromonas* and *Fusobacterium*. In total, 15 bacterial genera were preferentially abundant on the feet of Merino sheep with footrot, only four of which were previously reported to be abundant on the feet of British breed sheep with footrot in the U.K. There was no significant difference in the composition of the bacterial communities in footrot lesions, irrespective of score, which suggests that the same bacterial genera may be of importance to both the early and latter stages of the disease process.

Experimental models are used extensively in the study of ovine footrot. Indoor, pen-based models are typically favoured as they enable investigators to manipulate environmental conditions. Bacterial challenge methods often involve bandaging of the foot to enhance transmission of *D. nodosus*. It is apparent that these models do not accurately represent the environment in which footrot is naturally expressed, nor the manner in which *D. nodosus* in naturally transmitted. The aim of the study presented in Chapter 7 was to develop a pasture-based experimental model incorporating a low-intervention challenge method. Three alternative challenge methods were shown to be effective in a pasture-based system: application of the inoculum to the interdigital skin with a cotton swab or transfer pipette, or direct application of a liquid suspension of the *D. nodosus* to the pasture. The experimental model presented in this

study reproduces the environmental conditions in which footrot is naturally transmitted, and the manner in which *D. nodosus* is naturally transmitted between sheep in an infected flock.

List of publications, conference proceedings, and poster presentations

Refereed first-author publications (included in this thesis):

1. **McPherson, A. S.**, Dhungyel, O. P., Whittington, R. J. 2017, 'Evaluation of genotypic and phenotypic protease virulence tests for *Dichelobacter nodosus* infection in sheep', *Journal of Clinical Microbiology*, vol. 55, no. 5, p. 1313-1326.

   This publication constitutes Chapter 3 of this thesis. I am the first author of this paper. I co-designed the study with the co-authors, performed the experiments, collected and analysed the data, and wrote drafts of the manuscript. Professor R.J. Whittington is the corresponding author.

2. **McPherson, A. S.**, Dhungyel, O. P., Whittington, R. J. 2018, 'Detection and serogrouping of *Dichelobacter nodosus* infection using direct PCR from lesion swabs to support outbreak-specific vaccination for virulent footrot in sheep', *Journal of Clinical Microbiology* (accepted manuscript), doi: 10.1128/JCM.01730-17.

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Authorship confirmation

Student: Further to the statements provided above, in cases where I am not the corresponding author of a refereed publication, permission to include the published material has been granted by the corresponding author.

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28.02.2018

Supervisor: As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Professor Richard J. Whittington

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McPherson, A. S., Dhungyel, O. P., Whittington, R. J. 2017, 'Laboratory diagnosis of ovine footrot – an update', Holbrook Landcare Network Healthy Hooves workshop, August 23, 2016, Albury.

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Abbreviations

A	adenine
AFS	average foot score
AHO	animal health officer
AprB2	acidic protease isoenzyme B2
aprB2	acidic protease isoenzyme B2 allele
AprB5	acidic protease isoenzyme B5
aprB5	acidic protease isoenzyme B5 allele
AprV2	acidic protease isoenzyme V2
aprV2	acidic protease isoenzyme V2 allele
AprV5	acidic protease isoenzyme V5
aprV5	acidic protease isoenzyme V5 allele
A. pyogenes	*Arcanobacterium pyogenes*
AUD	Australian dollars
BDD	bovine digital dermatitis
BLAST	basic local alignment search tool
BOM	Australian Bureau of Meteorology
bp	base pair
BprB	basic protease isoenzyme B
bprB	basic protease B isoenzyme allele
BprV	basic protease isoenzyme V
bprV	basic protease isoenzyme V allele
BSA	bovine serum albumin
B. ureolyticus	*Bacteroides ureolyticus*
C	cytosine
cfu	colony forming units
CI	confidence interval
C. minuttisimum	*Corynebacterium minutissimum*
CODD	contagious ovine digital dermatitis
cPCR	conventional polymerase chain reaction
C. pyogenes	*Corynebacterium pyogenes*

Ct	threshold cycle
CV	coefficient of variation
DNA	deoxyribonucleic acid
D. nodosus	*Dichelobacter nodosus*
dNTP	deoxynucleotide triphosphate
dsDNA	double-stranded DNA
DSe	diagnostic sensitivity
DSp	diagnostic specificity
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin unit
F	forward primer
FAM	6-carboxyfluorescein
F. equinum	*Fusobacterium equinum*
F. necrophorum	*Fusobacterium necrophorum*
FISH	fluorescent *in situ* hybridisation
g	grams
g (italicised)	g-force
G	guanine
GLMM	generalized linear mixed model
HA	hoof agar
ID	interdigital dermatitis
intA	integrase A gene
L	litres
LB	lysis buffer
LDA	linear discriminant analysis
LEfSe	linear discriminant analysis effect size
LF	left forefoot
LH	left hind foot
LOD	limit of detection

m	metres
M	molar
MFS	maximum foot score
mg	milligram
$MgSO_4$	magnesium sulphate
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
mins	minutes
mL	millilitres
mm	millimetre
mM	millimolar
MQW	Milli-Q water
mSTM	modified Stuart's transport medium
NA	not applicable
NaOH	sodium hydroxide
ng	nanograms
NGS	next generation sequencing
nM	nanomoles
No.	number
NSW	New South Wales
NUF	number of underrun feet
N.Z.	New Zealand
OID	ovine interdigital dermatitis
OIE	Office International des Epizooties (World Organization for Animal Health)
OTU	operational taxonomic unit
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
PCoA	principle coordinate analysis
PCR	polymerase chain reaction
PCR-SSCP	polymerase chain reaction single strand

	conformational polymorphism
P. disiens	*Prevotella disiens*
PEPY	per ewe per year
pg	picograms
P. gingivalis	*Porphyromonas gingivalis*
P. levii	*Porphyromonas levii*
pnpA	polynucleotide phosphorylase gene
P. zoogleoformans	*Pseudomonas zoogleoformans*
QIIME	quantitative insights into microbial ecology
qPCR	quantitative real-time polymerase chain reaction
R	reverse primer
REML	restricted maximum likelihood
RF	right forefoot
RH	right hind foot
ROX	6-carboxy-X-rhodamine
rpoD	RNA polymerase sigma-70 factor gene
rRNA	ribosomal ribonucleic acid
s	seconds
SA	South Australia
S. penortha	*Spirochaeta penortha*
SNP	single nucleotide polymorphism
spp.	species
T	thymine
Tas.	Tasmania
TAS	trypticase arginine serine
TBE	Tris boric acid EDTA
TFS	total foot score
T. vincentii	*Treponema vincentii*
TWFS	total weighted foot score
U	units

U.K.	United Kingdom
UNG	uracil N-glycosylase
U.S.A.	United States of America
vap	virulence-associated protein
vrl	virulence-associated locus
WA	Western Australia
w/v	weight/volume
X^2	chi-squared
μL	microliter
μM	micrometre
°C	degrees centigrade

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Chapter 1

Literature review

1.1 Introduction

Footrot is a transmissable, mixed bacterial disease of the feet of cloven-hoofed animals (Beveridge, 1941). The disease initially presents as a mild to severe dermatitis of the interdigital skin, and may progress to separation of the sole and horny epidermis from the underyling dermal tissues (referred to as "underrunning") (Beveridge, 1941). The essential causative agent, *Dichelobacter nodosus* (previously referred to as organism K, *Fusiformis nodosus*, *Bacteroides nodosus* (Beveridge, 1938b, 1941; Dewhirst et al., 1990; Mraz, 1963), is an obligate parasite of the ruminant hoof (Beveridge, 1941).

Footrot is primarily of concern to the sheep meat and wool industries, but *D. nodosus* infection has been reported in goats (Egerton, 1989b; Egerton et al., 2002; Ghimire et al., 1999; Piriz Duran et al., 1990b; Piriz et al., 1991; Wani et al., 2015; Zhou and Hickford, 2000a; Zhou et al., 2009b), cattle (Egerton, 1989b; Egerton and Laing, 1978; Egerton and Parsonson, 1966; Laing and Egerton, 1978; Stewart, 1979; Thorley et al., 1977), deer (Egerton, 1989b; Skerman, 1983), and pigs (Piriz et al., 1996). *D. nodosus* infection has also been reported in wild ungulates including the Alpine Ibex (*Capra ibex*) (Belloy et al., 2007), and European Mouflon (*Ovis aries musimon*) (Belloy et al., 2007; Nattermann et al., 1993).

Footrot presents as a spectrum of clinical manifestations but for descriptive and regulatory purposes outbreaks are usually consigned to discrete categories using objective measures of disease severity, such as foot scores (Egerton, 1971; Egerton, 1989a). Three clinical forms of the disease have been described in the literature: virulent, intermediate, and benign. *D. nodosus* strains are also classified as virulent, intermediate, or benign according to their *in vitro* phenotypic and genotypic characteristics (Egerton, 1989a; Every, 1982; Frosth et al., 2015; Kortt et al., 1983; Liu and Yong, 1997; Palmer, 1993; Stäuble et al., 2014a; Stewart, 1979). Virulent footrot is responsible for considerable production losses and is a major economic and animal welfare concern in many countries where sheep are reared (Hardi-Landerer et al., 2017; Lane et al., 2015; Nieuwhof and Bishop, 2005; Stewart, 1989; Wassink et al., 2010). In contrast,

production losses associated with benign footrot are negligible (Egerton and Parsonson, 1969; Glynn, 1993; Lane et al., 2015).

Footrot is prevalent in most sheep-rearing countries and has been reported in Australia (Beveridge, 1941), Bulgaria (Ivanov et al., 1977), Canada (Morck et al., 1994), Denmark (Forbes et al., 2014), France (Stäuble et al., 2014b), Germany (Younan et al., 1999), Greece (Colaghis, 1971), India (Bhat et al., 2012; Sreenivasulu et al., 2013), Nepal (Egerton et al., 2002), New Zealand (Zhou and Hickford, 2000a), Norway (Vatn et al., 2012), Portugal (Jimenez et al., 2003), Spain (Piriz Duran et al., 1990a), Sweden (König et al., 2011), Switzerland (Stäuble et al., 2014b), the United Kingdom (Green and George, 2008), and the United States (Gradin et al., 1993). Footrot was once prevalent in Nepal (Egerton et al., 2002) and Bhutan (Gurung et al., 2006b) but has since been eradicated

The aetiopathogenesis of footrot is complex; the clinical manifestations of *D. nodosus* infection are the result of interactions between host and pathogen, and the environment in which these interactions occur (Egerton et al., 1969; Emery et al., 1984; Maboni et al., 2017; Roberts and Egerton, 1969). Infection with *D. nodosus* does not necessarily result in clinical footrot; consequently, clinical expression is not always a reliable indicator of the presence of *D. nodosus* (Egerton et al., 1969; Frosth et al., 2015; Locher et al., 2015; Roberts and Egerton, 1969), and the clinical severity of an outbreak does not always reflect the virulence of the infecting *D. nodosus* strain(s) (Egerton, 1989a; Stewart and Claxton, 1993). As such, *in vitro* virulence testing of the infecting *D. nodosus* strain(s) is often necessary to determine their virulence. Several genotypic and phenotypic virulence tests have been developed to differentiate virulent and benign strains of *D. nodosus*, including the gelatin gel (Palmer, 1993) and elastase (Stewart, 1979) tests, the zymogram (Every, 1982), gene probes (Liu and Yong, 1993a), the *intA* PCR (Cheetham et al., 2006), and two real-time polymerase chain reaction (PCR) tests targeting the *aprV2*/*aprB2* alleles (Frosth et al., 2015; Stäuble et al., 2014a). Of these, only the elastase test is capable of differentiating intermediate strains of *D. nodosus* (Stewart, 1979).The level of agreement between these tests and the clinical severity of an outbreak varies, and there is often poor agreement between different diagnostic tests (Claxton, 1986a; Dhungyel et al., 2013b; Links and Morris, 1996; Liu et al., 1994; Liu and Yong, 1993b; Palmer, 1993; Stewart et al., 1986).

1.2 Forms of footrot

Three discrete clinical forms of footrot are described in the literature: virulent, benign, and intermediate.

1.2.1 Virulent footrot

Virulent footrot is a chronic, debilitating form of the disease characterised by underrunning of the sole and hoof capsule in a large proportion of sheep in an infected flock (Beveridge, 1941; Egerton, 1989a). Production losses can be substantial, and are commensurate with the clinical severity of an outbreak (Lane et al., 2015; Marshall et al., 1991b; Nieuwhof and Bishop, 2005; Stewart, 1989; Wassink et al., 2010). Where environmental conditions are favourable, there is a rapid spread of the infection and a rapid development of underrun lesions (Stewart, 1989). Typically both digits and more than one foot are affected, and the lesion is covered with a layer of grey-white necrotic material with a characteristic fetid odour (Beveridge, 1941; Stewart, 1989). In chronic cases, the hoof capsule can become long and irregular, though the cause of this is unknown (Beveridge, 1941); Stewart (1989) speculated that irregular growth of the hoof may be due to increased bloodflow to the affected digits, but this has not been proven experimentally. There is evidence that an inability to bear weight on the affected limb may contribute to the growth of a long, misshapen hoof by reducing the rate at which the hoof capsule is worn down (Smith et al., 2014). There does not appear to be any significant difference in hoof growth rates between the front and hind feet, nor between medial or lateral digits (Shelton et al., 2012).

Profound lameness is characteristic of virulent footrot; animals may carry the affected limb, kneel when grazing, or spend prolonged periods in ventral recumbency if multiple limbs are affected (Beveridge, 1941; Stewart, 1989). This can lead to sternal ulceration and eventual death due to bacteraemia (Beveridge, 1941; Stewart, 1989). Lameness is most pronounced in animals with advanced lesions, but animals may be noticeably lame with lesser lesions (Beveridge, 1941). Virulent footrot has significant impacts on bodyweight, wool growth, and wool quality, due to the inappetance of affected sheep (Marshall et al., 1991b; Stewart et al., 1984). In Australia, flystrike (myiasis) by the Australian sheep blowfly (*Lucilia cuprina*) of the affected digits can occur (Stewart, 1989). Bodystrike may also occur if there has been transfer of necrotic material to the wool (Stewart, 1989).

1.2.2 Benign footrot

Benign footrot presents as a mild or moderately severe interdigital dermatitis (Egerton and Parsonson, 1969; Glynn, 1993). Underrun lesions may be evident in a small proportion of affected animals (typically <1%) (Egerton, 1989a). Ridging of the horn of the posterior axial wall of the hoof may be evident on feet with chronic lesions (Stewart, 1989). Benign footrot can cause lameness but this is usually transient and has a negligible impact on production (Marshall et al., 1991b; Stewart, 1989).

In contrast to virulent footrot, foot lesions resolve rapidly with the application of topical antibacterial treatments, such as foot-bathing, or with the advent of hot, dry environmental conditions (Egerton and Parsonson, 1969). In Australia, benign footrot is endemic in many areas and while this form of the disease is easy to control, it is extremely difficult to eliminate from a flock (Egerton and Parsonson, 1969). Mixed infections of virulent and benign strains are common (Dhungyel et al., 2013a; Egerton and Parsonson, 1969), and benign strains often persist in flocks from which virulent footrot has previously been eliminated (Egerton and Parsonson, 1969). In Australia, benign footrot is estimated to cost the sheep meat and wool industries approximately $12 million per annum (Lane et al., 2015). Given that benign footrot is typically thought to have very little economic significance, this estimate is surprising; however, as intermediate footrot is not differentiated from benign footrot by animal health agencies in Australia, this estimate probably captures the economic impact of both the benign and intermediate forms of the disease.

1.2.3 Intermediate footrot

Intermediate footrot is described in the literature but is not differentiated by animal health agencies in Australia (Buller and Eamens, 2014). Intermediate footrot has variously been referred to as sub-virulent footrot, severe benign footrot, and active benign footrot (Claxton, 1986a; Stewart, 1989). Clinically, intermediate footrot is poorly defined and the descriptions of intermediate footrot in the literature cover a spectrum of clinical manifestations (Abbott and Egerton, 2003a; Abbott and Egerton, 2003b; Stewart, 1989). In general terms, the clinical severity of an outbreak of intermediate footrot is described as somewhere between that of virulent footrot and benign footrot (Stewart, 1989). Production losses associated with intermediate footrot are commensurate with the clinical severity of the outbreak; where

environmental conditions favour progression of the disease, the proportion of sheep that present with severe, underrun lesions can begin to approach that of virulent footrot (Stewart, 1989).

Intermediate footrot has not been the focus of any recent studies and remains poorly understood. Isolates with intermediate phenotypic characteristics have been reported (Claxton, 1986a; Stewart, 1979, 1989); however an intermediate genotype has not been identified (Kennan et al., 2014). Intermediate footrot was previously the target of control programmes in New South Wales as it was thought that intermediate footrot could progress to virulent footrot when environmental conditions were favourable; however, this was eventually disproven (Abbott and Egerton, 2003a). Unlike benign footrot, for which there are no reports of the disease being eliminated, intermediate footrot can be eliminated through the application of topical antibacterial solutions and repeated examination and culling of sheep with active lesions, with or without vaccination (Abbott and Egerton, 2003b).

1.3 Scoring systems

Objective measures of disease severity are necessary to study the impact, treatment, control, and epidemiology of footrot (Egerton, 1989a; Egerton and Roberts, 1971; Marshall et al., 1991b; Stewart et al., 1982; Stewart et al., 1985b; Walker, 1988; Woolaston, 1993). Objective measures are also useful for the measurement of natural resistance and may help to inform breeding decisions (Conington et al., 2008; Conington et al., 2002). To this end, a number of objective scoring systems have been devised to grade the severity of disease at the foot, sheep, and flock levels. A simple scoring system to describe the severity of foot lesions was first devised by Egerton and Roberts (1971). Briefly, clinically health feet are assigned a score of 0; mild lesions restricted to the interdigital skin are given a score of 1; where the interdigital inflammatory lesion is severe, a score of 2 is given; where underrunning of the posterior sole and soft horn of the heel is observed, a score of 3 is given; where the underrunning extends to the abaxial wall, a score of 4 is given. This system is reported to have a high level of repeatability (Phythian et al., 2016).

This system has been extended in subsequent studies, and score 3 further divided into scores 3a, 3b, and 3c according to the extent of underrunning (Stewart et al., 1982; Stewart et al., 1985b). A scoring system of 0 to 5, with no sub-division of score 3, has also been reported and applied in

the field (Walker, 1988; Woolaston, 1993). According to this system, score 5 denotes lesions with extensive necrosis and separation the sole and abaxial hoof wall (Walker, 1988).

A number of systems have been devised to provide a single disease severity score for each animal, inclusive of all four feet: Egerton and Roberts (1971) used a total foot score (TFS) which was the sum of all four individual foot scores; Woolaston (1993) and Lee et al. (1983) employed an average foot score (AFS), which was equivalent to TFS/4; the maximum foot score (MFS) and number of underrun feet (NUF) have also been to describe disease severity (Marshall et al., 1991b; Stewart et al., 1985a; Stewart et al., 1982; Stewart et al., 1983). Subsequently, Whittington and Nicholls (1995b) argued that these systems do not adequately differentiate sheep with severe lesions from those with mild lesions, and devised a weighted scoring system that provides a measure of the number of feet with lesions and the number of underrun lesions. In this system, Score 3 lesions are assigned a weighted score of 9, score 4 lesions are assigned a weighted score of 16, and scores 1 and 2 are given no weighting. A total weighted foot score (TWFS) is then calculated for each affected sheep that provides a clear indication of how severely affected an individual sheep is.

Single metrics have also been used to describe disease severity at the group or flock level, such as the number or proportion of sheep with foot lesions (Egerton and Burrell, 1970), the number or proportion of feet with lesions (Every and Skerman, 1982), the average TFS for an affected flock (Egerton and Roberts, 1971), the combined TFS of all sheep in an affected flock (Egerton and Roberts, 1971), and the average TWFS (Whittington and Nicholls, 1995b).

In Australia, for descriptive and regulatory purposes, outbreaks of virulent and benign footrot are differentiated according to the prevalence of score 4 lesions; however, the cut-point used to differentiate virulent and benign footrot differs between States. In New South Wales, a cut-point of 10% score 4 lesions is used (Buller and Eamens, 2014; Egerton, 1989a). In Victoria and South Australia, a cut-point of 1% score 4 lesions is used (Buller and Eamens, 2014; Riley and Buchanan, 2003). In Western Australia, diagnosis is based entirely on the outcomes of laboratory virulence tests (Buller and Eamens, 2014; Higgs, 2003), which are discussed in detail later in this review. In Tasmania, where there is no control program, the diagnostic criteria are unclear (Middleton, 2003). In Queensland, where footrot is rare, the diagnostic criteria are unclear, with a combination of flock examination and laboratory tests described (Fraser, 2003). Intermediate

footrot was previously recognised by regulatory authorities in some, but not all, Australian States (Abbott and Egerton, 2003a; Buller and Eamens, 2014; Stewart and Claxton, 1993). However, a 1992 Animal Health Committee (AHC) on the *Coordination of Footrot Control* in Australia recommended that in order to harmonise diagnostic approaches between States, footrot should be classified as virulent or benign only, and that outbreaks previously classified as intermediate should be deemed virulent (Stewart and Claxton, 1993).

A number of locomotion scoring systems have also been devised to describe and monitor lameness (Kaler et al., 2009; Ley et al., 1989; Welsh et al., 1993). For instance, a seven-point visual locomotion scale was devised by Kaler et al. (2009), ranging from 0 (normal locomotion) to 6 (unable to stand). Factors such as posture, the ability to bear weight on all four limbs, stride length, the number of limbs affected, and ability to rise were considered when assigning a score.

1.4 Economic and production impacts

Footrot is an economic burden in many sheep-rearing countries (Lane et al., 2015; Nieuwhof and Bishop, 2005; Wassink et al., 2010). In Australia, for example, virulent footrot is estimated to cost the sheep meat and wool industry approximately $AUD33 million per annum, primarily due to production losses (Lane et al., 2015). Benign footrot is estimated to cost the industry an additional $AUD12.1 million per annum (Lane et al., 2015). The prevalence of footrot has decreased considerably in some parts of Australia following the success of State-based control programmes such as the NSW Footrot Strategic Plan (Scott-Orr and Seaman, 2006). Prior to the implementation of the NSW programme, footrot was estimated to cost the NSW sheep meat and wool industry alone $AUD42.6 million per annum (Stewart, 1989). In States where footrot is a notifiable disease, flocks infected with virulent strains of *D. nodosus* are subjected to compulsory elimination programmes, with costs met by the producer. The cost of eliminating virulent footrot from a flock can exceed $AUD10 per sheep (Allworth, 1990). In the U.K., where footrot is endemic and a major cause of lameness (Winter et al., 2015), the disease is estimated to cost the sheep industry approximately £24 million per annum due to production losses and the cost of treatment and preventive measures (Nieuwhof and Bishop, 2005; Wassink et al., 2010). In N.Z., the annual economic impact of footrot in flocks that had experienced one or more outbreaks of footrot was estimated to be $10,293 per flock (Greer, 2005). Costs associated with prevention

and control accounted for $4,545, the majority of which ($2,564) was associated with labour costs. Production losses, predominantly associated with deaths and culling, accounted for $5,652.

1.5 Aetiopathogenesis

1.5.1 Aetiology

Footrot has a complex mixed bacterial aetiology. The clinical manifestations of the disease are a consequence of synergistic interactions between the essential causative agent, *D. nodosus*, and the bacterial community of the foot following environmental predisposition (Egerton et al., 1969; Roberts and Egerton, 1969). The bacterial community of the hoof is diverse; their interactions with *D. nodosus* have not been described comprehensively. Historically, investigators have primarily focussed on bacterial taxa that can be cultured on common growth media, or those with distinct cell morphologies, such as *Fusobacterium* and Spirochaetes, which are easily identifiable in smears and histological sections.

Fusobacterium necrophorum, a constituent of the normal gastrointestinal flora of ruminants (Langworth, 1977), is thought to be essential for initiation and progression of the disease (Egerton et al., 1969; Roberts and Egerton, 1969). *F. necrophorum* is excreted into the environment in faeces and is common on healthy feet (Roberts and Egerton, 1969). However, in warm, moist environmental conditions the interdigital skin becomes soft and macerated, and *F. necrophorum* rapidly invades the superficial layers of the epidermis (Egerton et al., 1969; Roberts and Egerton, 1969). *F. necrophorum* is able to invade the epidermis and proliferate as it expresses a leukocytic toxin that inhibits leukocyte activity and prevents them from accessing the site of infection, enabling microbial proliferation (Roberts, 1967b). Thereafter, toxins released by *F. necrophorum*, and irritants discharged by dead immune cells, trigger a severe inflammatory response that leads to progressive destruction of the epidermal matrix (Egerton et al., 1969; Piriz Duran et al., 1990a). *D. nodosus* then invades the devitalised epidermis, accompanied or closely followed by *F. necrophorum* (Egerton et al., 1969). Recent studies have confirmed the association between *F. necrophorum* and the development of lesions: comparing feet with severe lesions to those with mild lesions or healthy feet, Frosth et al. (2015) found that the odds of developing severe footrot were increased 4.9 times when *F. necrophorum* was present.

There are two subspecies of *F. necrophorum*: *necrophorum* and *funduliforme* (formerly referred to as biovar A and biovar B, respectively) (Nagajara et al., 2005). Both subspecies can be present in a flock (Frosth et al., 2015). Subspecies *necrophorum* expresses more leukotoxin than subspecies *funduliforme* and is reported to be more pathogenic (Nagajara et al., 2005; Tan et al., 1996). Recent studies have demonstrated that severe footrot lesions are more likely to develop when subsp. *necrophorum* is present: Frosth et al. (2015) reported that the odds of developing severe footrot were increased 9.5 times when subsp. *necrophorum* was present, and only 2.5 times when subsp. *funduliforme* was present. The prevalence of each subspecies varies between countries: *F. necrophorum* subsp. *necrophorum* is reported to be more prevalent in U.K. flocks (Maboni et al., 2016), while subsp. *funduliforme* is reported to be more prevalent in Swedish flocks (Frosth et al., 2015). In one study, only *F. necrophorum* subsp. *necrophorum* was detected in specimens of lesion material collected from sheep, goats and cattle from farms in N.Z. (Zhou et al., 2009b).

Recently, the status of *F. necrophorum* as a primary or secondary invader has been debated: Witcomb et al. (2014) reported that *F. necrophorum* was more likely to be detected in specimens collected from severe, underrun lesions than in those collected from mild interdigital lesions, and that whilst there was an association between increasing *D. nodosus* load and the development of an interdigital lesion, there was no association between *F. necrophorum* load and the development of an interdigital lesion. Furthermore, although *F. necrophorum* was detected in severe, underrun lesions, the bacterium was not associated with the development of such lesions (Witcomb et al., 2014). Subsequently, Witcomb et al. (2015) reported that while *D. nodosus* load was significantly higher in feet with interdigital lesions and severe, underrun lesions than on healthy feet, *F. necrophorum* load was significantly higher in feet with severe, underrun lesions only. In both cases, the authors concluded that although *F. necrophorum* undoubtedly enhances disease severity, it might not be essential for initiation of the disease process. These findings were supported by Maboni et al. (2016), who reported that *F. necrophorum* load, determined by qPCR, was higher in interdigital skin biopsies collected from severe, underrun lesions than in mild lesions or on healthy feet. However, qPCR may not have been an appropriate method of detection for the stated aim of the work. Given that qPCR detects both viable and non-viable organisms, it is possible that a higher *F. necrophorum* load was detected in the latter stages of disease due to the gradual accumulation of dead organisms in the lesion, rather than the

organisms proliferating at an increased rate. This may partially explain why *F. necrophorum* was detected in severe, underrun lesions but was not associated with the development of severe lesions. Furthermore, it should not be assumed that a high bacterial load is necessary for initiation of the disease process *per se*, given that the minimum bacterial load required for initiation of the disease process has not been determined, and the infectivity of *F. necrophorum* is dramatically enhanced by the presence of other bacterial species such as *Corynebacterium pyogenes* and *Escherichia coli* (Roberts, 1967a; Roberts, 1967b). Additionally, all specimens were collected from the interdigital skin, rather the active margin of the developing lesion, which is found beneath the underrun horn and sole in the latter stages of disease. Finally, the distribution of *D. nodosus* and *F. necrophorum* in infected epidermal tissue is not homogenous and the bacterial load detected in a specimen may vary considerably depending on which part of the lesion is sampled. For instance, Egerton et al. (1969) reported that the distribution of *D. nodosus* and *F. necrophorum* in the epidermal tissue is irregular and that in recently-infected tissues there are often foci of growth of *D. nodosus* and *F. necrophorum*. Thus the bacterial load detected via qPCR could vary considerably between specimens collected from different lesions of the same score.

Further, the findings of the aforementioned studies are at odds with those of earlier studies, which have demonstrated that footrot does not commence without the presence of *F. necrophorum*, even with environmental predisposition (Beveridge, 1941; Egerton et al., 1969; Gregory, 1939; Roberts and Egerton, 1969). Collectively, these earlier studies indicate that *D. nodosus* is unable to invade the epidermis without prior colonisation and devitalisation of the epidermis by *F. necrophorum*. For instance, Roberts and Egerton (1969) reported that challenge with *D. nodosus* in the absence of faecal contamination failed to induce footrot, even when sheep were predisposed via water maceration. Interestingly, colonisation with *F. necrophorum* during the early stages of disease also appears to influence the long-term persistence of lesions: Roberts and Egerton (1969) challenged penned sheep with *D. nodosus* alone, a mixture of *D. nodosus* and *F. necrophorum*, a mixture of *D. nodosus* and *C. pyogenes*, or a combination of all three organisms. When returned to the field under hot, dry environmental conditions, lesions only persisted in sheep challenged with *D. nodosus* and *F. necrophorum* or a combination of all three organisms.

There is little doubt that *D. nodosus* leads the invasion into the deeper layers of the epidermal matrix, accompanied or followed closely by *F. necrophorum* (Egerton et al., 1969); however, invasion of the epidermal matrix by *D. nodosus* appears to induce little or no inflammatory response (Egerton et al., 1969). In contrast, invasion of the epidermis by *F. necrophorum* is associated with an acute inflammatory response (Egerton et al., 1969). As such, it could be argued that in the absence of the inflammatory stimulus provided by *F. necrophorum*, the clinical manifestations of footrot may be mild. It must also be conceded that gastrointestinal microorganisms other than *F. necrophorum* may also contribute to the disease process. For instance, Nattermann et al. (1993) reported that footrot could be induced by application of mixed cultures of *D. nodosus* and *Porphyromonas levii*. The possible significance of *Porphyromonas* is discussed in more detail later in this review.

Since *D. nodosus* was identified as the essential transmitting agent (Beveridge, 1941) and the importance of *F. necrophorum* was identified (Egerton et al., 1969; Roberts and Egerton, 1969), investigators have largely focussed on these two organisms. Several other constituents of the bacterial community of the foot are thought to contribute to the disease process both directly and indirectly, but their role is less well defined than that of *F. necrophorum*. For instance, *C. pyogenes* is thought to contribute to the disease process indirectly by means of synergistic interactions with *F. necrophorum* (Roberts, 1967a; Roberts, 1967b). *C. pyogenes* has been shown to enhance the infectivity of *F. necrophorum* through the production of nutrient factors (Roberts, 1967a). This has been demonstrated *in vitro*: growth of *F. necrophorum* on an agar medium under anaerobic conditions is enhanced when *C. pyogenes* has been grown aerobically on a different area of the same plate for 24-48 hours prior to the plate being inoculated with *F. necrophorum* (Roberts and Egerton, 1969). *C. pyogenes* is reported to be abundant in the superficial layers of the footrot lesion: Egerton et al. (1969) reported that aerobic cultures of lesion material were dominated by *C. pyogenes* and other diphtheroid bacteria. The proliferation of *C. pyogenes* and other aerobic bacteria in the superficial layers of the epidermis is also thought to aid the disease process by removing oxygen, and eliminating hydrogen peroxide through the production of catalase (Parsonson et al., 1967), thereby creating a favourable environment for strict anaerobes such as *D. nodosus* and *F. necrophorum* to establish and proliferate.

Synergistic interactions have been reported between *F. necrophorum* and other bacterial species, including *Arcanobacterium pyogenes*, *Dermatophilus congolensis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (Brook and Walker, 1986; Smith et al., 1991; Smith et al., 1989; Timoney et al., 1988). There is evidence that *D. nodosus* also enhances the growth and invasiveness of *F. necrophorum* through the provision of a filterable nutrient factor (Roberts and Egerton, 1969). These interactions can have a dramatic effect on the infectivity of *F. necrophorum*. For instance, suspension of *F. necrophorum* in 100 μL of supernatant from an *E. coli* broth culture reduced the infective dose of *F. necrophorum* from $>10^6$ organisms to <10 organisms (Smith et al., 1991).

Spirochaetes are frequently detected in footrot lesions but their pathogenic significance has not been defined (Beveridge, 1941; Collighan et al., 2000; Collighan and Woodward, 1997; Demirkan et al., 2001; Dopfer et al., 1997; Egerton et al., 1969; Naylor et al., 1998; Rasmussen et al., 2012; Roberts and Egerton, 1969). Spirochaetes were investigated as a potential causative agent prior to the identification and characterisation of *D. nodosus*. A novel spirochaete was isolated from specimens of lesion material by Beveridge (1936), which he named *Spirochaeta penortha*. The organism was ubiquitous in footrot lesions but was not thought to be essential to the disease process. Thereafter, Beveridge (1941) concluded that although spirochaetes are not essential to the disease process, they probably increase disease severity, as challenge with *D. nodosus* and *S. penortha* induced lesions of greater severity than challenge with *D. nodosus* alone. Several species of *Treponema* have been identified in footrot lesions; for instance, a *Treponema* species closely related to *T. vincentii* has been detected in sheep with virulent footrot (Collighan et al., 2000). Recent studies have cast doubt on the significance of *Treponema*: Maboni et al. (2016) reported that the rate of detection of *Treponema* in tissue biopsies of healthy and disease feet was low, and that *Treponema* was present on a similar number of healthy and diseased feet; Frosth et al. (2015) reported that there was no significant association between disease severity and the presence of *Treponema*. Although *Treponema* spp. are not thought to be necessary for expression of the disease, they are implicated in the pathogenesis of other mixed bacterial diseases of the ruminant hoof, such as bovine digital dermatitis (BDD) (Dhawi et al., 2005) and contagious ovine digital dermatitis (CODD) (Duncan et al., 2014; Moore et al., 2005b). As such, it is possible that they may play an accessory role in the pathogenesis of ovine footrot.

Porphyromonas is common in footrot lesions of sheep and goats (Jimenez et al., 2003; Maboni et al., 2016; Piriz Duran et al., 1990b), and has been implicated as a potential causative agent (Nattermann et al., 1993), but its role has not been shown experimentally. *Porphyromonas* is a member of the normal gastrointestinal flora and is excreted into the environment in faeces, so detection of the organism in footrot lesions is not surprising. However, *Porphyromonas* is regarded as a 'keystone' species in both BDD and human periodontal disease, both of which are mixed bacterial diseases that share several common aetiological agents with ovine footrot. For example, *P. gingivalis*, a constituent of the normal oral flora, is regarded one of three primary aetiological agents of human periodontal disease (Darveau et al., 2012). The synergistic reactions between members of the normal bacterial community that occur in cases of human periodontal disease and footrot are similar in many respects; *P. gingivalis* is able to manipulate the host's immune response and inhibit leukocyte-mediated killing mechanisms (Wang et al., 2010), leading to unrestrained proliferation of other members of the normal bacterial community and a state of dysbiosis. Discussing these changes, Darveau et al. (2012) describe a progressive, qualitative shift in the bacterial community of the oral cavity in which some taxa become dominant and other members can no longer be detected. This is remarkably similar to reported changes in the bacterial community of the hoof in cases of ovine footrot (Maboni et al., 2017) and in cases of BDD (Krull et al., 2014).

Several other bacterial species have been noted in footrot lesions, but their significance is yet to be determined. *A. pyogenes*, a commensal organism of the mucosal surfaces of the urogenital and respiratory tracts of ruminants (Jost et al., 2005), has been detected in footrot lesions and has been isolated from mixed infections of the feet of fallow deer (Lavin et al., 2004). Several *Prevotella* species were isolated from the feet of Merinos with virulent footrot in Portugal (Jimenez et al., 2003), the most frequent of which were *P. disiens* and *P. zoogleoformans*. In Spain, *Prevotella* was isolated from 44.4% (96/216) of cases of ovine footrot (Hurtado et al., 1998). *Prevotella* has also been isolated from the coronary band lesions of free-living fallow deer (Lavin et al., 2004). *Bacteroides* has been identified in footrot lesions, however the role of *Bacteroides* in the disease process has not been investigated. An organism closely related to *B. ureolyticus* was isolated from specimens of lesion material by Zhou et al. (2009a). *Bacteroides* are abundant in bovine digital dermatitis lesions (Berg and Loan, 1975), so it is possible that they are of significance in ovine footrot.

The microbial community of the ovine hoof is extremely diverse; identifying which species are of significance to disease is challenging. For instance, Piriz Duran et al. (1990b) isolated 582 anaerobic microbes belonging to 50 species from the feet of goats with virulent footrot in Spain. The genera most frequently isolated were *Bacteroides* (80%), *Peptostreptococcus* (63.6%), *Megasphaera* (40%), *Fusobacterium* (29.2%), *Clostridium* (22.5%), *Propionibacterium* (12.5%), and *Eubacterium* (11.7%). Piriz Duran et al. (1990a) reported that organisms belonging to the genera *Bacteroides*, *Peptostreptococcus*, *Tissierella*, *Fusobacterium*, and *Megasphaera* were frequently isolated from the footrot lesions of sheep in Spain.

1.5.2 Microbiome of the footrot lesion

The advent of next-generation sequencing (NGS) and 16S ribosomal RNA (rRNA) amplicon-based metagenomics has enabled bacterial communities to be examined in greater detail. These technologies have been employed to characterise the communities present on the feet of healthy and footrot-affected sheep and to identify bacterial genera that are of potential pathogenic significance. The first such study, undertaken by Calvo-Bado et al. (2011), examined the bacterial community of the ovine hoof using pyrosequencing of a variable region of the bacterial 16S rRNA gene coupled with cloning and Sanger sequencing. Sheep were selected from three geographically distant flocks (A, B, and C) located in the south-west of England for inclusion in the study. Flock A was clinically healthy with no evidence of interdigital dermatitis or footrot, Flock B presented with interdigital dermatitis (ID) only, and Flock C presented with virulent footrot (severe, underrun lesions). Three clinically healthy sheep were selected from Flock A. Three sheep with healthy feet and two sheep with interdigital dermatitis were selected from Flock B. Two sheep with healthy feet, three sheep with interdigital dermatitis, and two sheep with underrun lesions were selected from Flock C. Biopsies were collected from the interdigital skin of all four feet *post mortem* using a 5 mm core borer to a depth of 8 mm. Deooxyribonucleic acid (DNA) was prepared from each tissue biopsy, the DNA pooled for each sheep, and a variable region of the bacterial 16S rRNA gene was amplified using the 27F and 1525R universal primers, and cloned. Plasmid DNA was then purified and sequenced in one direction using the 27F primer.

The authors reported that there was a core bacterial community of 187 operational taxonomic units (OTUs) that were common to all three groups. There was inter-flock variation in the

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bacterial communities of healthy feet, but the composition of the bacterial communities of diseased feet were more similar. The most abundant genera associated with disease were *Macrococcus*, *Micrococcus*, and *Staphylococcus*. *Corynebacterium* was most abundant in mild interdigital lesions, and *Peptostreptococcus* was most abundant on healthy, intact interdigital skin. *D. nodosus* was not detected through sequencing, probably because there is a single nucleotide polymorphism (SNP) in the *D. nodosus* 16S rRNA gene region that 27F primer binds to (Myers et al., 2007).

In a more recent study, Maboni et al. (2017) used next-generation Illumina sequencing to characterise the bacterial communities of the feet of clinically healthy sheep and sheep with mild and severe footrot lesions. DNA was prepared from post-slaughter interdigital skin biopsies collected with disposable 6 mm biopsy punches. Feet were classified into three categories based on disease status: healthy, presenting with mild ID (mild interdigital lesion with no evidence of underrunning), or presenting footrot (moderate to severe ID lesion and underrunning). Analysis of the bacterial community was undertaken with specimens collected from 40 healthy feet, 30 feet with mild ID and 36 feet with moderate to severe disease. DNA was extracted from each sample, the V3-V4 region of the 16S rRNA gene was amplified using the universal primers 341F and 534R, and the PCR product was sequenced on an Illumina MiSeq. The authors reported significant differences between the microbial populations of healthy feet and feet with moderate to severe disease at the phylum, family, and genus level, and a significant difference between the populations of healthy feet and feet with mild ID at the phylum and genus levels. The dominant families on diseased feet were *Moraxellaceae* (20-36%) and *Corynebacteriaceae* (14-20%). The abundance of the family *Mycoplasmataceae* was significantly greater on feet with moderate to severe disease than on healthy feet or feet with mild ID. The abundance of the families *Spirochaetaceae* and *Fusobacteriaceae* was also significantly higher on feet with moderate to severe disease. *Flavobacteriaceae* and *Staphylococcaceae* were significantly more abundant on healthy feet than on diseased feet. The most abundant genera in samples collected from healthy feet and feet with mild ID were *Corynebacterium* (26% and 31%, respectively), *Psychrobacter* (26% and 19%, respectively) and *Acinetobacter* (11% and 8%, respectively). In contrast, the microbial populations of feet with moderate to severe disease were dominated by *Mycoplasma* (20%), *Corynebacterium* (19%), *Psychrobacter* (18%) and *Treponema* (14%). *D. nodosus*

abundance was low in all cases (0.5-1.9%), but significantly higher in feet with mild ID and moderate-to-severe disease compared to healthy feet.

1.5.3 Host susceptibility

There is considerable variation in host susceptibility to *D. nodosus* infection. Five potential tiers of variation were described by Egerton and Raadsma (1991): (i) variation between breeds; (ii) variation between strains within a breed; (iii) variation between bloodlines within a strain; (iv) variation between sire lines within a bloodline; and (v) variation between individuals. The extent of variation between individuals in a flock was elaborated by Egerton et al. (1983), who observed a flock of Merino sheep from mid-winter to early summer for three successive years following experimental challenge with *D. nodosus*. Susceptibility was determined by the rate at which clinical manifestations appeared with the advent of spring, and the rate at which lesions resolved with the advent of summer. Four levels of susceptibility were noted: (i) no clinical manifestations at any point; (ii) clinical manifestations appeared in early spring and did not resolve prior to the final examination in early summer; (iii) clinical manifestations appeared late in spring and resolved prior to the final examination; and (iv) clinical manifestations were apparent before the advent of spring. Variation between individuals within the same breed has been reported following the administration of vaccines targeting *D. nodosus*, with some individuals failing to respond to vaccination (Bhardwaj et al., 2014).

It is widely acknowledged that Merinos are more susceptible to footrot than British breeds or cross-breeds (Beveridge, 1941; Emery et al., 1984; Skerman, 1982; Stewart et al., 1985b; Youatt, 1837), but the basis of this susceptibility is contentious. Following immunisation with a monovalent whole-cell vaccine, Skerman et al. (1982) reported that the antibody response persisted for longer in Romneys (20 weeks) than in Merinos (5 weeks), which suggests that British breeds may be able to mount a more effective humoral immune response. Differences in the physical characteristics of the epidermis have also been proposed as a contributing factor: Emery et al. (1984) reported that disease outcomes were similar for Merinos and Romneys when the interdigital skin was lightly scarified prior to experimental challenge with *D. nodosus*, which suggests that the epidermis of the Romney is a more effective physical barrier than that of the Merino.

Genetic markers have been identified that appear to correlate with susceptibility to *D. nodosus* infection. For instance, an association between major histocompatibility complex (MHC) haplotype and susceptibility has been reported: Escayg et al. (1997) noted that that the MHC gene *DQA2* had immunomodulatory activity and that sheep with the *DQA2-E* (*1101*) allele were more susceptible to infection; Ennen et al. (2009) also reported an association between *DQA2* and susceptibility to *D. nodosus* infection. In Greece, Gelasakis et al. (2013) reported that Chios dairy sheep with the *1101* allele were 9% more likely to be susceptible to footrot than sheep with other variants of the *DQA2* gene.

1.5.4 Environmental factors

Environmental factors, particularly moisture and temperature, govern the transmission and expression of *D. nodosus* infection (Egerton et al., 1983). Moisture is an essential predisposing factor, as *D. nodosus* is unable to invade healthy, dry feet (Beveridge, 1941; Cross, 1978; Depiazzi et al., 1998; Graham and Egerton, 1968; Gregory, 1939; Marsh and Tunnicliff, 1934; Mohler and Washburn, 1904; Murnane, 1933; Thomas, 1957, 1962a; Whittington, 1995). Prolonged exposure to moisture leads to softening and maceration of the interdigital skin, which facilitates invasion of the superficial layers of the epidermis by a complex mixture of bacteria, including *D. nodosus* (Beveridge, 1941; Egerton et al., 1969; Thomas, 1962a). Free moisture also appears to be necessary for transmission of the infection, but the reason for this is uncertain (Beveridge, 1941).

Lush pasture and soft, marshy ground favour expression and transmission of the disease (Beveridge, 1941; Cross, 1978; Egerton and Parsonson, 1969; Glynn, 1993; Graham and Egerton, 1968; Gregory, 1939; Mohler and Washburn, 1904; Murnane, 1933; Shahan, 1942; Stewart, 1989; Woolaston, 1993; Youatt, 1837), but the disease may also express in less favourable conditions. For instance, Whittington (1995) reported that infection was transmitted on long, unimproved pasture dominated by long Kangaroo grass (*Themeda australis*), and suggested that abrasion of the interdigital skin by rough, mature grasses may be a predisposing factor.

The range of environmental conditions in which the disease is transmitted and expressed have not been reported in great detail, but estimates of the minimum rainfall and temperature

requirements were provided by Graham and Egerton (1968). Briefly, the authors examined the spread of footrot on four farms located on the Central Tablelands of NSW, Australia, across a period of eight years, and reported that outbreaks predominantly occurred in spring when there was rainfall of 50 mm or more per month during the preceding winter. Outbreaks rarely occurred in the summer months where rainfall was similar to the long-term average, however outbreaks did occur when there was higher than average rainfall (≥125 mm), which maintained sufficient pasture growth to facilitate transmission and expression of the disease. Outbreaks occurred less frequently in autumn, although evenly distributed rainfall of 60 mm or more per month during the preceding summer and the autumn months was sufficient for outbreaks to occur. Brief periods of high rainfall did not prompt outbreaks; even distribution of rainfall appears necessary to maintain soil moisture and support pasture growth, particularly during summer and autumn (Graham and Egerton, 1968). Heavy dews may also support outbreaks of footrot when pasture is dense and is capable of retaining moisture throughout the day; Graham and Egerton (1968) reported that outbreaks commenced in spring when the average daily air temperature was ≥10°C. This finding was supported by Cross (1978), who reported that footrot did not express when the air temperature was <10°C following challenge of sheep with *D. nodosus* in an pen-based experimental model, even with sufficient moisture and scarification of the interdigital skin. Mild air temperatures are important for optimal growth and invasiveness of *D. nodosus*: Beveridge (1941) reported that optimal growth of *D. nodosus* occurred at 37°C, slowed at 25°C and ceased below 20°C. A fall in ambient temperature has also been reported to cause a fall in the temperature of the extremities of sheep, which may provide a less favourable environment for *D. nodosus* (Graham and Egerton, 1968). In the U.K., however, footrot was reported to express throughout the year, even when temperatures were <10°C (Green et al., 2007; Ridler et al., 2009).

Paddock topography can also affect transmission and expression of footrot, as it has an effect on soil moisture and pasture growth (Stewart et al., 1984). Paddocks with an even topography favour transmission of infection moreso than paddocks with an uneven topography with alternating moist and dry areas (Beveridge, 1941; Stewart et al., 1984). Mechanical damage due to grass seeds (Beveridge, 1934; Glynn, 1993), abrasian by mature grasses (Whittington, 1995), crop stubble (Shahan, 1942), stones (Beveridge, 1941), frosts (Graham and Egerton, 1968), and

penetration of the epidermis by *Strongyloides* larvae (Beveridge, 1934; Cross, 1978) may also predispose sheep to infection.

The survival, and therefore transmission, of *D. nodosus* in the environment may be influenced by soil type and other environmental factors. *D. nodosus* was reported to survive for 7 to 14 days in soil, faeces, or pasture (Beveridge, 1941; Stewart, 1989; Stewart and Claxton, 1993), but more recent studies have indicated that *D. nodosus* might be capable of surviving for longer periods. For instance, *D. nodosus* has been reported to survive in hoof trimmings for up to six weeks (Winter, 2009). *D. nodosus* was also shown to survive for up to 24 days in soil supplemented with ground hoof, when incubated at 5°C under laboratory conditions (Cederlöf et al., 2013).

Recently, Muzafar et al. (2016) evaluated the survival *D. nodosus* in clay, sandy and sandy loam soil microcosms. The soil was inoculated with *D. nodosus* and incubated aerobically in the dark at 25°C or 5°C for a period of 40 days. Survival of *D. nodosus* was evaluated by microbiological culture, qPCR and fluorescent microscopy. There was a biphasic decline in viable *D. nodosus* cells, with a relatively rapid decline up to day 6 and a slower rate of decline from days 7-14. Survival of *D. nodosus* was higher at 5°C than at 25°C and was significantly higher at both temperatures in clay soil than sandy soils. Soil structure influenced soil desiccation, with open structured sandy soils drying faster than closed or partially closed structured soils, which is likely to have contributed to the survival of *D. nodosus*. Surprisingly, *D. nodosus* survived for longer than 30 days in all cases, which is much longer than previous estimates; however, this was under laboratory conditions and is unlikely to reflect survival rates under natural conditions. Further investigation is required to demonstrate that the bacterium remains infective during this period, and in sufficient numbers to facilitate transmission and to susceptible sheep.

1.6 Differential diagnosis

There are several disorders of the hoof, both infectious and non-infectious, that bear some resemblance to footrot (Stewart, 1989). Some of these also share common aetiological agents with footrot, which can add further confusion (Stewart, 1989). A brief description of some of these is provided below.

1.6.1 Ovine Interdigital Dermatitis (OID)

OID is caused by invasion of the epidermis by *F. necrophorum* following prolonged exposure to warm, moist environmental conditions (Parsonson et al., 1967). *C. pyogenes* is also abundant in OID lesions and may help to enhance the infectivity of *F. necrophorum* (Parsonson et al., 1967). OID resembles benign footrot and the early stages of virulent footrot and is difficult to distinguish by visual observation alone (Stewart, 1989); however, interdigital dermatitis can be differentiated from footrot by determining the presence or absence of *D. nodosus* in smears prepared from specimens of lesion material (Parsonson et al., 1967). In contrast to footrot, OID rarely causes lameness and resolves rapidly with the advent of dry weather (Stewart, 1989). The expression of OID requires persistent moisture, so the disease is only prevalent where environmental conditions favour expression of the disease. In a survey of 35,520 sheep from 90 flocks in western Victoria, Australia, inflammation of the interdigital skin was observed in 70% (63/90) of the flocks, but *D. nodosus* was only detected in 54% (34/63) of these flocks, which suggests that OID was present in 46% of the flocks surveyed (Morgan et al., 1972).

1.6.2 Foot abscess

Foot abscess (also referred to as infective bulbar necrosis) is caused by a mixed infection of *F. necrophorum* and *C. pyogenes* (Roberts et al., 1968; Stewart, 1989; West, 1989). As with footrot and OID, foot abscesses typically occur when sheep are maintained on boggy pasture (Roberts et al., 1968; Stewart, 1989; West, 1989). Foot abscesses are most commonly observed in the hind feet of rams and overweight, pregnant ewes (Roberts et al., 1968). Foot abscess is regarded as a possible complication of OID in which the infection extends to the digital cushion (Roberts et al., 1968). In contrast to footrot and OID, foot abscesses occur sporadically and do not usually affect a large proportion of a flock (Stewart, 1989; West, 1989). Foot abscess can cause profound lameness and the affected limb may be carried (Stewart, 1989; West, 1989). During the early stages, there may be a sinus in the interdigital space from which pus can be expressed (Stewart, 1989). In chronic cases, there may be noticeable inflammation and swelling above the coronet, and sinuses may appear along the coronary border from which pus can be expressed (Stewart, 1989).

1.6.3 Toe abscess

Toe abscess is distinct to foot abscess but is also caused by a mixed infection of *F. necrophorum* and *C. pyogenes* (Stewart, 1989). Toe abscess usually affects the front feet and follows mechanical damage of the horn and injury to the laminae (Roberts et al., 1968). Toe abscess may also occur secondary to shelly toe (Stewart, 1989). Toe abscess causes severe lameness and the affect limb is usually carried (Stewart, 1989).

1.6.4 Strawberry footrot

Strawberry footrot is caused by *Dermatophilus congolensis* and is rarely reported in Australia (Stewart, 1989; Thomas, 1962b). Strawberry footrot presents as pronounced, granulomatous swellings between the coronary band and the knee (Stewart, 1989). The disease typically resolves without treatment (Stewart, 1989).

1.6.5 Laminitis (founder)

Laminitis is a nutritional or metabolic disease. The disease primarily affects lambs following overfeeding on grains or concentrates (Stewart, 1989; Thomas, 1962b). The disease presents as inappetance and depression, and severe lameness in one or more feet caused by aseptic inflammation of the laminae of the hooves (Stewart, 1989).

1.6.6 Shelly-Toe

Shelly-toe is characterised by separation of the abaxial hoof wall from sole of the hoof, forming a cavity that fills with mud, faeces and organic matter (Stewart, 1989). Shelly-toe can be confused with virulent footrot due to the separation of the hoof wall, but is the result of mechanical damage to the hoof and is not infectious. Shelly-toe rarely causes lameness and is of little economic significance (Stewart, 1989).

1.7 Clinical differentiation of virulent, intermediate, and benign footrot

When environmental conditions are favourable for disease expression, clinically virulent, intermediate and benign footrot can be differentiated by examination of a representative sample of an infected flock, as described by Egerton (1989a). Briefly, the feet of at least 100 randomly-selected sheep from the affected flock or mob must be inspected to establish the prevalence of severely underrun (score 4) lesions (Egerton, 1989a). A score (0 to 4) is assigned to each foot to

grade the lesion severity (Egerton and Roberts, 1971). In Australia, virulent and benign footrot are differentiated according to the prevalence of score 4 lesions, but the cut-point used to differentiate virulent and benign footrot differs between States. For example, in New South Wales an outbreak is regarded as virulent where more than 10% of sheep present with score 4 lesions (Egerton, 1989a) or benign if the prevalence is <1%. In Victoria and South Australia, a cut-point of 1% score 4 lesions is used used (Buller and Eamens, 2014; Riley and Buchanan, 2003). Intermediate footrot is no longer differentiated by most regulatory authorities (Buller and Eamens, 2014; Stewart and Claxton, 1993). Scoring systems are described in more detail elsewhere in this review.

1.8 Bacteriology of *D. nodosus*

The essential transmitting agent, *D. nodosus* (formerly organism K, *Fusiformis nodosus*, *Bacteroides nodosus*) (Beveridge, 1938b, 1941; Dewhirst et al., 1990; Mraz, 1963), is a large, non-sporing, Gram-negative, rod-shaped bacterium approximately 3-10 µM in length and 0.8-1.2 µM in width, with characteristic swollen termini (Beveridge, 1938b, 1941). Enlarged termini are more likely to be observed in organisms isolated from lesions than in organisms in culture (Beveridge, 1941), and the organism is likely to be smaller in culture, approximately 2-4 µM in length and 0.6-1.0 µM in width (Beveridge, 1941)

When stained with Loeffler's methylene blue, prominent red-staining granules are evident along the length of the organism (Beveridge, 1941). The name of the organism reflects this morphological feature; the word 'nodosus' is a Latin adjective meaning 'full of knots'. *D. nodosus* is an obligate anaerobic bacterium: optimum growth occurs in an atmosphere with 5-10% carbon dioxide, but the organism can tolerate up to 80% carbon dioxide (Beveridge, 1941). The organism is temperature-sensitive: growth is most rapid at 37°C (Beveridge, 1941), slows at 25°C (Beveridge, 1941), and ceases below 10°C (Graham and Egerton, 1968). The optimal pH range for *D. nodosus* is 7.4-7.6, and while the organism can tolerate pH 8 or pH 9, no growth occurs beyond pH 5 (Beveridge, 1941).

D. nodosus is reported to survive in the environment for up to 14 days, but is only thought to remain infective for up to seven days (Beveridge, 1941; Gregory, 1939). There are circumstances in which *D. nodosus* can sur*D. nodosus* was reported to survive in hoof trimmings for up to six

weeks (Beveridge, 1941; Winter, 2009). More recent studies indicate that *D. nodosus* may be capable of surviving in soil for longer than 14 days at low temperature. For instance, (Cederlöf et al., 2013; Gregory, 1939). *D. nodosus* was able to be cultured from soil supplemented with hoof powder for up to 24 days when incubated in a microcentrifuge tube at a temperature of 5°C, but only up to 7 days when incubated at a temperature of 15°C (Cederlöf et al., 2013). However,

1.9 Virulence factors of *D. nodosus*

1.9.1 Extracellular proteases

Virulent strains of *D. nodosus* expresss three subtilisin-like extracellular proteases: acidic protease isoenzyme 2 (AprV2), acidic protease isoenzyme 5 (AprV5) and a basic protease (BprV), encoded by the genes *aprV2*, *aprV5*, and *bprV*, respectively (Billington et al., 1996; Lilley et al., 1992). Benign strains secrete the analogous proteases AprB2, AprB5, and BprB, encoded by the genes *aprB2*, *aprB5*, and *bprB*, respectively (Billington et al., 1996; Kortt and Stewart, 1994; Lilley et al., 1992). All three proteases contribute to degradation of the hoof, and there is evidence of synergistic interactions between the three proteases, either at the processing or substrate level (Kennan et al., 2010).

AprV2 was shown to be essential for virulence *in vivo* through the construction of an *aprV2* gene mutant of virulent *D. nodosus* strain VCS1703A (Kennan et al., 2010); pathogenicity testing in penned sheep demonstrated that the *aprV2* mutant was effectively avirulent. Virulence was restored following complementation with the wild-type *aprV2* gene. Elastase testing revealed that the *aprV2* mutant was unable to digest elastin, indicating that AprV2 has elastolytic activity and may form the basis of the elastase test. Interestingly, complementation of the *aprV2* mutant with the wild-type *aprB2* gene restored virulence but did not restore elastase activity, suggesting that although the elastase activity of AprV2 is a useful indicator of virulence, elastase activity *per se* may not be necessary for virulence *in vivo*.

Sequence analysis of *aprV2* and *aprB2* has shown that the two alleles differ by a two base-pair substitution (TA/CG) at positions 661/662, resulting in a single amino acid change (Kennan et al., 2010; Riffkin et al., 1995). AprV5 has the most proteolytic activity of the three extracellular proteases of *D. nodosus* (Kennan et al., 2010) and is thought to be required for optimal processing and activation of AprV2 and BprV via cleavage of the C-terminal extension and pro-

domain (Han et al., 2012). However, neither AprV5 nor BprV are essential for virulence (Kennan et al., 2010).

1.9.2 Fimbriae (pili)

D. nodosus possesseses an abundance of fine, filamentous appendages called fimbriae or pili, which enable the bacterium to colonise and invade the epidermis (Billington et al., 1996; Egerton et al., 1969; Han et al., 2008). *D. nodosus* exhibits twitching motility, which enables the bacterium to translocate across the surface of a solid medium, such as agar (Han et al., 2008; Kennan et al., 2001; Mattick, 2002; Whitchurch, 2006). This occurs via extension and retraction of the pili (Kaiser, 2000; Merz et al., 2000; Skerker and Berg, 2001). Fimbriae are a key virulence factor of *D. nodosus*; they are essential for adherence to epithelial cells, expression of extracellular proteins, and natural competence (Han et al., 2008; Kennan et al., 2001). Fimbriae are the primary surface (K) antigen of *D. nodosus*, and they are responsible for the K agglutination reaction (Egerton, 1973), which forms the basis of the slide and tube agglutination tests that are used to assign *D. nodosus* to serogroups.

The ability of *D. nodosus* to translocate across a solid medium is reported to correlate with the degree of piliation (Stewart and Claxton, 1993; Stewart et al., 1986). Virulent strains of *D. nodosus* are often heavily piliated, and are thus able to transmigrate further across an across a solid medium than benign strains, which are less heavily piliated (Stewart et al., 1986). The degree of piliation does not appear to be a reliable virulence indicator, however, and is known to vary between strains of the same virulence type, and can differ depending on the choice of growth medium (Stewart et al., 1986). There is also thought to be a relationship between the degree of piliation, colony morphology, and virulence, given that virulent isolates are able to migrate further across an agar plate and form larger colonies. However, colony morphology is an unreliable indicator of virulence and may be influenced by extrinsic factors such as media composition and number of passages (Stewart et al., 1986), as discussed previously.

Fimbriae are composed of a single repeating subunit protein, encoded by the *fimA* gene (Mattick et al., 1984). The subunit protein consists of a positively-charged leader sequence, which is cleaved from the mature subunit protein, a highly-conserved amino-terminal domain, and a highly variable carboxy-terminal domain, which constitutes approximately 70% of the mature

fimbrial subunit protein (Dalrymple and Mattick, 1987). The fimbriae of *D. nodosus* are classified as type IV fimbriae based on a number of physical and genetic characteristics, including the structure of the subunit protein, and their location at the terminus of the bacterium (Mattick et al., 1991). *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Moraxella bovis*, and *Moraxella nonliquifaciens* also possess type IV fimbriae (Dalrymple and Mattick, 1987). This has been exploited for the synthesis of fimbrial protein for use in vaccines through the construction of recombinant *P. aeruginosa* strains, which are easier to culture than *D nodosus* (Mattick et al., 1987).

The biogenesis and function of the fimbrial machinery is highly complex and involves a large number of genes; 20 putative fimbrial biogenesis genes have been identified in the *D. nodosus* genome, along with ten putative fimbrial regulatory genes (Myers et al., 2007).

1.9.3 Virulence-associated genomic regions

Virulent strains of *D. nodosus* possess two genetic elements, *vap* (virulence-associated protein) and *vrl* (virulence-related locus), which are associated with virulence but do not encode any confirmed virulence factors (Katz et al., 1992; Liu and Yong, 1993a; Myers et al., 2007; Rood et al., 1994). It has been suggested that these elements may have a regulatory role as they are inserted in, or adjacent to, global gene expression regulators (Myers et al., 2007). One of the *vap* regions was found to contain an integrase gene, *intA*, that is similar to integrases present in bacteriophages ϕR73, P4, and Sf6 (Cheetham et al., 1995), indicating that *D. nodosus* may have acquired the *vap* regions by integration of a bacteriophage (Cheetham et al., 1995). This is consistent with the findings of Myers et al. (2007) who reported that approximately 20% of the *D. nodosus* genome was acquired by lateral transfer of genetic material. The identification of loci homologous to the *vrl* region of *D. nodosus* in other bacterial species further supports this conclusion (Myers et al., 2007).

The *vap* and *vrl* regions of *D. nodosus* were identified via the construction of recombinant *E. coli* clones containing fragments of genomic DNA from virulent *D. nodosus* prototype strain A198 (A1001). The recombinant *E. coli* clones were screened with genomic DNA from virulent strain A198 (A1001) and benign strain C305 in dot-blot hybridisation assays, and twelve genetic regions were identified (Katz et al., 1991). Plasmids containing fragments of these gene regions

were used as probes to screen genomic DNA prepared from virulent and benign *D. nodosus* field strains. Three of these plasmids, pJIR313 and pJIR314B (both of which represented fragments of the *vrl* region) and pJIR318 (which represented the *vap* region) were preferentially associated with virulent strains.

Screening of *D. nodosus* field isolates has indicated that the *vap* regions are present in approximately 95% of virulent or high-intermediate strains, 88% of intermediate strains, and 38% of benign or low intermediate strains (Katz et al., 1991; Rood et al., 1996). The *vrl* region appears to have a stronger association with virulence, as it present in approximately 77% of virulent or high-intermediate strains, but only 13% of intermediate strains and 7% of benign or low-intermediate strains (Katz et al., 1991; Rood et al., 1996).

The number of *vap* copies in the genome varies between strains; for example, there are three copies (referred to as *vap* regions 1 to 3) of the *vap* region in prototype virulent strain A198 (A1001) (Katz et al., 1991; Katz et al., 1994). The *vrl* element is a large, 27 kb genomic region that is present in single copy in the genome of most virulent strains of *D. nodosus* (Haring et al., 1995; Rood et al., 1994).

1.10 Laboratory diagnosis

Clinical differentiation of virulent, intermediate and benign footrot is relatively straightforward when the disease is fully expressed; however, during the early stages of disease or when environmental conditions are do not favour expression of the disease, it can be difficult to differentiate virulent and benign footrot by visual observation alone. In such cases, laboratory tests may assist diagnosis, though a definitive diagnostic test has not yet been developed.

1.10.1 Smears

D. nodosus is readily identifiable in Gram-stained smears of lesion material (Beveridge, 1941). Lesion material is collected from the interdigital lesion or underrun horn with a cotton-tipped swab and spread in a thin layer on a glass microscope slide. The smear is air-dried, fixed, and Gram-stained. The smear is then examined under oil at 1,000 x magnification using a light microscope (Buller and Eamens, 2014; Stewart and Claxton, 1993). This is a simple test that can be used to differentiate footrot from other disorders of the hoof that have a similar clinical presentation. However, virulent and benign *D. nodosus* strains cannot be differentiated via

examination of smears, thus microbiological culture and phenotypic virulence testing of the infecting strain(s) is necessary to establish the virulence of the infecting *D. nodosus* strain(s) where the clinical diagnosis is uncertain.

1.10.2 Isolation of *D. nodosus*

Phenotypic virulence and serotyping tests require the isolation of *D. nodosus* from specimens of lesion material (Buller and Eamens, 2014). Lesion material is a complex matrix and may be composed of necrotised skin, inflammatory exudates, blood, soil, faeces, and organic material. Lesion material is also laden with environmental and commensal microorganisms (Beveridge, 1941; Calvo-Bado et al., 2011; Egerton et al., 1969; Maboni et al., 2017). As such, isolating *D. nodosus* is challenging and requires specialised equipment and training.

Specimens of lesion material are collected from the interdigital lesion, or underrun hoof capsule or sole, using a cotton swab, scalpel blade, or wooden applicator stick into a transport medium that is suitable for the preservation of fastidious microorganisms, such as modified Stuart's Transport Medium (mSTM) (Amies, 1967). Material collected from deep within the lesion is most likely to yield viable *D. nodosus* and is less likely to be contaminated with environmental organisms (Stewart and Claxton, 1993). A simple method for isolating *D. nodosus* from specimens of lesion material was developed by Thorley (1976): lesion material is inoculated onto 4% agar plates (Thomas, 1958) supplemented with ground hoof material (hoof agar; HA) in a checkerboard pattern and incubated anaerobically for up to 72 hours (Stewart and Claxton, 1993; Thorley, 1976). Because of the high percentage of agar, *D. nodosus* is able to translocate across the surface of plate away from the inoculation site, but other organisms are not (Thorley, 1976). *D. nodosus* colonies are easy to differentiate on 4% HA as they have a flat, spreading appearance, with concentric zones and a characteristic fimbriate edge (Stewart et al., 1986). One or more *D. nodosus* colonies are then picked from the primary culture plate with a sterile inoculation loop and inoculated onto a second HA plate (Stewart and Claxton, 1993). This process is repeated until a pure culture of each *D. nodosus* isolate is obtained.

1.10.3 Detection of *D. nodosus* by PCR

PCR tests may be used alongside microbiological culture for the detection of *D. nodosus*, or direct testing of specimens of lesion material (Calvo-Bado et al., 2011; Frosth et al., 2015; Frosth

et al., 2012; La Fontaine and Rood, 1996; Maboni et al., 2016; Moore et al., 2005b; Stäuble et al., 2014a; Stäuble et al., 2014b; Witcomb et al., 2014). Historically, direct (culture-independent) detection of *D. nodosus* has been hampered by the presence of PCR inhibitors in the sample matrix, but this has been overcome to some extent through the use of modern DNA extraction methods, and several direct testing methodologies have been developed (Calvo-Bado et al., 2011; Frosth et al., 2012; Maboni et al., 2016; Moore et al., 2005a; Witcomb et al., 2014). However, the diagnostic performance of these direct testing procedures varies.

Several target genes have been reported for the detection of *D. nodosus*. A conventional PCR assay targeting a variable region of the *D. nodosus* 16S ribosomal RNA (rRNA) gene was developed by La Fontaine et al. (1993), and adapted by Moore et al. (2005a). More recently, a qPCR assay targeting the 16S rRNA gene was developed by Frosth et al. (2012), and reported to be more sensitive than the conventional PCR test developed by Moore et al. (2005a). The 16S rRNA gene is suitable for the detection of *D. nodosus* as there are three copies of the ribosomal operon in the *D. nodosus* genome (La Fontaine and Rood, 1996), thus the sensitivity of assays targeting this gene is likely to be higher than tests targeting single-copy genes.Tests have also been developed for the detection and quantification of *D. nodosus* that target single-copy genes: Calvo-Bado et al. (2011) developed a real-time quantitative PCR assay targeting the RNA polymerase sigma-70 factor gene (*rpoD*), Cheetham et al. (2006) developed a conventional PCR assay targeting the polynucleotide phosphorylase gene (*pnpA*), and Stäuble et al. (2014b) developed a real-time quantitative PCR assay targeting the *pnpA* gene. PCR tests developed for serotyping (Dhungyel et al., 2002) and virulence testing (Cheetham et al., 2006; Frosth et al., 2015; Stäuble et al., 2014a) may also be used for detection of *D. nodosus* (Dhungyel et al., 2013a; Greber et al., 2016; Locher et al., 2015).

1.10.4 Serology

A humoral immune response occurs following exposure to *D. nodosus* (Whittington et al., 1990), and there is a positive correlation between antibody level and lesion severity (Whittington, 1990). As such, the detection of antibodies against *D. nodosus* antigens using an enzyme-linked immunosorbent assay (ELISA) has been proposed as an alternative or adjunct to microbiological culture for the identification of sheep infected with or previously exposed to *D. nodosus*, particularly those with sub-clinical infections (Whittington and Marshall, 1990). Serological tests

have a number of limitations, however, and antibody responses vary in response to the duration and severity of lesions (Whittington, 1995; Whittington, 1996; Whittington and Egerton, 1994; Whittington and Nicholls, 1995a, b). For instance, Whittington and Egerton (1994) reported that a satisfactory diagnostic sensitivity (>90%) was not achieved until five or six weeks after exposure to *D. nodosus*, and that severe, active lesions must persist for three weeks or more for the majority (>95%) of sheep in a flock to seroconvert. The diagnostic performance of an ELISA varies according to the stage and severity of disease: Whittington and Nicholls (1995a) reported that lesion severity had the greatest impact on antibody levels during the early stages of disease, but after several weeks the duration of severe lesions became more important. An ELISA can be used to identify sheep that have previously been exposed to *D. nodosus*, however there is a rapid fall in serum antibody levels after the disease has resolved and past exposure can only be reliably detected for up to eight weeks (Whittington and Egerton, 1994). Nevertheless, ELISAs have practical applications; for instance, the efficacy of therapeutic agents can be assessed by monitoring antibody levels, with a decline in antibody levels indicative of the infection being eliminated (Whittington and Egerton, 1994). Pilus ELISA has been used to identify sheep infected with *D. nodosus* as part of a control programme in Nepal (Dhungyel et al., 2001). Exogenous factors such as vaccination, passive transfer of antibodies, and cross-reactivity between antigens can affect the diagnostic specificity of an ELISA; for instance, Whittington et al. (1992) reported that the humoral immune response stimulated by the administration of a commercial multivalent vaccine targeting the fimbrial antigen of *D. nodosus* is detectable for up to seven weeks post-vaccination, and Whittington and Egerton (1994) reported elevated antibody levels in flocks that had been vaccinated three years prior and had no subsequent exposure to *D. nodosus*. Furthermore, non-specific antibodies to core bacterial lipopolysaccharides have been detected in sheep of one-to-two years in age (Whittington, 1996; Whittington and Egerton, 1994). Specificity can be enhanced, however, by using purified *D. nodosus* pilus antigens in place of other cell wall components that are likely to cross-react (Whittington et al., 1997).

1.11 Virulence testing

1.11.1 Phenotypic virulence tests

1.11.1.1 Colony morphology

There is evidence that colonies produced by virulent and benign strains of *D. nodosus* on HA differ in their morphologies (Depiazzi et al., 1991; Stewart et al., 1986; Thorley, 1976). Two basic colony types are reported in the literature: fimbriate and non-fimbriate, which are associated with virulent and benign strains, respectively. Fimbriate colonies typically have a central zone with a beaded appearance, a granular mid-zone, a peripheral zone with a texture resembling ground glass, and a fimbriate edge. Non-fimbriate colonies have a smooth central dome and a non-fimbriate edge (Thorley, 1976).

Colonies produced by virulent strains grow faster and spread further on HA than those produced by benign strains (Depiazzi et al., 1991; Thorley, 1976). This is partly due to the degree of piliation of virulent and benign strains, and the different abilities of virulent and benign strains of *D. nodosus* to translocate across the surface of a solid medium (Depiazzi et al., 1991; Thorley, 1976). Colony morphology is reported to be variable, however, and is not thought to be a reliable indicator of virulence (Stewart, 1979; Stewart et al., 1986). For example, colony morphology has been reported to vary in response to repeated sub-culturing (Skerman et al., 1981; Thorley, 1976), variations in media composition and agar concentration (Stewart et al., 1986; Thorley, 1976).

1.11.1.2 Elastase test

The basic principle of the elastase test is that virulent strains of *D. nodosus* express protease with elastolytic activity, while benign isolates express protease that has weak or no elastolytic activity (Stewart, 1979). Briefly, each *D. nodosus* isolate is inoculated in a linear streak onto an agar plate infused with insoluble elastin particles and incubated anaerobically (Stewart, 1979). The plates are examined at four-day intervals for a total of 28 days. Elastase activity manifests as a concentric zone of hydrolysis (clearing) of the insoluble elastin particles around the colonies (Stewart, 1979). The diameter of the zone of clearing varies but can extend up to 12 mm away from the linear colonies (Stewart, 1979). Virulent isolates exhibit marked elastase activity within 8-12 days, while benign strains may exhibit weak elastase activity at 24-28 days or not at all

(Stewart, 1979). The elastase test provides both a quantitative and a temporal measure of elastase activity, and can be used to differentiate virulent and intermediate isolates. Intermediate isolates are also elastase positive but tend to exhibit delayed elastase activity at approximately 14-21 days (Claxton, 1986a). This suggests that the difference between virulent and intermediate strains is most likely quantitative rather than qualitative in regards to the expression of elastase.

There is a strong correlation between elastase activity and the clinical characteristics of an outbreak (Claxton, 1986a; Dhungyel et al., 2013b; Liu et al., 1994; Liu and Yong, 1993a, b; Stewart, 1979), which suggests that elastase activity is an important virulence marker. Correlation between elastase activity and clinical expression is not perfect, however, and isolates exhibiting elastase activity at 7-days have been isolated from flocks with clinically benign footrot (Liu et al., 1994). The elastase test has a high qualitative and quantitative repeatability, however the expression of elastase appears to be more variable for isolates that exhibit weak, delayed elastase activity (20-28days) than those that exhibit marked elastase activity at 7-10 days (Links and Morris, 1996). Interestingly, increased elastase activity has been reported following sheep passage (Stewart, 1979), and it has been suggested that *in vitro* elastase activity is probably an indicator of minimum elastase activity only (Links and Morris, 1996).

1.11.1.3 Gelatin gel test

The gelatin gel test differentiates virulent and benign strains of *D. nodosus* according to protease stability: protease expressed by virulent strains of *D. nodosus* is heat-stable, whilst protease expressed by benign strains is relatively heat-labile (Palmer, 1993). Briefly, *D. nodosus* is cultured in trypticase-arginine-serine (TAS) broth, and a portion of the supernatant is heated at 68°C for periods of 8 and 16 mins. Aliquots of the heat-treated and unheated supernatant are deposited into wells on a sheet of agar infused with gelatin. Protease activity manifests as hydrolysis (clearing) of the gelatin. The zones of hydrolysis are measured and thermostability is determined by the percentage change in protease activity of the heat-treated protease in comparison to the unheated protease. Protease heated for 16 mins provides greater resolution (Links and Morris, 1996). The gelatin gel test developed by Palmer (1993) is a modified version of the degrading proteinase thermostability test developed by Depiazzi and Richards (1985), in which samples of TAS culture supernatant were added to HEPES buffer and heated for 15 mins

at 40°C, filtered, and incubated for 15 mins at 70°C. Hide powder azure was included as a substrate, and protease activity measured with a spectrophotometer.

The level of agreement between the gelatin gel test and clinical severity is known to vary (Depiazzi et al., 1991; Dhungyel et al., 2013b). The gelatin gel test is known to be highly sensitive to laboratory conditions: a temperature change of 2°C during the incubation step can make result in a false-positive or false-negative result (Palmer, 1993). The gelatin gel test cannot differentiate virulent and intermediate strains of *D. nodosus*, as intermediate strains of *D. nodosus* also express heat-stable protease.

1.11.1.4 Electrophoretic zymogram

Both virulent and benign strains of *D. nodosus* express extracellular proteases. The electrophoretic zymogram differentiates virulent and benign strains of according to the relative electrophoretic mobility of their proteases. This test cannot differentiate intermediate strains (Liu et al., 1994; Stewart et al., 1986). Three basic banding patterns were reported by Every (1982): virulent strains produced a zymogram pattern with four bands, while benign isolates had five bands (Gordon et al., 1985). Virulent strains could be further divided into Type I and Type II strains according to the electrophoretic mobility of the fourth band (Gordon et al., 1985). Type I strains possessed a fourth band that was more mobile, with a molecular weight similar to band five of benign isolates, while Type II strains possessed a fourth band that was less mobile, with a molecular weight between bands three and five of benign strains (Gordon et al., 1985). Benign isolates can be differentiated from virulent strains by the presence of two bands (bands one and two) that are less mobile than any of the four bands produced by virulent strains (Gordon et al., 1985). However, these bands can be difficult to detect (Stewart and Claxton, 1993). Subsequent work identified further banding patterns: Depiazzi et al. (1991) reported one isoenzyme pattern associated with strains that produce thermostable protease, and four patterns associated with strains that produce thermolabile protease. All five patterns were composed of three isoenzymes, one of which was common to all groups. The remaining two isoezymes formed pairs that were separated by a relative electrophoretic mobility of 3-4%. There is a good degree of correlation between zymogram patterns and protease stability (Depiazzi et al., 1991; Palmer, 1993; Stewart et al., 1986), although conflicting results have been reported (Palmer, 1993).

1.11.2 Genotypic virulence tests

Phenotypic virulence tests are expensive and laborious to perform, and diagnostic performance of these tests can be poor (Dhungyel et al., 2013b). Molecular virulence tests have been developed in an attempt to improve the accuracy, speed, and cost of testing. In some circumstances molecular tests may reduce or eliminate the need for prior microbiological culture.

1.11.2.1 Gene probes

Two virulence-associated regions (*vap* and *vrl*) were identified in the *D. nodosus* genome in genetic hybridisation experiments (Katz et al., 1991). Gene probes targeting these regions have been evaluated as potential diagnostic tools. Rood et al. (1996) evaluated the plasmids pJIR313, pJIR314B and pJIR318 as potential gene probes. The probes were compared with conventional phenotypic virulence tests using 771 *D. nodosus* field isolates with virulent, intermediate and benign phenotypes. Dot-blot hybridisations were carried out, and the *D. nodosus* field isolates were classified into four categories:

- Category 1: Hybridised with all three probes
- Category 2: Hybridised to pJIR318 only
- Category 3: Did not hybridise to any of the probes
- Category 4: Hybridised to pJIR318 and pJIR314B but not pJIR313

These experiments revealed that the *vap* and *vrl* regions appeared to have some, but not absolute, correlation with virulence. A total of 201 isolates were assigned to Category 1, approximately 88% of which were phenotypically virulent or high-level intermediate. In contrast, only 6% of Category 1 isolates were phenotypically benign or low-level intermediate. The majority of isolates that failed to hybridise with any of the probes (Category 3) were phenotypically benign or low-level intermediate. Of the 141 isolates in Category 2, 70% were phenotypically intermediate, 31% were benign, and 18% were virulent. There was good agreement between the gene probe tests and the elastase test: approximately 88% of virulent isolates (7-days elastase positive) were in Category 1, but 6.3% of isolates in Category 2 and 5.6% of isolates in Category 3 were also classified as virulent by the elastase test. Isolates in Category 2 were predominantly elastase positive at 14 days or more or elastase-negative, and the majority of Category 3 isolates were phenotypically benign. There was good agreement between the gene probe tests and the

gelatin gel test and zymogram: 95% of Category 1 isolates were gelatin-gel stable (virulent) and 98% of Category 1 isolates had a virulent isoenzyme pattern. There was also good agreement between the gene probe tests and colony morphology. The gene probe tests were converted into *vap*-specific and *vrl*-specific conventional PCR tests by Rood et al. (1996), with commensurate results.

1.11.2.2 *intA* PCR

The *intA* PCR test targets a virulence-associated gene (*intA*) that is adjacent to a virulence-associated gene region (*vap*) in some strains of *D. nodosus*. The *vap* regions are described in more detail elsewhere in this review (Cheetham et al., 2006). The *intA* PCR test was developed as an adjunct test to be used in circumstances where there was a discrepancy between clinical diagnosis and the results of the gelatin gel test (gel stable, field benign) (Cheetham et al., 2006). Southern blotting was used to analyse DNA prepared from 595 *D. nodosus* isolates from 124 farms in NSW with virulent or benign footrot for the presence or absence of the *intA* gene (Cheetham et al., 2006). The results were compared with clinical diagnosis and the gelatin gel test. There was moderate agreement with the gelatin gel test: *intA* was detected in 75.3% of stable isolates from flocks with virulent footrot, and 93.2% of stable isolates from flocks with benign footrot. The gene probes were adapted into a conventional PCR assay, and applied to 221 of the isolates assessed by Southern blotting. The results were generally in agreement with that of the gene probe assays. However, the fact that stable, *intA* positive isolates were identifed in a large number of flocks suggests that neither test is able to accurately identify strains capable of causing clinically severe disease.

1.11.2.3 *aprV2/B2* qPCR

D. nodosus expresses three extracellular proteases, the detection of which forms the basis of current phenotypic virulence tests (Palmer, 1993; Stewart, 1979). One of those expressed by virulent strains, AprV2 (encoded by the gene *aprV2*) is essential for virulence. Benign strains of *D. nodosus* express the analogous protease AprB2 (encoded by the gene *aprV2*). These genes differ by a two-base-pair substitution. Two qPCR tests targeting this two-bp substitution have been developed in Europe (Frosth et al., 2015; Stäuble et al., 2014a). Both tests were reported to be capable of identifying *D. nodosus* and differentiating virulent and benign strains in a single

test. The *aprV2/B2* qPCR has recently been used in eradication programmes in Switzerland to identify flocks infected with virulent strains of *D. nodosus*, particularly those with sub-clinical infections (Greber et al., 2016; Locher et al., 2015).

In summary, a few molecular virulence tests have been reported, but these tests typically perform poorly when applied in a field setting. These tests classify strains as virulent or benign according to the presence or absence of a single target gene. The failure of these tests to accurately identify virulent strains of *D. nodosus* suggests that virulence is probably polygenic. The spectrum of virulence reported for field isolates indicates that these genes may also have a cumulative effect.

1.12 Genomics of *D. nodosus*

D. nodosus is classified as a member of the *Cardiobacteriaceae* family within the gamma subdivision of the class *Proteobacteria* (Dewhirst et al., 1990). The gamma subdivision of *Proteobacteria* consists of only three species: *D. nodosus*, *Cardiobacterium hominum*, and *Suttonella indologenes* (Dewhirst et al., 1990). *D. nodosus* was previously assigned to the *Bacteroides* genus (Mraz, 1963), but was reassigned to the genus *Dichelobacter* based on analysis of the 16S rRNA gene (Dewhirst et al., 1990).

D. nodosus has a relatively small genome consisting of a single circular chromosome of 1,389,350 bp, which is predicted to encode 1,299 genes (Myers et al., 2007). *D. nodosus* has one of the smallest genomes of any anaerobic bacterial species that has been sequenced and one of the smallest genomes of any non-intracellular bacterial pathogens (Myers et al., 2007). The *D. nodosus* genome contains eight major regions of sequence variability (Kennan et al., 2014). Examination of these regions has revealed evidence of extensive lateral gene transfer (Kennan et al., 2014; Myers et al., 2007). Nineteen-percent of genes in the *D. nodosus* genome were located in variable regions with atypical trinucleotide composition (Myers et al., 2007). Phylogenetic analyses of these genes revealed that 65% of the genes located in these regions did not cluster with the gamma-Proteobacteria, which provides further evidence of gene transfer events (Myers et al., 2007). There is also evidence that recombination events have occurred between virulent and benign strains (Kennan et al., 2014).

Interestingly, the genome of *D. nodosus* reveals very little evidence of major genome reduction, which is a consequence of reductive evolution. This occurs when there is a close evolutionary

relationship between a bacterial pathogen and its host (Moran, 2002): in the process of adapting to a specific host or specific environment, genes relating to superfluous metabolic pathways or transport mechanisms are shed, whilst genes relating to core processes such as cell replication are conserved (Moran, 2002). In contrast, genes related to metabolic pathways are typically diverse in free-living bacteria as they are required to tolerate environmental fluctuations and adapt to a range of different hosts or ecological niches (Moran, 2002). Bacterial pathogens that are highly adapted to a specific ecological niche require fewer metabolic capabilities as they are the able to obtain intermediate metabolites from the host. For example, *D. nodosus* lacks a number of amino acid biosynthesis pathways, as the bacterium is able to break down host proteins to obtain the amino acids it requires (Myers et al., 2007). There is little evidence of gene-family expansion in the *D. nodosus* genome, which is consistent with adaption to a specific ecological niche (Jordan et al., 2001). Extensive gene-family expansion is evidence of a broad host-range, as expansion is driven by the need to adapt to different environments (Jordan et al., 2001).

The sequence of the *aprV2* gene, a key virulence marker of *D. nodosus*, is highly conserved with the exception of a two-bp substitution that distinguishes the *aprV2* and *aprB2* alleles (Kennan et al., 2010; Riffkin et al., 1995). The *aprV5* and *bprV* genes are more variable (Kennan et al., 2014), but the functional significance of this variability has not been investigated. Genes involved in fimbrial biogenesis are highly conserved, which reflects the important role of fimbriae. None of the fimbrial biogenesis genes are exclusive to virulent or benign isolates and therefore have no relationship with virulence (Kennan et al., 2014).

Whole-genome sequencing of 103 *D. nodosus* isolates from eight different countries revealed that the genome is highly conserved across geographic regions; all isolates shared >95% sequence identity with the reference genome of virulent *D. nodosus* strain VCS1703A (Kennan et al., 2014). Analysis of 31,627 single-nucleotide polymorphisms identified in the *D. nodosus* genome revealed that *D. nodosus* exists as a globally conserved, bimodal population consisting of two genetically distinct clades (Kennan et al., 2014). Clade I correlates with a virulent genotype and phenotype, whilst Clade II correlates with a benign genotype and phenotype (Kennan et al., 2014). The existence of two very distinct genetic clades suggests that intermediate genomic states might not exist, however isolates with intermediate phenotypes were not included in this study. Interestingly, when the genomes of 38 Australian *D. nodosus* isolates

were analysed separately, the Clade I/II division was less distinct (Kennan et al., 2014). Instead, the isolates formed clusters that correlated with geographic region from which they originated. However, given that only 38 Australian isolates were sequenced by Kennan et al. (2014), it is difficult to estimate the true genetic diversity of the *D. nodosus* strains circulating in Australian flocks. Analysis of a larger number of Australian *D. nodosus* isolates would provide greater insights, but this has not been reported.

1.13 Serological diversity of *D. nodosus*

D. nodosus strains are divisible into a number of serologically distinct groups (serogroups) due to the antigenic and sequence diversity of the carboxy-terminal domain of the fimbrial subunit protein (Claxton, 1986b; Claxton et al., 1983; Dalrymple and Mattick, 1987; Elleman, 1988). The Australian serogrouping system recognises 10 *D. nodosus* serogroups (A to I, and M), most of which consist of a number of sub-types (serotypes) (Claxton, 1986b; Claxton et al., 1983; Ghimire et al., 1998). Serogroup B is the most diverse serogroup, consisting of six serotypes (Bhat et al., 2012; Claxton, 1986b). The Australian serogroup classification is supported by immunological studies, which have demonstrated that immunisation against a specific *D. nodosus* serogroup provides protection against homologous challenge, but limited or no protection against heterologous challenge (Dhungyel et al., 2013a; Dhungyel et al., 2008; Egerton, 1979; Egerton et al., 2002; Gurung et al., 2006a; Stewart et al., 1985a; Stewart et al., 1982; Stewart et al., 1991). Multiple *D. nodosus* serogroups may be present in a flock. In Australia, for example, up to seven serogroups have been reported in a single flock, and up to four serogroups on a single foot (Claxton et al., 1983; Dhungyel et al., 2013a). It is not usually necessary to differentiate sub-types within a common serogroup for the purposes of immunisation, however, as there is sufficient cross-protective immunity between sub-types within a common serogroup (Elleman et al., 1990; Stewart et al., 1991). This typing system is also supported by the success of serogroup-specific vaccination programmes, which have been used successfully to control and eliminate virulent footrot from entire flocks and entire geographic regions in Australia, Nepal, and Bhutan (Dhungyel et al., 2013a; Dhungyel et al., 2008; Egerton et al., 2002). *D. nodosus* strains are further divisible into two classes based on the arrangement of the fimbrial gene region: Class I, which consists of serogroups A, B, C, E, F, G, I, and M, and Class II, which consists of serogroups D and H (Ghimire et al., 1998; Hobbs et al.,

1991; Mattick et al., 1991). The fimbrial gene region of Class I strains contains the *fimB* gene, which is located downstream of *fimA* (Hobbs et al., 1991). The fimbrial gene region of Class II strains contains the *fimC*, *fimD*, *and fimZ* genes (Hobbs et al., 1991).

Alternative serotyping systems have been proposed: in the U.K., Thorley and Day (1986) reported the identification of 17 serotypes using the tube agglutination test, and proposed that the Australian classification system be expanded to include serogroups J to R (Day et al., 1986). In the U.S., Gradin et al. (1993) identified 21 serotypes (designated I-XXI), also using the tube and cross-tube agglutination tests. Most of the novel serotypes reported in the U.K. and U.S. are probably sub-types within the 10 serogroups currently recognised by the Australian classification scheme, and the identification of 17 and 21 distinct serotypes, respectively, is probably a consequence of the typing method used by the authors. The slide agglutination test categorises *D. nodosus* isolates into serogroups based on the presence of major, group-specific fimbrial antigens, whilst the tube agglutination test further distinguishes sub-types within each of the major groups according to the presence of minor, type-specific fimbrial antigens (Claxton, 1989). Three of the serotypes identified in U.S. are reported to be antigenically distinct to the 10 serogroups currently recognised under the Australian classification scheme (Gradin et al., 1993), which possibly indicates the existence of serogroups that are yet to be characterised. Novel *D. nodosus* serogroups have not been reported elsewhere, however, thus the existence of serogroups other than those included in the Australian serotyping system is yet to be proven.

Spontaneous serogroup conversion has been reported and is thought to be due to genetic recombination events between the *fimA* genes or gene regions of different *D. nodosus* strains belonging to different serogroups (Kennan et al., 2003). Spontaneous serogroup conversion has not been proven to occur in the field, but unique fimbrial genes sequences have been reported in field isolates that may potentially be the result of recombination between fimbrial subunit genes from different serogroups (Zhou and Hickford, 2000b). It is postulated that genetic recombination events between fimbrial subunit genes, driven by the need to evade the host's immune response, has in fact given rise to serogroup and serotype diversity (Kennan et al., 2003). *D. nodosus* is known to be naturally competent, and homologous recombination appears to occur readily (Kennan et al., 1998; Kennan et al., 2001), thus it is possible that recombination events could occur between different serogroups. Recombination events could occur between

virulent and benign strains of *D. nodosus* (Kennan et al., 2003), resulting in new virulent serogroups.

The prevalence of each serogroup is known to vary between geographic regions; however, it is very difficult to obtain accurate prevalence data due to the limitations and biases of the current culture-based diagnostic methods, and the different typing methods used in different countries. Serogroup B is thought to be most prevalent in Australia: in a survey of 1267 Australian *D. nodosus* field isolates from, serogroup B had an abundance of 28.2% (Claxton et al., 1983). Serogroups A, D, F, G and H had a similar abundance of 10 to 15%, and serogroups C and E had an abundance of 5 to 6% (Claxton et al., 1983). As part of an outbreak-specific vaccine trial, Dhungyel et al. (2013a) typed isolates from 12 Australian flocks and reported that the prevalence of serogroups A, D, C, D, E, G, and H were similar (58 to 75% of flocks), whilst serogroups F and I were less common (0 and 25% of flocks, respectively). Serogroup B and H appear to be the most prevalent serogroups in the U.K.: in a survey of 58 U.K. flocks, Thorley and Day (1986) reported that serogroup H was most prevalent. Hindmarsh and Fraser (1985) reported that serogroup B was most prevalent in the U.K., followed by serogroup H. More recent studies indicate that serogroup B and H remain the dominant serogroups in U.K. flocks: Moore et al. (2005a) reported that serogroup was H most prevalent (47.0%), followed by serogroup B (22.6%) and serogroup F (14.8%). Studies have reported serogroup B (Cagatay and Hickford, 2005; Chetwin et al., 1991), serogroup D (Kingsley et al., 1986), serogroup E (Cagatay and Hickford, 2005) are prevalent in N.Z. Serogroup A was reported as most prevalent in Norway (Gilhuus et al., 2013).

To date, serogroup M has only been detected in Australia, Nepal, Norway, N.Z. and the U.K. (Chetwin et al., 1991; Day et al., 1986; Dhungyel et al., 2015; Ghimire et al., 1998; Gilhuus et al., 2013). The prevalence of serogroup M in Australia is unknown as the serogroup was not included in testing panels until it was identified in south-eastern Australia (Dhungyel et al., 2015). Interestingly, although serogroup M is rarely reported in Australia, in a retrospective survey of *D. nodosus* isolates collected from 59 Australian flocks, Chetwin et al. (1991) detected serogroup M in five flocks, which suggests that the serogroup is probably present in flocks on mainland Australia but is not often detected. Serogroup M isolates are known to cross-react with serogroup F antisera (Chetwin et al., 1991), however, and there is no PCR test for serogroup M at

present, so it is possible that isolates belonging to serogroup M might be incorrectly assigned to serogroup F.

1.14 Serotyping

In order to target the appropriate *D. nodosus* strain(s) with a mono- or bivalent vaccine, in each outbreak of footrot the infecting *D. nodosus* strain(s) must be isolated from specimens of lesion material, purified and serotyped using a slide agglutination test (Claxton et al., 1983; Egerton, 1973) or PCR amplification of serogroup-specific variable regions of the *fimA* gene (Dhungyel et al., 2002; John et al., 1999). In Australia, the slide agglutination test is the traditional serotyping test (Claxton et al., 1983; Stewart and Claxton, 1993); however, the serogroup-specific PCR assays published by Dhungyel et al. (2002) are now recommended by animal health agencies (Buller and Eamens, 2014). Alternative serotyping methods have been reported based on either PCR amplification of the *fimA* gene followed by cloning and sequencing (Cagatay and Hickford, 2005, 2006), or PCR-Single Strand Conformational Polymorphism (PCR-SSCP) analysis (Cagatay and Hickford, 2011) of Class I and Class II-specific regions of the *fimA* gene.

1.15 Control and elimination of footrot

The basic principles of controlling footrot were recognised long before footrot was even accepted as a transmissible disease. For instance, prior to Beveridge (1938a) and Gregory (1939) describing the fundamental principles for the control of footrot in sheep, the following practices were suggested in the literature:

- In an affected flock each foot of each sheep should be carefully inspected on a regular basis to identify sheep with active lesions (Marsh and Tunnicliff, 1934; Mohler and Washburn, 1904; Murnane, 1933; Youatt, 1837)
- Sheep with active lesions should be segregated and managed separately (Mohler and Washburn, 1904; Murnane, 1933; Youatt, 1837)
- Morbidity is reduced by running sheep on elevated, drier pasture or providing access to elevated, dry spots on which sheep can camp (Marsh and Tunnicliff, 1934; Mohler and Washburn, 1904; Murnane, 1933; Youatt, 1837)

- Overgrown hoof should be trimmed to expose pockets of necrotic material, and the foot treated with a topical antibacterial solution (Marsh and Tunnicliff, 1934; Mohler and Washburn, 1904; Murnane, 1933; Youatt, 1837)

- Purchased sheep should be quarantined for a period prior to being introduced to the main flock (Mohler and Washburn, 1904; Youatt, 1837)

- Contaminated paddocks should be rested to prevent transmission to uninfected sheep (Marsh and Tunnicliff, 1934; Mohler and Washburn, 1904; Youatt, 1837)

The principles outlined by Beveridge (1938a) and Gregory (1939) for the control and elimination of footrot form the basis of current control and elimination programmes.

- Control efforts should be focussed on the summer months. The infected flocks should be examined, and any sheep with active lesions should be segregated. These sheep should be treated and returned to the main flock no less than a month after the lesions have resolved. Alternatively, these may be sold to slaughter.

- As a precautionary measure, all sheep should be run through a foot-bath containing an antibacterial solution, such as copper sulphate, following examination.

- The length of time that a contaminated paddock should be spelled before introducing clean sheep differs according to environmental conditions. Where environmental conditions are favourable for transmission of the disease, a minimum spelling period of two weeks is required. Where environmental conditions are hot and dry, a brief spelling period may be sufficient as *D. nodosus* is unlikely to remain viable in the environment and the hoof is less susceptible to infection. A spelling period of five days is sufficient to decontaminate yards and laneways.

- Destocking should be considered where a flock is run on irrigated pasture and a high proportion of the flock is affected. In such circumstances, where complete destocking is not an option, an intensive control programme may be necessary to reduce disease prevalence, involving foot-bathing all affected sheep every two-to-three days

These principles were expanded upon by Egerton (1989a), who delineated a control phase and an elimination phase. The control phase is devoted to reducing the prevalence of sheep with active lesions using treatments such as foot-bathing, with or without foot-paring, and vaccination. The

elimination phase is devoted to eliminating the infection through further foot-bathing coupled with selective or non-selective culling.

There is evidence that the suitability and efficacy of traditional methods of control, such as foot-bathing and foot-trimming, varies between countries according to local climatic conditions. In the U.K., for example foot trimming, with or without foot-bathing, was associated with an increased prevalence of lameness (Green et al., 2007; Kaler and Green, 2009; Wassink et al., 2003; Winter and Green, 2017; Winter et al., 2015). Foot-bathing was effective, but only when used to treat mild interdigital lesions (Winter et al., 2015). The authors specify that foot-trimming was associated with lameness only when bleeding occurred following trimming, which suggests that physical damage of the foot due to excessive trimming was responsible for an increased prevalence of lameness, not trimming *per se*. Cost-benefit analysis of different treatment measures, measured as cost per ewe per year (PEPY), indicated that increased costs due to lameness PEPY of £0.90 and £2.96 were associated with routine foot-bathing and routine foot-bathing, respectively. Recent studies indicate that under U.K. conditions, foot-trimming is ineffective at best. For example, Winter et al. (2015) reported that if bleeding is adjusted for, there is no association between foot-trimming and the prevalence of lameness, which suggests that foot trimming does not reduce the prevalence of lameness in a flock.

In Norway, an elimination programme based on traditional practices such as field examination, targeted culling, foot-bathing, rotation through clean paddocks, and repeated examinations has been successful, with virulent *D. nodosus* eliminated from 65-70% of infected flocks (Vatn, 2016; Vatn et al., 2012). Footrot continued to spread to new flocks, but modelling indicated that the spread and economic burden of the disease would have been greater had the elimination programme not been implemented (Asheim et al., 2015; Groneng et al., 2015). In Switzerland, virulent strains of *D. nodosus* were successfully eliminateded from 28 flocks using a procedure based on careful paring of overgrown hoof, weekly foot-bathing in stand-in foot-baths for a period of 10 minutes per sheep, culling of non-responders, and thorough follow-up examination and PCR testing (Greber et al., 2016).

1.16 Vaccination

Natural infection with *D. nodosus* is known to elicit an antibody response (Whittington et al., 1990; Whittington and Nicholls, 1995a), but the magnitude of this response is not sufficient to provide protection against re-infection (Beveridge, 1941). Vaccination is an effective tool for controlling and eliminate footrot, however, having been used to eliminate footrot from individual flocks and entire geographic regions (Dhungyel et al., 2013a; Dhungyel et al., 2008; Egerton et al., 2002). Footrot vaccines also have therapeutic activity, and can be used to treat sheep with clinical footrot (Egerton and Roberts, 1971). There are, however, a number of challenges associated with vaccination against virulent footrot: immunity is known to be serogroup-specific, with little or no cross-protection offered between serogroups (Stewart et al., 1991), and multiple serogroups may be present in a flock. In Australia, for example, up to seven serogroups have been detected in a single flock, and up to four serogroups have been detected on a single foot (Claxton et al., 1983; Dhungyel et al., 2013a). Multivalent vaccines containing fimbrial antigens representing nine serogroups (A-I) have been trialled but provide only partial protection for a brief period of time (Raadsma et al., 1994; Schwartzkoff et al., 1993). This is due to the phenomenon of antigenic competition, in which there is a poorly characterised interaction between structurally similar antigens and the immune system (Hunt et al., 1994). As a consequence of antigenic competition, the antibody response to one antigen is dominant whilst the response to the remaining antigens is suppressed; in some cases, this competition can result in the antibody response to all antigens being suppressed to some extent (Hunt et al., 1994).

In contrast, outbreak-specific vaccination using mono- or bi-valent vaccines targeting only those serogroups that are present in an infected flock can successfully control and eliminate virulent footrot. To target the appropriate *D. nodosus* strains, in each outbreak of virulent footrot the infecting *D. nodosus* strain(s) must be isolated and serotyped and the appropriate serogroup-specific vaccine(s) administered (Dhungyel et al., 2013a). Where three or more serogroups are identified in a flock, sequential administration of mono- and bivalent vaccines can be undertaken with an inter-vaccination interval of three-months, to avoid antigenic competition (Dhungyel and Whittington, 2009). This approach has been used to eradicate virulent footrot from Nepal (Egerton et al., 2002) and Bhutan (Gurung et al., 2006a), and to control and eliminate virulent footrot from individual flocks in Australia (Dhungyel et al., 2013a; Dhungyel et al., 2008).

Chapter 2

General materials and methods

2.1 Reagents

2.1.1 Preparation of Milli-Q water (MQW)

Milli-Q water was produced using a Milli-Q® Biocel Ultrapure Water Purification System (Millipore, Billerica, U.S.A.). The specifications of the Milli-Q water are as follows:

Resistivity at 25°C: 18.2

Total organic carbon: 5 to 10 parts per billion

Pyrogens < 0.001 EU/mL

Bacteria < 1 colony-forming unit (cfu)/mL

2.1.2 Preparation of modified Stuart's Transport Medium (mSTM)

Sodium glycerophosphate (Merck, Frenches Forest, Australia)	5 g
Sodium thioglycollate (Ajax Chemicals, Sydney, Australia)	0.5 g
Sodium chloride (Amresco, Solon, U.S.A.)	0.005 g
Methylene blue solution (0.02%)	5.0 mL
Cysteine hydrochloride (anhydrous) (BD, Cockeysville, U.S.A.)	0.205 g
L-cysteine (Sigma Aldrich, Castle Hill, Australia)	0.5 g
Agar (Difco; BD, Cockeysville, U.S.A)	2 g
MQW	500 mL

L-cysteine was weighed into a 5 mL serum vial (Techno Plas, St Marys, South Australia) and dissolved in 3 mL of 6M NaOH solution. Thereafter, the dissolved L-cysteine and all other reagents were dissolved in water, and the pH adjusted to 7.4 with 10M NaOH. The agar was weighed into a 2 L flask, and the pH adjusted solution added to the flask. The flask was autoclaved at 121°C for 20 mins, and approximately 5 mL of the solution was dispensed into 5 mL serum vials (Techno Plas, St Marys, South Australia) while still hot. The solution was stored at 4°C until required.

2.1.3 Preparation of ground ovine hoof

Sheep feet were collected from the abattoir and autoclaved at 121°C for 15 mins to enable the hoof to be peeled away. The hoof was cut into narrow strips and dried in the sun. The dried hoof clippings were ground in a hammer mill until powdery. The hoof powder was stored in a screw-cap jar at room temperature until required.

2.1.4 Preparation of 2% and 4% hoof agar medium

Polypeptone peptone (BD, Cockeysville, U.S.A.)		10 g
Beef extract (BD, Cockeysville, U.S.A.)		4 g
Yeast extract (BD, Cockeysville, U.S.A.)		1 g
Sodium chloride (Amresco, Solon, U.S.A.)		5 g
Ground ovine hoof		15 g
Agar (Difco; BD, Cockeysville, U.S.A.)	2%	20 g
	4%	40 g
Milli-Q water		1 L

Hoof agar medium (Thomas, 1958) was made with an agar concentration of 2% (2% HA) or 4% (4% HA). The other ingredients and manner of preparation was otherwise identical. Four percent HA plates were used for the primary isolation of *D. nodosus* from specimens of lesion material and for sub-culturing. Two percent HA plates were used for sub-culturing.

Agar and ground ovine hoof were weighed into a conical flask. The peptone, beef extract, yeast extract, and sodium chloride were first dissolved in 1 L of Milli-Q water. The pH of the solution was adjusted to 7.8-8.0 using 10M NaOH, and added to the conical flask. The mouth of the conical flasks was covered with aluminium foil, and autoclaved at 121°C for 15 mins. Thereafter, the medium was cooled to 50-60°C, and poured into sterile plastic Petri dishes (approximately 30 mL per dish). Once set, the plates were dried at 55°C for 30-40 minutes. The plates were stored in sealed plastic bags at 4°C prior to use.

2.1.5 Preparation of phosphate buffered saline (PBS) (1x)

PBS tablet (Amresco, Solon, U.S.A)   1 tablet

MQW   100 mL

The reagents were added to a 500 mL flask. The pH of the solution was adjusted to 7.4 using 10M NaOH. The solution was autoclaved at 121°C for 15 mins and stored at room temperature prior to use.

2.1.6 Preparation of phosphate buffered saline (PBS) (10x)

Sodium chloride (Amresco, Solon, U.S.A.)   80 g

Potassium chloride (Sigma Aldrich, Castle Hill, Australia)   2 g

Disodium hydrogen phosphate (Bacto Laboratories, Sydney, Australia)   11.5 g

Potassium dihydrogen orthophosphate (Ajax Chemicals, Sydney, Australia)   3.1 g

MQW   1 L

The reagents were added to a 2 L flask. The pH of the solution was adjusted to 7.4 using 10M NaOH. The solution was autoclaved at 121°C for 15 mins and stored at room temperature prior to use.

2.1.7 Preparation of phosphate-buffered saline (PBS) with 0.05% formalin

Formalin (Sigma Aldrich, Castle Hill, Australia)   12.5 mL

PBS 1 x   1 L

2.1.8 Preparation of trypticase serine arginine (TAS) broth

Trypticase peptone (BD, Cockeysville, U.S.A.)   15 g

Polypeptone peptone (BD, Cockeysville, U.S.A.)   5 g

Beef extract (BD, Cockeysville, U.S.A.)   5 g

Yeast extract (BD, Cockeysville, U.S.A.)   2 g

L-Arginine HCl (BD, Cockeysville, U.S.A.)   5 g

DL Serine (BD, Cockeysville, U.S.A.)	1.5 g
MgSO$_4$.7H$_2$O (BD, Cockeysville, U.S.A.)	2 g
MQW	1 L

The reagents were weighed out into a 2 L flask, and the MQW added. The pH was adjusted to 7.8-8.0 with 10M NaOH and autoclaved at 121°C for 30 mins and stored at 4°C prior to use. Note: if the broth had been stored for any length of time, it was boiled before use to remove any undissolved oxygen prior to use.

2.1.8 Preparation of TBE buffer (10 x)

Tris base (Amresco, Solon, U.S.A)	108 g
Boric acid (Progen, London, U.K.)	55 g
0.5 M EDTA, pH 8.0	40 mL
MQW	made up to 1 L

The reagents were weighed out into a 2 L flask, and the MQW added.

2.1.9 Preparation of agarose gels for visualisation of PCR products

Table 2.1: Quantities of reagents for preparation of agarose gels.

	Quantity		
Reagent	**Medium 2%**	**Large 2%**	**Medium 3%**
Agarose (Difco; BD, Cockeysville, U.S.A)	2 g	2.25 g	3.0 g
10 x TBE buffer	100 mL	125 mL	100 mL
RedSafe	6 μL	6 μL	6 μL

The agarose was weighed into a 500 mL conical flask. The TBE was added to the flask, and stirred gently to mix. The conical flask was placed in the microwave, and heated for approximately 90 s to dissolve the agarose. The flask was removed from the microwave periodically and stirred to avoid the solution bubbling up and spilling out of the flask. Once the agarose was dissolved, the flask was removed from the microwave. RedSafe was added to the

flask, and the solution was stirred. The solution was then poured into the tray of a gel tank and left to set at room temperature for 30 mins.

2.1.10 *D. nodosus* reference strains

D. nodosus reference strain A1001 (A198) and field strain JIR3528 were used as virulent and benign controls, respectively.

2.1.11 Foot lesion scoring system

Scores were assigned to each foot of each sheep using the scoring system devised by Egerton and Roberts (1971), as described in Table 2.1

Table 2.2: Scoring system used to grade the severity of footrot lesions, as devised by Egerton and Roberts (1971).

Foot score	Clinical description
0	Healthy interdigital skin with no evidence of inflammation, hyperkeratosis or inflammatory exudate
1	Interdigital dermatitis, mostly restricted to the posterior interdigital skin, characterized by hyperkeratosis and a small amount of inflammatory exudate
2	More extensive interdigital dermatitis; interdigital skin is erythematous with a moderate amount of inflammatory exudate; hair loss; erosion of the interdigital skin extends to the soft horn of the posterior abaxial wall of the hoof
3	Extensive erosion of the interdigital skin, characterized by erythema, hyperkeratosis, hair less and necrosis; separation of the soft horn and sole (underrunning), beginning at the heel and partially extending toward the axial wall of the hoof
4	Underrunning of the hard horn and sole, extending to the axial wall of the hoof; the underlying connective tissue is covered with grey-white necrotic exudate with a fetid odor

2.2 Isolation of *D. nodosus* from foot swabs

Foot swabs were transported in mSTM. Upon receipt at the laboratory, each foot swab was removed from the mSTM and streaked onto a 4% HA plate (Thomas, 1958) in a checkerboard pattern, and incubated in an anaerobic jar with an anaerobic gas pack (GasPak, BD, New Jersey, U.S.A.) and an anaerobic indicator (Oxoid, Hampshire, U.K.) at 37°C for 72 hours (Stewart and Claxton, 1993). Thereafter, individual *D. nodosus* colonies were picked from the primary culture using a sterile inoculation loop and sub-cultured onto 2% HA and incubated under the conditions described above. This process was repeated until individual colonies of *D. nodosus* were obtained.

2.3 Slide agglutination test

Each *D. nodosus* isolate was serotyped using the slide agglutination test as described previously (Stewart and Claxton, 1993). Briefly, individual *D. nodosus* colonies were sub-cultured on 2% HA (Stewart and Claxton, 1993) and incubated under the conditions described above. Each pure culture was harvested by flooding the surface of the agar plate with 500 mL of PBS, pH 7.4, with 0.5% w/v formalin, scraping the *D. nodosus* colonies from the surface of the agar with a sterile scalpel blade, and collecting the suspended culture into a 1.5 mL screw-cap microcentrifuge tube (SSIBio, Lodi, U.S.A.). Each suspension was mixed for 10 s in a vortex mixer and visually assessed to ensure an even suspension. Antisera, which were prepared in rabbits for each of the 10 *D. nodosus* prototype serogroup antigens as described previously (Claxton et al., 1983) and stored at -20°C, were brought to room temperature. Twenty-microlitres of the harvested *D. nodosus* suspension were mixed with 5 μL of undiluted rabbit antiserum on a clean glass microscope slide. The slide was gently rocked for 10 s and examined. A reaction was regarded as positive when a substantial coarse agglutination reaction was observed within 10 s of the serum and *D. nodosus* suspension being mixed. A slide agglutination test result was classified as ambiguous when a very fine or delayed agglutination reaction was observed, and negative if no reaction was observed.

2.4 Gelatin gel test

The gelatin gel test differentiates virulent and benign strains of *D. nodosus* on the basis that the extracellular proteases expressed by deemed virulent strains are heat-stable whilst those

expressed by deemed benign strains are heat-labile (Palmer, 1993). Briefly, a pure culture of *D. nodosus* was grown in trypticase-arginine-serine (TAS) broth (Skerman, 1975) under anaerobic conditions for 2-4 days at 37°C to achieve a concentration of 10^8 cells per mL, as determined by spectrophotometry. Thereafter, the broth cultures were diluted in 1.0 mL of HEPES buffer in glass test tubes, mixed, and a 20 µL aliquot of each diluted culture was deposited in the top well of the gelatin gel. The remainder of each diluted culture was placed into a water bath at 68°C. After 8 mins of incubation, a second 20 µL aliquot of the diluted culture was deposited in the middle row of the gelatin gel. After 16 mins of incubation, a third 20 µL aliquot of diluted culture was deposited in the bottom row of the gelatin gel. Known stable (A1001) and unstable (JIR3528) *D. nodosus* strains were included as controls. Thereafter, the gels were incubated for 18 hours in a moist chamber at 37°C. The gel was developed by flooding with hot (60-70°C) saturated ammonium sulphate solution. When the gel appeared milky-white, it was rinsed with tap water. Proteolysis was indicated by a zone of clearing around the well containing the sample inoculums. The diameter of the widest point of the zone of clearing was measured using callipers. If the diameter of the zone of clearance of the sample heated for 16 mins was ≥50% of the diameter of the un-heated sample, the protease expressed by the isolate was defined as stable (virulent). If the diameter of the zone of clearance of the sample heated for 16 mins was <50% of the diameter of the un-heated sample, the protease expressed by the isolate was defined as unstable (benign). A valid gelatin gel test was one in which there was a <50% reduction in the diameter of the zone of clearance of the stable control, and a ≥50% reduction in the diameter of the zone of clearance of the unstable control.

2.5 Elastase test

The principle of the elastase test is that deemed virulent *D. nodosus* isolates express an extracellular protease that hydrolyses elastin within 10-12 days. Deemed benign isolates show delayed elastolytic activity (~28 days) or none at all (Stewart, 1979). Pure cultures of *D. nodosus* were sub-cultured onto one quarter of an elastin agar plate in a linear streak, with three pure cultures sub-cultured on each plate. In the remaining quarter, an isolate with known elastase activity (virulent *D. nodosus* type strain A1001; elastase-positive at 4-8 days post-inoculation) was inoculated as a virulent control. Plates were incubated in anaerobic jars containing an anaerobic gas pack (Gas Pak; BD, Cockeysville, U.S.A.) and an anaerobic indicator at 37°C.

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Plates were examined after 4, 8, 12, 16, 20, 24, and 28 days of incubation, and re-incubated anaerobically after each examination. An isolate was classified as elastase-positive (virulent) if growth was observed and a zone of clearing was observed around the inoculum within 12 days post-inoculation. If a zone of clearing was observed after this point or not at all, the isolate was classified as elastase-negative (benign). A valid elastase test was one in which the virulent type strain was elastase-positive at 4-8 days post-inoculation, and there was no growth of bacteria other than *D. nodosus* on the elastin agar plate.

2.6 DNA extraction

2.6.1 Extraction of DNA from pure cultures of *D. nodosus* by boiling and centrifugation

Pure cultures were harvested from HA plates by flooding the surface of the plate with sterile, nuclease free water, scraping the growth from the surface of the plate with a sterile scalpel blade, collecting the suspended culture with a 1.0 mL pipette, and transferring into a 1.5 mL screw-cap microcentrifuge tube (SSIBio, Lodi, U.S.A.). The microcentrifuge tube was incubated for 10 mins at 100°C in a dry heat block to lyse the cells. The microcentrifuge tube was centrifuged at 3,000 x g for 3 mins to pellet the cellular debris. The supernatant, which contained the extracted DNA, was aspirated and transferred to a new 1.5 mL screw-cap microcentrifuge tube and stored at -20°C.

2.6.2 Extraction of DNA from pure cultures of *D. nodosus* with the Wizard Genomic DNA Purification kit

DNA was extracted from pure cultures of *D. nodosus* using the Wizard Genomic DNA Purification kit (Promega, Madison, U.S.A.), in accordance with the protocol for Gram-negative bacteria. Briefly, a pure culture of *D. nodosus* was harvested with a cotton-tipped swab and deposited in microcentrifuge tube with 600 μL of nuclei lysis solution, incubated at 80°C in a dry heat block for 60 mins, then cooled to room temperature. Three-micro-litres of RNase solution was added to each microcentrifuge tube and the tubes were gently mixed by inversion, incubated for 60 mins at 37°C in a dry heat block, and cooled to room temperature. Thereafter, 200 μL of Protein Precipitation Solution was added to each microcentrifuge tube and vortexed at high speed for 20 s to mix the solution with the cell lysate, before being incubated on ice for 5 mins. Each microcentrifuge tube was then centrifuged at 16,000 x g for 3 mins, and the supernatant aspirated

and transferred to a clean 1.5 mL microcentrifuge tube containing 600 µL of room-temperature isopropanol, and gently mixed by inversion until thread-like strands of DNA became visible. Each tube was then centrifuged at 16,000 x g for 2 mins to pellet the DNA. The supernatant was poured off and 600 µL of room temperature 70% ethanol added, and the tube inverted several times to wash the DNA pellet. Each microcentrifuge tube was centrifuged at 16,000 x g for 2 mins, and the ethanol carefully aspirated. Each tube was then drained and left to air-dry for 15 mins. Thereafter, 100 µL of DNA Rehydration Solution was added to each microcentrifuge tube, and incubated overnight at 4°C. The resuspended DNA was stored in 20 µL aliquots at -20°C.

2.6.3 Direct extraction of DNA from cotton foot swabs

Direct extraction of DNA from cotton foot swabs was undertaken using a magnetic bead DNA purification kit (BioSprint 96 One-For-All Vet Kit, Qiagen, Hilden, Germany), following the manufacturer's instructions. Briefly, swabs were placed into a 1.5 mL screwcap microcentrifuge tube containing 300 µL lysis solution (Buffer RLT; Qiagen, Hilden, Germany), and incubated at room temperature overnight. Thereafter, the microcentrifuge tube was vortexed for 30 s, the swab discarded, and 100 µL of the lysate transferred to a deep 96-well plate. Forty microliters of proteinase K and 900 µL of a magnetic bead solution (consisting of 300 µL of isopropanol, 300 µL of Buffer RLT, 2.7 µL of carrier RNA, and 25 µL of MagAttract Suspension G) were added to each well. Three additional deep 96-well plates were prepared: the first contained 700 µL Buffer AW1 per well; the second and third both contained 500 µL of Buffer RPE per well. A standard 96-well elution plate was also prepared containing 75 µL of Buffer AWE per well. All plates were run on an automated magnetic particle processor (MagMAX Express96; Invitrogen, Carlsbad, U.S.A), with the BS96 Vet 100 protocol (Qiagen, Hilden, Germany). The 75 µL of eluate from each well was transferred to individual 0.2 mL tubes, and stored at -20°C.

2.7 Multiplex cPCR amplification of the *fimA* gene

The *fimA* gene of *D. nodosus* was amplified using the multiplex conventional PCR (cPCR) assay published by Dhungyel et al. (2002). A common forward primer and nine serogroup-specific reverse primers were used (Table 2.3), run as three triplex assays: (i) serogroups A, B and C; (ii) serogroups D, E and F; (iii) serogroups G, H and I. Each 20 µL reaction volume consisted 2x Multiplex Mastermix (Qiagen, Hilden, Germany), 5x Q Solution (Qiagen, Hilden, Germany), 0.4 µM of the forward primer and 0.2µM of each of three reverse primers, and 1.0 µL of DNA

template. Details of each primer are provided in Table 2.3. Reaction conditions consisted for an initial denaturation step of 95°C for 10 mins, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 90 s, and a final extension phase of 72°C for 5 mins. Amplification was performed in a Bio-Rad T100 thermal cycler (Bio-Rad, Gladesville, Australia). DNA extracted from pure cultures of each of the appropriate *D. nodosus* serogroup prototype strains, and sterile nuclease-free water, were included in each run as positive and negative controls, respectively. PCR product was visualised on a 2% agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, South Korea), and viewed under ultraviolet light. A successful PCR run was defined as one in which: (i) there was amplification of the positive controls, indicated by the presence of three amplicons of the appropriate molecular weights on the 2% agarose gel, and (ii) there was no amplification of the negative control.

2.8 qPCR amplification of the *D. nodosus* 16S rRNA gene

The *D. nodosus* 16S rRNA gene was amplified using the qPCR assay published by Frosth et al. (2012). Each sample was analysed in duplicate in a 20 μL reaction volume. Each 20μL reaction volume contained 1 x PerfeCTa qPCR FastMix, UNG, Low Rox (Quanta BioSciences Inc., Gaithersburg, U.S.A.), 0.1 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, U.S.A.), 0.4 μM of each primer, 0.15 μm of the TaqMan probe, and 2.5 μL of template DNA. Primer details are provided in Table 2.3. PCR amplification was performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, U.S.A.) in fast mode. Cycling conditions consisted of an initial denaturation step of 95°C for 10 mins, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. DNA extracted from *D. nodosus* prototype strain A1001, and sterile nuclease free water, were included in each run as positive and negative controls, respectively. Fluorescence signals were analysed using the threshold automatically set by the 7500 software (v2.0.4).

Table 2.3: List of PCR primers/probes common to two or more chapters of this thesis.

Primer	Direction	Gene	Sequence (5'-3')	Product size (bp)	Reference	Chapters
16S Ac	F	16S rRNA	CGGGGTTATGTAGCTTGC	783	La Fontaine et al. (1993)	
16S-C	R		TCGGTACCGAGTATTTCTACCCAACACC	-		
16Sf	F	16S rRNA	CGGGGTTATGTAGCTTGCTATG	84	Frosth et al. (2012)	
16sr	R		TACGTTGTCCCCCACCATAA	-		
16Sprobe	Probe		FAM-TGGCGGACGGGTGAGTAATATATAGGAATC-QSY	-		
FP	F	*fimA* (all)	CCTTAATCGAACTCATGATTG	-	Dhungyel et al. (2002)	
RA	R	*fimA* (A)	AGTTTCGCCTTCATTATATTT	415		
RB	R	*fimA* (B)	CGGATCGCCAGCTTCTGTCTT	283		
RC	R	*fimA* (C)	AGAAGTGCCTTTGCCGTATTC	325		
RD	R	*fimA* (D)	TGCAACAATATTTCCCTCATC	319		
RE	R	*fimA* (E)	CACTTTGGTATCGATCAACTTGG	363		
RF	R	*fimA* (F)	ACTGATTTCGGCTAGACC	241		
RG	R	*fimA* (G)	CTTAGGGGTAAGTCCTGCAAG	279		
RH	R	*fimA* (H)	TGAGCAAGACCAAGTAGC	409		
RI	R	*fimA* (I)	CGATGGGTCAGCATCTGGACC	189		

Chapter 3

Evaluation of genotypic and phenotypic protease virulence tests for *Dichelobacter nodosus* infection in sheep

3.1 Introduction

Footrot is a major economic and animal welfare burden in many sheep-producing countries, including Australia, the U.K., and the United States (Gradin et al., 1993; Lane et al., 2015; Nieuwhof and Bishop, 2005; Wassink et al., 2010). The disease is highly contagious and is transmitted between sheep via contaminated pasture and soil (Beveridge, 1941). The essential transmitting agent, *Dichelobacter nodosus* (formerly *Fusiformis nodosus*, *Bacteroides nodosus*) (Beveridge, 1941; Dewhirst et al., 1990), is a fastidious, strictly anaerobic bacterium and an obligate parasite of the ruminant hoof (Beveridge, 1941). Footrot initially presents as a mild to severe dermatitis of the interdigital skin, and may progress in susceptible sheep to separation of the sole and horn of the hoof ("underrunning") when warm, moist environmental conditions enable progression of the disease (Graham and Egerton, 1968; Stewart et al., 1986). For descriptive and regulatory purposes, two clinical forms of footrot are recognised by most Australian regulatory authorities: virulent and benign (Stewart and Claxton, 1993). A third clinical form, intermediate (also referred to as low-virulent), is also described in the literature but is no longer recognised as a distinct form by most Australian regulatory authorities (Stewart and Claxton, 1993). The clinical severity of an outbreak of footrot is partially determined by the virulence of the infecting *D. nodosus* strain(s), which are also classified as virulent or benign according to their *in vitro* phenotypic and genotypic characteristics (Stewart and Claxton, 1993).

The severity of footrot lesions is described using a simple scoring system devised by Egerton and Roberts (1971). Briefly, mild lesions restricted to the interdigital skin are given a score of 1; if the interdigital lesion is severe, a score of 2 is given; where underrunning of the posterior sole and soft horn of the heel is observed, a score of 3 is given; if the underrunning extends to the abaxial hoof wall, a score of 4 is given. In most States in Australia, classification of an outbreak of footrot as clinically virulent or benign is based on the proportion of infected sheep with at least one score 4 lesion (Egerton, 1989a; Stewart and Claxton, 1993). This classification system acknowledges that both virulent and benign strains of *D. nodosus* are capable of inducing severe, underrun lesions in a proportion of susceptible sheep under favourable environmental conditions,

an observation that is frequently overlooked in the literature. The cut-point used to differentiate virulent and benign footrot differs between States. In New South Wales, for example, an outbreak is regarded as virulent where more than 10% of sheep present with score 4 lesions (Buller and Eamens, 2014; Egerton, 1989a) or benign if the prevalence is <10%. In Victoria and South Australia, a cut-point of 1% score 4 lesions is used (Buller and Eamens, 2014; Riley and Buchanan, 2003).

Clinical diagnosis of virulent footrot is relatively straightforward when the disease is fully expressed; however, it can be difficult to differentiate virulent and benign footrot by examination alone during the initial stages of infection, or where environmental conditions do not enable the disease to fully express (Egerton, 1989a; Stewart and Claxton, 1993). The initial stages of clinically virulent footrot may also resemble other non-transmissible diseases of the hoof, such as OID, which are not associated with infection by *D. nodosus* (Parsonson et al., 1967). Control measures, such as antiseptic foot bathing, may also complicate diagnosis of virulent footrot by masking or suppressing expression of the disease. In such cases, laboratory identification and virulence testing of the infecting *D. nodosus* strain(s) may assist diagnosis. It should be noted that in Western Australia diagnosis of virulent footrot is based entirely on the results of laboratory virulence tests, irrespective of clinical severity.

D. nodosus expresses three extracellular proteases, the detection of which forms the basis of current phenotypic protease virulence tests (Palmer, 1993; Stewart, 1979). The elastase test, which measures temporal and quantitative variations in activity of the extracellular proteases expressed by virulent and benign *D. nodosus* strains (Stewart, 1979), currently is not widely used in Australia but has been shown to correlate well with clinical diagnoses (Dhungyel et al., 2013b; Links and Morris, 1996). The gelatin gel test, which measures differences in the thermostability of extracellular proteases expressed by virulent and benign *D. nodosus* strains (Palmer, 1993), is used by most Australian regulatory authorities, but can be unreliable (Cheetham et al., 2006; Dhungyel et al., 2013b; Links and Morris, 1996).

Current phenotypic virulence tests require microbiological culture of *D. nodosus* from specimens of lesion material, a specialised process that is difficult and laborious (Stewart and Claxton, 1993). There is evidence of discrepancies between phenotypic virulence tests and clinical observations, with phenotypically virulent *D. nodosus* isolates identified amongst isolates

obtained from clinically benign outbreaks. For instance, in a survey of 595 *D. nodosus* isolates collected from 37 flocks in NSW, Cheetham et al (2006) reported that a considerable number (63.0%; 233/370) of isolates collected from outbreaks of clinically benign footrot were phenotypically virulent as they produced heat-stable proteases or proteases of equivocal heat-stability. Liu et al. (1994) reported the isolation of *D. nodosus* strains classified as virulent by both the elastase test and the gelatin gel test from flocks with clinically benign footrot, and Stewart (1979) demonstrated that *D. nodosus* isolates classified as benign by the elastase test were capable of inducing severe, underrun lesions in penned sheep. These discrepancies may reflect the lack of reproducibility of the tests themselves, which are highly dependent on culture conditions (Palmer, 1993; Stewart, 1979), or may be the result of other host, pathogen or environmental factors that are yet to be determined. There is also evidence of discrepancies between the different phenotypic virulence tests. Dhungyel et al (2013b) undertook a comparative study in which 2851 *D. nodosus* isolates were subjected to both the elastase and gelatin gel tests. The level of agreement between the two tests was slight (kappa statistic = 0.12), as 84.3% (857/1017) of *D. nodosus* isolates classified as benign by the elastase test were classified as virulent by the gelatin gel test. Links and Morris (1996) reported a near-perfect level of agreement (kappa statistic = 0.82) between the gelatin gel test and the elastase test; however 20.8% (99/476) of elastase-negative *D. nodosus* isolates were classified as virulent by the gelatin gel test.

Virulent strains of *D. nodosus* secrete three subtilisin-like extracellular proteases: acidic protease isoenzyme 2 (AprV2), acidic protease isoenzyme 5 (AprV5) and a basic protease (BprV), encoded by the genes *aprV2*, *aprV5*, and *bprV*, respectively (Billington et al., 1996; Lilley et al., 1992). Benign strains secrete the analogous proteases AprB2, AprB5, and BprB, encoded by the genes *aprB2*, *aprB5*, and *bprB*, respectively (Billington et al., 1996; Kortt and Stewart, 1994; Lilley et al., 1992). All three proteases contribute to degradation of the hoof, and there is evidence of synergistic interactions between the three proteases, either at the processing or substrate level (Kennan et al., 2010).

AprV2 was shown to be essential for virulence *in vivo* through the construction of an *aprV2* gene mutant of virulent *D. nodosus* strain VCS1703A (Kennan et al., 2010); pathogenicity testing in penned sheep demonstrated that the *aprV2* mutant was effectively avirulent. Complementation

with the wild-type *aprV2* gene subsequently restored virulence. Elastase testing revealed that the *aprV2* mutant had little or no detectable elastase activity (Kennan et al., 2010). In contrast, wild-type elastase activity was maintained in the *aprV5*, *bprV*, and *aprV5bprV* mutants, indicating that the AprV2 protease is involved in the digestion of elastin, either directly or indirectly (Kennan et al., 2010). Interestingly, complementation of the *aprV2* mutant with the wild-type *aprB2* gene restored virulence but did not restore elastase activity, suggesting that although the elastase activity of AprV2 is a useful indicator of virulence, elastase activity may not be necessary for virulence *in vivo*.

Sequence analysis of *aprV2* and *aprB2* has shown that the two alleles differ by a two base-pair substitution (TA/CG) at positions 661/662, resulting in a single amino acid change (Kennan et al., 2010; Riffkin et al., 1995). Recently, two competitive quantitative real time polymerase chain reaction (qPCR) tests targeting this two-bp substitution were developed in Europe (Frosth et al., 2015; Stäuble et al., 2014a). Both tests are reported to be capable of identifying *D. nodosus* and differentiating virulent and benign strains. One of these tests has been adopted in Victoria and Western Australia and has been the subject of media interest following press releases from the Departments of Primary Industry in each state (DAFWA, 2015; Rawlin, 2016).

In Australia, virulent footrot is a notifiable disease in most jurisdictions, but legislative approaches and the means by which footrot is controlled vary. In NSW, flocks with clinically virulent footrot are quarantined, and must undergo a compulsory elimination programme, with costs met by the producer. The estimated cost of eliminating virulent footrot from a flock may exceed $10/head per annum until the disease has been eliminated (Allworth, 1990). Western Australia and South Australia both have similar legislative approaches involving movement restrictions. In Tasmania, however, virulent footrot is not a notifiable disease and infected flocks are not subject to such restrictions. The means by which outbreaks of footrot are designated as virulent or benign also differ between jurisdictions. In NSW, for example, diagnosis of virulent footrot is primarily based on examination of sheep; laboratory tests may be used to assist with a diagnosis, but cannot be the sole basis of a diagnosis (Buller and Eamens, 2014; Stewart and Claxton, 1993). In contrast, in Western Australia diagnosis of virulent footrot is based entirely on the results of laboratory virulence testing, irrespective of the clinical severity of an outbreak (Buller and Eamens, 2014).

Given the potential negative economic impacts of a false-positive result, if the *aprV2/B2* qPCR is to be used alongside or in place of existing phenotypic virulence tests for the diagnosis of virulent and benign footrot in Australia, it is critical that the assay be validated using samples collected from Australian sheep flocks with clinically virulent and benign footrot, classified using the appropriate case definitions (Egerton, 1989a). As such, the aim of this study was to evaluate the *aprV2/B2* qPCR for use in Australia by subjecting the test to the important initial steps in the validation pathway outlined in Chapter 1.1.6 of the World Organization for Animal Health (OIE) *Terrestrial Manual*, including comparison of the qPCR test with clinical diagnosis and standard phenotypic virulence tests (OIE, 2016).

3.2 Materials and Methods

3.2.1 Flock selection

Forty Australian sheep flocks were included in the present study, including 24 flocks with clinically virulent footrot and 16 flocks with clinically benign footrot (Table 3.1). Fifteen flocks were located in Tasmania, 14 in South Australia, 10 in NSW, and one in Western Australia (Table 3.1). Lesion swabs were collected from flocks 1-32 and 34-40 between June 2014 and August 2016. Lesion swabs were collected from Flock 33 in 2006 during the course of a previous study (Dhungyel et al., 2008); this flock was included because the entire flock of 1716 sheep was examined by the authors on several occasions, including the time at which the lesion swabs were collected.

3.2.2 Examination and clinical diagnosis

Three approaches (Methods 1, 2, and 3) to examination and diagnosis were used during this study (see below). The approach used on each farm is indicated in Table 3.1. The choice of approach depended on whether the mob(s) were examined by the authors or an animal health officer, the number of sheep or mobs presented for examination by the producer, and on prior diagnostic investigations. On each farm, sheep were placed in dorsal recumbency and each foot was carefully inspected, as described by Stewart and Claxton (1993). A score was assigned to each foot according to the scoring system devised by Egerton and Roberts (1971), as described in Table 2.1. For Methods 1 and 2, which are described in detail below, diagnosis of clinically virulent and benign footrot was based on the prevalence of score 4 lesions observed in ≥100

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randomly selected sheep, as described by Egerton (1989a). According to this system, outbreaks are classified as benign when the prevalence of score 4 lesions is <1%, intermediate when the prevalence is 1-9%, and virulent when the prevalence is ≥10%. However, to align with the dichotomous classification system used in New South Wales, which does not recognise intermediate footrot (Buller and Eamens, 2014), benign and intermediate outbreaks were grouped together, such that outbreaks were classified as clinically benign where score 4 lesions were observed in <10% of the flock or mob, or clinically virulent where score 4 lesions were observed in ≥10% of the flock or mob.

Method 1: A sample of at least a total of 100 sheep (see Table 3.1) was randomly selected and examined by the author from either one mob (where only one mob on the farm were observed with clinical signs of lameness, or where foot lesions were previously observed in one mob only during routine husbandry procedures) or two mobs (where at least two mobs on a farm were observed with clinical signs of lameness, or foot lesions were previously observed in two or more mobs during routine husbandry procedures). A flock history was provided by the producer at the time of examination. Lesion swabs were collected at the time of examination by the author.

In all flocks that appeared to have clinically benign footrot, the following additional criteria were all applied to support the classification as benign:

    i.   The flock/mob must have been examined on two or more previous occasions by the authors or an experienced animal officer, according to the system described by Stewart and Claxton (1993). The disease must have been classified as clinically benign on each occasion, according to the system of Egerton (1989a). The retrospective foot score data were examined by the author.

    ii.   Environmental conditions must have been deemed favourable for transmission and expression of the disease in the two weeks prior to each of the examinations (average daily air temperature ≥10°C, consistent rainfall) (Graham and Egerton, 1968). Climatic data recorded at the nearest Bureau of Meteorology weather station were accessed.

    iii.   The flock history did not suggest clinically virulent footrot, nor was there any evidence at the time of examination of virulent footrot having been present in the

flock previously, i.e. old lesions (such as damage to the axial hoof wall indicative of underrun lesions having been present).

iv.   Topical treatments that may suppress or mask the severity of disease, such as antiseptic foot bathing, had not been used in the four weeks preceding each examination.

v.   The sheep were all Merino, which are naturally susceptible to footrot (Emery et al., 1984).

Method 2: A small number of animals were examined by an experienced AHO for the purpose of collecting lesion swabs. The flock had been examined by the same AHO on two or more previous occasions, and a clinical diagnosis made using Method 1. As such, there was an interval between the time at which the clinical diagnosis was made and the time at which the lesion swabs were collected. The animal health officer informed the author of his or her clinical diagnosis but did not provide the retrospective foot score data.

Method 3: Sheep in a "hospital mob" were examined by the author or an experienced AHO. Between 10 and 60 sheep from each hospital mob were examined on each farm, as indicated in Table 3.1. The sheep were separated from the parent flock(s) by the producer because they had the most severe clinical signs of lameness or because they were the only sheep in the parent flock(s) with foot lesions. The sheep had not been examined previously by the author or an experienced animal health officer, and retrospective foot scores were not available. However, a flock history was obtained from the producer describing the progression of the disease since it was first introduced to the flock. Clinical diagnosis was based on the severity of clinical disease observed in the hospital mob, the number of sheep with score 4 lesions separated from the parent flocks(s), the size of the parent flock (and therefore an estimate of apparent prevalence of sheep with severe lesions was possible) and the flock history. Lesion swabs were collected at the time of examination by the author or AHO.

Table 3.1: Details of the Merino flocks from which samples were collected during this study. Field diagnosis was made using one of three approaches, as described above.

Farm	State	Operator	Diagnostic approach	Season at time of examination	No. mobs examined	No. sheep examined	Sheep with score 4 lesions	Clinical diagnosis	No. sheep with lesions sampled	No. swabs tested directly	No. isolates collected
1	SA	AHO	2	Winter	1	NA	≥10%	Virulent	11	12	12
2	NSW	Author	3	Winter	NA	54	23	Virulent	14	4	12
3	Tas.	Author	3	Winter	NA	51	11	Virulent	24	11	11
4	Tas.	Author	3	Winter	NA	52	40	Virulent	50	10	14
5	Tas.	AHO	3	Winter	NA	42	10	Virulent	20	23	23
6	Tas.	AHO	2	Winter	1	NA	≥10%	Virulent	20	23	23
7	Tas.	AHO	3	Winter	NA	33	26	Virulent	20	20	20
8	Tas.	AHO	3	Winter	NA	28	18	Virulent	20	19	19
9	Tas.	AHO	3	Winter	NA	13	7	Virulent	13	13	13
10	Tas.	AHO	3	Spring	NA	26	11	Virulent	20	15	15
11	Tas.	AHO	3	Spring	NA	20	5	Virulent	20	8	8
12	SA	AHO	2	Spring	1	NA	≥10%	Virulent	10	10	10
13	SA	AHO	2	Spring	1	NA	≥10%	Virulent	6	9	9
14	SA	AHO	2	Spring	1	NA	≥10%	Virulent	7	8	7
15	SA	AHO	2	Spring	1	NA	≥10%	Virulent	10	13	13
16	SA	AHO	3	Spring	NA	16	3	Virulent	16	16	16
17	Tas.	Author	3	Spring	NA	25	14	Virulent	25	15	15
18	Tas.	Author	3	Spring	NA	50	19	Virulent	50	29	29
19	NSW	Author	3	Summer	NA	51	15	Virulent	50	15	15
20	NSW	AHO	3	Summer	NA	50	4	Benign	14	14	14
21	Tas.	AHO	2	Summer	1	NA	≥10%	Virulent	11	11	11

Farm	State	Operator	Diagnostic approach	Season at time of examination	No. mobs examined	No. sheep examined	Sheep with score 4 lesions	Clinical diagnosis	No. sheep with lesions sampled	No. swabs tested directly	No. isolates tested
22	Tas.	AHO	2	Summer	1	NA	≥10%	Virulent	17	17	17
23	SA	AHO	2	Summer	1	NA	≥10%	Virulent	4	4	4
24	NSW	Author	3	Autumn	NA	20	0	Benign	20	20	21
25	NSW	Author	3	Winter	NA	NA	≥10%	Virulent	10	14	14
26	Tas.	AHO	2	Winter	1	NA	≥10%	Virulent	12	5	5
27	SA	Author	1	Spring	1	100	4	Benign	40	40	20
28	SA	Author	1	Spring	1	100	0	Benign	40	40	11
29	SA	Author	1	Spring	1	100	2	Benign	40	40	12
30	SA	Author	1	Spring	1	100	0	Benign	40	40	6
31	SA	Author	1	Spring	1	170	0	Benign	40	40	3
32	SA	Author	1	Spring	1	100	0	Benign	40	40	7
33	SA	AHO	1	Spring	1	1716	42	Benign	50	0	15
34	Tas.	Author	1	Winter	2	100	0	Benign	30	30	6
35	NSW	Author	1	Winter	2	120	0	Benign	21	21	10
36	NSW	Author	1	Winter	1	100	0	Benign	28	20	6
37	NSW	Author	1	Winter	1	100	0	Benign	22	20	1
38	NSW	Author	1	Winter	1	100	0	Benign	20	20	2
39	NSW	Author	1	Winter	1	100	0	Benign	25	23	0
40	WA	AHO	2	Winter	1	NA	0	Benign	30	27	0
								Total		758	469

3.2.3 Collection of lesion swabs

To ensure that lesion swabs were collected using a consistent methodology, all animal health officers were provided with instructions detailing the procedure for collecting a lesion swab. In each case, the interdigital skin or the active margin of an underrun lesion was swabbed with a sterile, cotton-tipped swab (CLASSIQSwabs; Copan Italia, Brescia, Italy). In most cases, two swabs were collected from each lesion: one was placed into mSTM for microbiological culture, and the other was placed into a 1.5 mL microcentrifuge tube (SSIBio, Lodi, U.S.A.) containing 500 µL of a lysis solution (Buffer RLT; Qiagen, Hilden, Germany, or Nuclei Lysis Solution; Promega, Madison, U.S.A.) for DNA extraction and direct (culture-independent) testing. All swabs were transported on ice. Swabs collected for microbiological culture were processed immediately upon receipt at the laboratory. Swabs collected for direct testing were stored at 4°C prior to DNA extraction, which was undertaken 24-48 hours after receipt.

3.2.4 Microbiological culture of *D. nodosus*

D. nodosus was isolated from each foot swab, as described in Chapter 2.2.

3.2.5 Archival samples

Lesion swabs were collected from Flock 33 in 2006. The entire flock had been examined on several occasions across a period of three years as part of a previous study (Dhungyel et al., 2008), and the disease was classified as clinically benign on each occasion based on Method 1. Individual *D. nodosus* isolates obtained in 2006 had been freeze-dried and stored at 4°C. For the present study, 15 randomly selected freeze-dried isolates were reconstituted in 200 µL PBS pH 7.4 (Astral Scientific, Taren Point, Australia); 100 µL of the suspension was spread-plated onto 4% HA for microbiological culture, as described previously. The remaining 100 µL was retained for DNA preparation.

3.2.6 Control strains

Virulent *D. nodosus* type strain A1001 and benign *D. nodosus* field strain JIR3528 were used as virulent (*aprV2*-positive) and benign (*aprB2*-positive) control strains, respectively.

3.2.7 DNA extraction

DNA was extracted from pure cultures using the Wizard Genomic DNA Purification kit (Promega, Madison, U.S.A.), as described in Chapter 2.6.2

Direct extraction of DNA from lesion swabs was undertaken using a magnetic bead DNA purification kit (BioSprint 96 One-For-All Vet Kit, Qiagen, Hilden, Germany), as described in Chapter 2.6.3

3.2.8 PCR identification of *D. nodosus*

D. nodosus was identified by real-time PCR amplification of a variable region of the *D. nodosus* 16S rRNA gene (Frosth et al., 2012), as described in Chapter 2.8. PCR product was visualised on 2% agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, South Korea), and viewed under ultraviolet light, as described previously (Dhungyel et al., 2013b). DNA extracted from *D. nodosus* strain A1001 was included in each run as a positive control.

3.2.9 Phenotypic virulence testing

1. Gelatin gel test
Each *D. nodosus* isolate was subjected to the gelatin gel test, as described in Chapter 2.4.

2. Elastase test
Each *D. nodosus* isolate was subjected to the elastase test, as described in Chapter 2.5.

3. aprV2/B2 qPCR

Primers, probes and master mixes reported by Stäuble et al. (2014a) and Frosth et al. (2015) (refer to Table 2.3) were ordered from Thermo Fisher Scientific Inc. Amplification was performed in a Stratagene Mx3000P thermocycler (Agilent Technologies, Santa Clara, CA). Reaction mixtures and cycling conditions were as described by Stäuble et al. (2014a). Reaction mixtures as described by Frosth et al. (2015) were used; however, as the thermocycler used in this study was unable to accommodate the fast-cycling conditions described by the authors, the cycling conditions recommended in the TaqMan Gene Expression Master Mix protocol were used, consisting of a UNG activation step of 2 min at 50°C an initial denaturation step of 10 min

at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s.

Virulent (*aprV2*-positive) and benign (*aprB2*-positive) controls were included in each run. A valid qPCR run was one in which: (1) there was amplification of both replicates of the *aprV2* and *aprB2* positive controls, with Ct values falling within the range of the standard curve (0.005 pg to 5000 pg); (2) there was no amplification of the *aprV2* and *aprB2* negative controls; and (3) given that the qPCR test was only used to determine the presence or absence of the two alleles during the present study, amplification efficiencies of 85 − 110% were accepted. The fluorescence threshold was initially set automatically for each run by the MxPro software (Agilent Technologies, Santa Clara, CA). However, to ensure that comparable Ct values were calculated for each run, the average fluorescence threshold was calculated for each target using the fluorescence threshold values set for all 20 runs, and applied retrospectively to each run.

Table 3.2: Primers used in this study.

Primer	Direction	Gene	Sequence (5'-3')	Product size (bp)	Reference
DnAprTM-L	F	*aprV2/B2*	CAATAGCCAAATTTCTTTAGATGGTGAT	126	Stäuble et al. (2014a)
DnAprTM-R	R		CAAGAGCTGTCGCTTCTTTCTTT		
DnAprTM-vMGB	Probe		FAM-CGGTGGTTATCCTGAT-MGBNFQ		
DnAprTM-bMGB	Probe		VIC-TGGTCGTCCTGATC-MGBNFQ		
*aprV2/B2*F	F	*aprV2/B2*	GAAGGCGACTGGTTTGATAACTG	71	Frosth et al. (2015)
*aprV2/B2*R	R		GAGCTGTCGCTTCTTTCTTTGC		
*aprV2*probe	Probe		FAM-ATGCGGTGGTTATCCT-MGBNFQ		
*aprB2*probe	Probe		VIC-ATGCGGTGGTCGTCCT-MGBNFQ		
DNO_1167F	F	*aprV2/B2*	AACACTACCGCGAACAATGG	661	This study (for Sanger Sequencing)
DNO_1167R	R		TCGCACCAACGCTTAATACG		

3.2.10 Analytical performance of the *aprV2/B2* qPCR

3.2.10.1 Analytical sensitivity

Amplification of the *aprV2* and *aprB2* alleles was analysed separately using serial dilutions of genomic DNA prepared from pure cultures of virulent *D. nodosus* type strain A1001 and benign *D. nodosus* field strain JIR3528, respectively. The limit of detection (LOD) and amplification efficiency was calculated for the *aprV2* and *aprB2* alleles for both qPCR tests. Data were collected from 20 individual experiments, with each reaction performed in duplicate ($n = 40$ data points per concentration). DNA template concentrations ranged from 0.0005 pg to 5000 pg of *D. nodosus* genomic DNA per reaction. The LOD was defined as the lowest concentration of genomic DNA at which amplification occurred for 50% of replicates (OIE, 2016).

3.2.10.2 Analytical specificity

The analytical specificity of each qPCR test was evaluated using DNA extracted from 15 bacterial species, along with the virulent and benign *D. nodosus* type strains (Table 3.4).

3.2.11 Diagnostic performance of each virulence test

The level of agreement between clinical diagnosis and laboratory diagnosis of virulent and benign footrot using the *aprV2*/*B2* qPCR was compared at the flock- and foot swab-levels. The elastase test and gelatin gel test were compared with clinical diagnosis at the flock- and isolate-levels. The laboratory diagnosis was virulent if one or more isolates were classified as virulent by a given laboratory virulence test.

3.2.11.1 Diagnostic sensitivity and specificity

i.   *aprV2*/*B2* qPCR test

At the flock level, diagnostic sensitivity (DSe) was defined as the percentage of clinically virulent flocks in which one or more swabs tested positive for the *aprV2* allele, whilst diagnostic specificity (DSp) was defined as the percentage of clinically benign flocks in which none of the swabs tested positive for the *aprV2* allele. At the foot swab-level, DSe was defined as the percentage of foot swabs collected from sheep in clinically virulent flocks that tested positive for the *aprV2* allele, whilst DSp was defined as the percentage of foot swabs collected from clinically benign flocks that tested negative for the *aprV2* allele.

ii.   Elastase test

At the flock-level, DSe was defined as the percentage of clinically virulent flocks from which one or more elastase-positive *D. nodosus* isolates were obtained, whilst DSp was defined as the percentage of clinically benign flocks from which no elastase-positive isolates were obtained. At the isolate-level, DSe was defined as the percentage of isolates obtained from clinically virulent flocks that were elastase-positive, whilst DSp was defined as the percentage of isolates obtained from clinically benign flocks that were elastase-negative.

iii.   Gelatin gel test

DSp was evaluated at both the flock- and isolate-level. At the flock level, DSp was defined as the percentage of clinically benign flocks from which only a heat-labile *D. nodosus* isolate was obtained. At the isolate-level, DSp was defined as the percentage of isolates obtained from clinically benign flocks that were heat-labile.

3.2.12 Repeatability

The between-run repeatability of the *aprV2/B2* qPCR test was determined for the *aprV2* and *aprB2* alleles, using three different concentrations of *D. nodosus* genomic DNA: 5000 pg, 50 pg, and 0.5 pg per reaction. The coefficient of variation (CV) was calculated for each concentration using Ct values collected across 20 qPCR runs, with each reaction performed in duplicate ($n = 40$ data points per concentration). The CV was calculated for each concentration of genomic DNA using the following formula:

$$CV = \frac{\text{Standard deviation of replicate}}{\text{Mean of replicates}} \text{ X } 100$$

3.2.13 Sanger sequencing

Sanger sequencing of the acidic protease 2 gene regions of five isolates was undertaken to confirm that the *aprV2/B2* qPCR developed by Frosth et al. (2015) was correctly identifying the *aprV2* and *aprB2* alleles. Two isolates from Farm 29 and two isolates from Farm 32, all of which were *aprV2*-positive, and one isolate from Farm 33, which was *aprB2*-positive, were sequenced. Forward and reverse primers were designed using the software package Primer3 (Koressaar and

Remm, 2007; Untergasser et al., 2012): aprF-AACACTACCGCGAACAATGG and aprR-TCGCACCAACGCTTAATACG, producing a 661-bp product. Primers were ordered from Sigma-Aldrich. Each 20 µL reaction mixture consisted of 1 x PCR Buffer, 0.2 mM of each dNTP, 3.0 mM of $MgCl_2$, 400 nM of each primer, 5U/µL of Taq polymerase, and 2.0 µL of template DNA. Amplification was performed in a Bio-Rad T100 thermocycler (Bio-Rad, Gladesville, Australia), and consisted of an initial denaturation step of 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 60s, followed by a final extension phase of 72°C for 5 mins. PCR product was purified with ExoSAP-IT (Affymetrix, Santa Clara, U.S.A.), and delivered to Garvan Molecular Genetics, Sydney, Australia, for sequencing. Upon receipt, the sequences were aligned against an *aprV2* gene sequence from virulent *D. nodosus* prototype strain A198 (A1001) (Accession number: L38395.1) using Clustal Omega (Sievers and Higgins, 2014).

3.2.14 Statistical analysis

The level of agreement between clinical and laboratory diagnoses were evaluated using Cohen's kappa statistic (Cohen, 1960). Kappa statistics were interpreted using the standards for strength of agreement proposed by Landis and Koch (1977): ≤0 = poor agreement, 0.01-0.20 = slight agreement, 0.21-0.40 = fair agreement, 0.41-0.60 = moderate agreement, 0.61-0.80 = substantial agreement, and 0.81-1.00 = almost perfect agreement. McNemar's Chi-Square Test for Paired Observations (McNemar, 1947) was performed to establish if there were statistically significant differences between the proportion of outbreaks classified as virulent by clinical or laboratory diagnosis using each of the three virulence tests. The results of each individual laboratory virulence test were also compared using this approach. All statistical analyses were conducted in Microsoft Excel 2010. Exact 95% binomial confidence intervals were calculated for diagnostic sensitivities and specificities in GenStat 16th Edition (©2000-2016 VSN International Ltd, Hemel Hempstead, U.K.).

3.3 Results

3.3.1 Flock selection

Forty Australian sheep flocks were selected for the present study, including 24 flocks with clinically virulent footrot and 16 flocks with clinically benign footrot (Table 3.1). A summary of lesion scores was available for 28 flocks. The distribution of lesion scores observed in each of these flocks is illustrated in Figure 1.1. The number of flocks with clinically virulent and benign footrot examined in each State is indicated in Table 3.3. Twenty flocks were examined by the authors and 20 were examined by experienced animal health officers. Sixteen flocks were examined during spring, 18 flocks during winter, five flocks during summer, and one flock during autumn. Lesion swabs were collected for direct testing from 40 flocks, but lesion swabs for microbiological culture were collected from only 38 flocks. The number of swabs collected from each flock for direct testing ranged from four to 40. The number of *D. nodosus* isolates obtained from each flock ranged from one to 29.

Table 3.3: Summary of the number of flocks examined in each Australian State, and the number of flocks in each State that were classified as clinically virulent or benign.

| State | Clinical Diagnosis | | Total |
	Virulent	Benign	
Tasmania	14	1	15
New South Wales	3	7	10
South Australia	7	7	14
Western Australia	0	1	1
Total	24	16	40
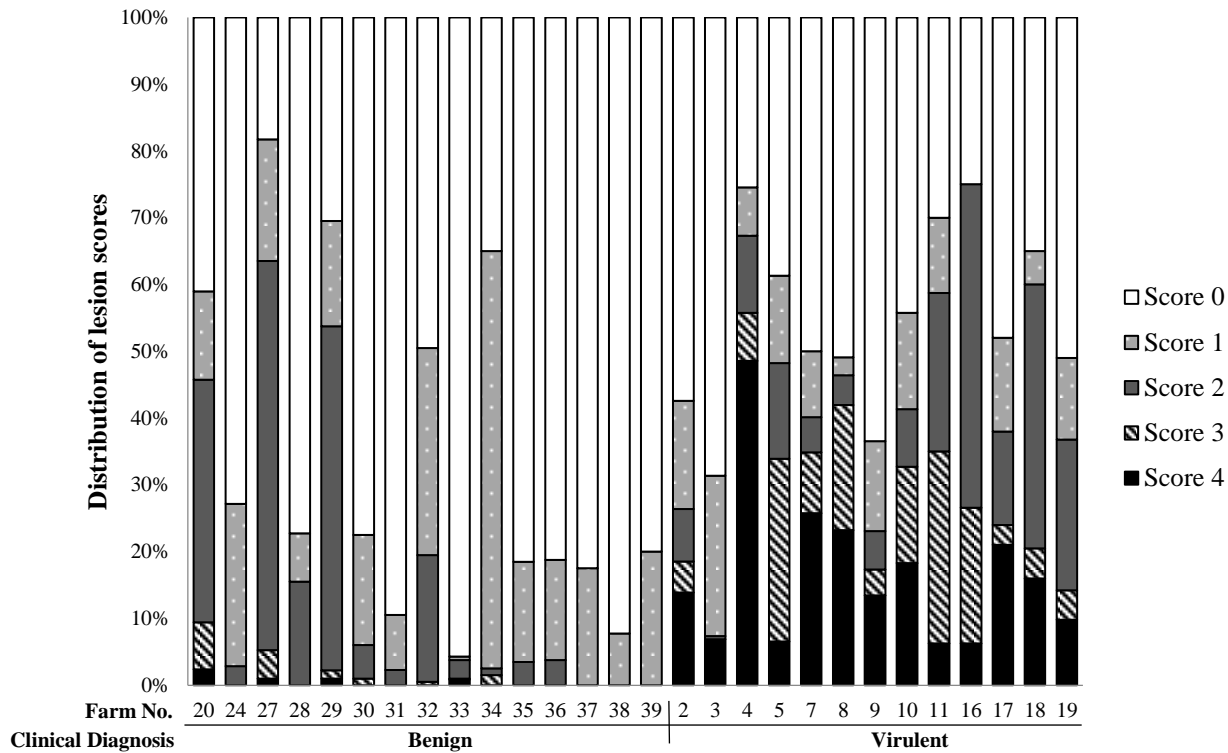
Figure 3.1: Summary of the proportion of feet with each lesion score for 28 of the flocks included in this study. Flocks are grouped according to clinical diagnosis. Lesion score summaries were not provided for 13 flocks examined by animal health officers using Method 3 (see Table 3.1). The number of sheep examined in each flock is indicated in Table 3.1.

3.3.2 Examination of flocks

Forty flocks were examined during the course of this study, and three approaches to clinical diagnosis were used (Table 3.1). Thirteen flocks were examined using Method 1, 11 were examined using Method 2, and 16 were examined using Method 3.

3.3.3 Analytical characteristics of the *aprV2/B2* qPCR

Amplification efficiencies and the LOD of the qPCR tests developed by Stäuble et al. (2014a) and Frosth et al. (2015) were calculated. Amplification efficiencies for *aprV2* and *aprB2* were 90.14 and 88.4, respectively, using the assay developed by Frosth et al. (2015). Amplification efficiencies for *aprV2* and *aprB2* were 87.8 and 91.4, respectively, using the assay developed by

Stäuble et al. (2014a). The LOD for *aprV2* and *aprB2* was 0.005 and 0.05 pg, respectively, for both qPCR tests (Figures 3.2 and 3.3).

The analytical specificity of the two qPCR assays was determined using genomic DNA extracted from pure cultures of 15 bacterial strains, along with genomic DNA extracted from pure cultures of virulent *D. nodosus* type strain A1001 and benign *D. nodosus* field strain JIR3528 (Table 3.5). Both assays were 100% specific for the *aprV2* and *aprB2* alleles, respectively, and no amplification occurred for the 15 other bacterial species.

The two qPCR tests were compared using 430 lesion swabs collected from 18 flocks (Table 3.5). The qPCR test developed by Frosth et al. (2015) detected the *aprV2* allele in 48 lesion swabs and the *aprB2* allele in 26 lesion swabs that the test developed by Stäuble et al. (2014a) did not. Consequently, a decision was made to proceed with the assay of Frosth et al. (2015) when undertaking a larger test evaluation.

3.3.4 Repeatability of the *aprV2/B2* qPCR

The repeatability of the *aprV2*/*B2* qPCR test developed by Frosth et al. (2015) was evaluated for the *aprV2* and *aprB2* alleles with three concentrations of genomic DNA per reaction (Table 3.4). The CV was similar for each of the three DNA concentrations for both the *aprV2* and *aprB2* alleles, and was <5%.

Table 3.4: Determination of the CV for the *aprV2* and *aprB2* alleles using three concentrations of genomic DNA extracted from virulent and benign *D. nodosus* type strains.

DNA concentration (pg/reaction)	Coefficient of variation% (95% CI)	
	aprV2	*aprB2*
5000	3.81 (3.12 – 4.90)	5.23 (4.28 – 6.72)
50	3.30 (2.70 – 4.24)	4.00 (3.28 – 5.14)
0.5	2.79 (2.29 – 3.59)	3.25 (2.67 – 4.18)
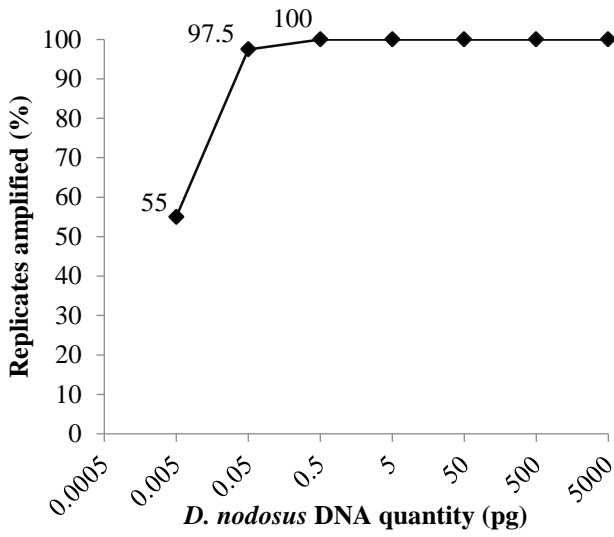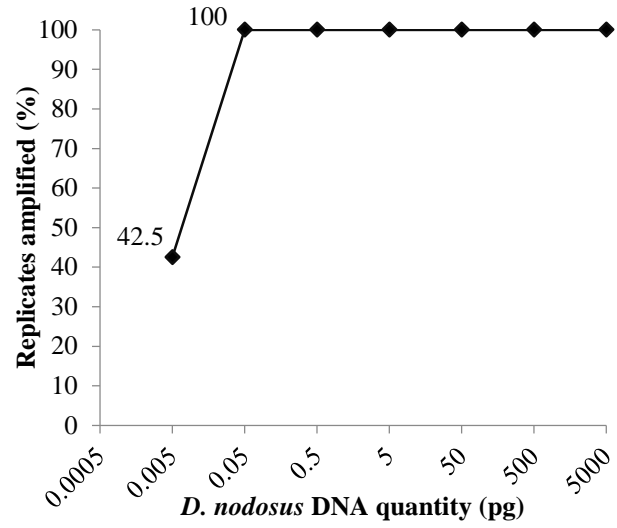
Figure 3.2: Limit of detection of the qPCR test developed by Frosth et al. (2015).

A = *aprV2*; B = *aprB2*.
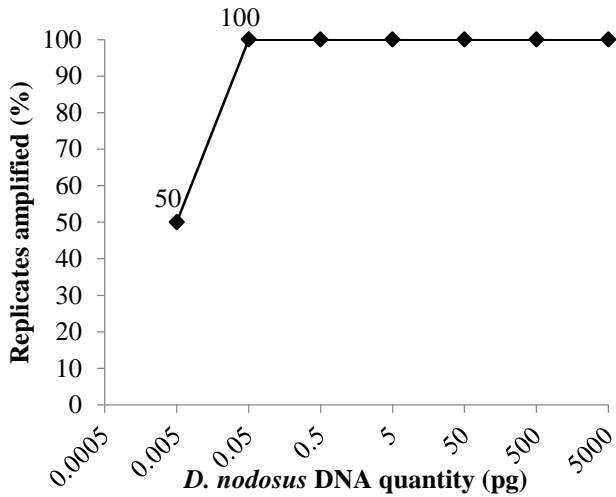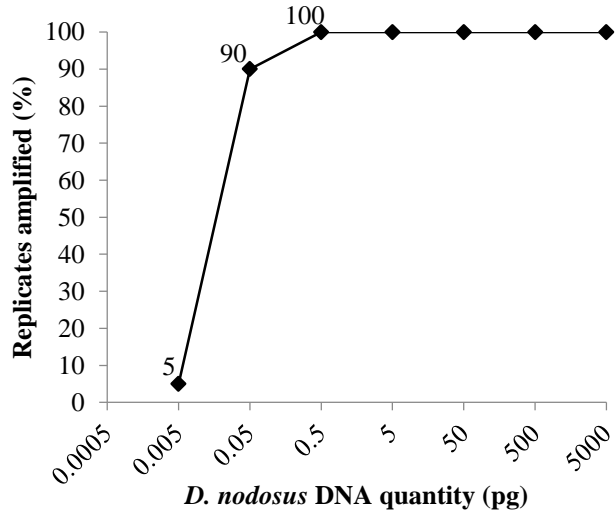
Figure 3.3: Limit of detection of the qPCR test developed by Stäuble et al. (2014a).

A = *aprV2*; B = *aprB2*.

Table 3.5: Evaluation of the analytical specificity of the *aprV2/V2* qPCR tests developed by Stäuble et al. (2014a) and Frosth et al. (2015) genomic DNA extracted from 15 bacterial species. Virulent (*aprV2*-positive) and benign (*aprB2*-positive) *D. nodosus* control strains were also included.

| | | | | qPCR test | | | |
| | | | | Stäuble et al. (2014a) | | Frosth et al. (2015) | |
Species	**Location**	**Host**	**ID no.**	*aprV2*	*aprB2*	*aprV2*	*aprB2*
Cardiobacterium hominis	NSW, Australia	Human	FD-3235	—	—	—	—
Corynebacterium ovis	NSW, Australia	Ovine	FD-2798	—	—	—	—
Dermatophilus congolensis	NSW, Australia	Ovine	FD-2839	—	—	—	—
Enterococcus uberis	NSW, Australia	Bovine	NA	—	—	—	—
Erysipelothrix rhusiopathiae	QLD, Australia	Ovine	FD-2825	—	—	—	—
Escherichia coli	NSW, Australia	Ovine	FD-2669	—	—	—	—
Fusobacterium necrophorum	NSW, Australia	Ovine	FD-2842	—	—	—	—
Klebsiella spp.	NSW, Australia	Bovine	NA	—	—	—	—
Moraxella bovis	NSW, Australia	Bovine	FD-2574	—	—	—	—
Nocardia spp.	NSW, Australia	Bovine	15-166	—	—	—	—
Pseudomonas auruginosa	NSW, Australia	Ovine	FD-2696	—	—	—	—
Salmonella Typhimurium	VIC, Australia	Porcine	NA	—	—	—	—
Staphylococcus aureus	NSW, Australia	Bovine	2793	—	—	—	—
Streptococcus B	NSW, Australia	Ovine	2438	—	—	—	—
Suttonella indologenes	NSW, Australia	Human	FD-3234	—	—	—	—
Dichelobacter nodosus A1001	NSW, Australia	Ovine	A1001	+	—	+	—
Dichelobacter nodosus JIR3528	NSW, Australia	Ovine	JIR3528	—	+	—	+

Table 3.6: Comparison of the *aprV2/B2* qPCR tests developed by Stäuble et al. (2014) and Frosth et al. (2015) using 430 foot swabs collected from 18 outbreaks of footrot. Additional information about each flock is provided in Table 3.1.

| | | | No. (%) swabs positive | | | | | |
| | | | Stäuble et al. (2014a) | | | Frosth et al. (2015) | | |
Flock no.	Field diagnosis	No. swabs tested	*aprV2*	*aprB2*	*aprV2/B2*	*aprV2*	*aprB2*	*aprV2/B2*
5	Virulent	18	18 (100)	0 (0)	0 (0)	18 (100)	0 (0)	0 (0)
6	Virulent	19	19 (100)	0 (0)	0 (0)	19 (100)	0 (0)	0 (0)
7	Virulent	18	17 (94.4)	1 (5.6)	0 (0)	17 (94.4)	1 (5.6)	0 (0)
8	Virulent	19	19 (100)	0 (0)	0 (0)	19 (100)	0 (0)	0 (0)
9	Virulent	13	10 (76.9)	0 (0)	3 (23.1)	10 (76.9)	0 (0)	3 (23.1)
10	Virulent	15	15 (100)	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)
11	Virulent	8	8 (100)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)
17	Virulent	15	15 (100)	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)
18	Virulent	29	29 (100)	0 (0)	0 (0)	29 (100)	0 (0)	0 (0)
24	Benign	21	18 (85.7)	3 (14.3)	0 (0)	18 (85.7)	3 (14.3)	0 (0)
25	Virulent	10	3 (30.0)	5 (50.0)	0 (0)	4 (40.0)	5 (50.0)	1 (10.0)
26	Virulent	5	4 (80.0)	0 (0)	0 (0)	4 (80.0)	0 (0)	0 (0)
27	Benign	40	40 (100)	0 (0)	0 (0)	40 (100)	0 (0)	0 (0)
28	Benign	40	40 (100)	0 (0)	0 (0)	40 (100)	0 (0)	0 (0)
29	Benign	40	32 (80.0)	0 (0)	2 (5.0)	38 (95.0)	0 (0)	2 (5.0)
30	Benign	40	29 (72.5)	0 (0)	0 (0)	34 (85.0)	0 (0)	0 (0)
31	Benign	40	5 (12.5)	0 (0)	0 (0)	37 (92.5)	0 (0)	3 (7.5)
32	Benign	40	3 (7.5)	1 (2.5)	14 (35.0)	3 (7.5)	1 (2.5)	36 (90)
	Total	**430**	**324**	**10**	**19**	**368**	**10**	**45**

3.3.5 Comparison of the *aprV2/B2* qPCR test of Frosth et al. (2015) with clinical diagnosis and standard phenotypic virulence tests at the flock-level.

3.3.5.1 Sensitivity and specificity of the *aprV2/B2* qPCR test.

The *aprV2/B2* qPCR test was compared with clinical diagnosis using lesion swabs collected from 40 Australian sheep flocks with clinically diagnosed footrot (Table 3.7). DNA was extracted directly from lesions swabs for analysis. An outbreak was more likely to be classified as virulent with the qPCR test than clinically ($P < 0.0009$). The level of agreement between clinical diagnosis and the *aprV2/B2* qPCR test beyond that expected by chance alone was fair (kappa statistic = 0.353). Clinical diagnosis and the qPCR test were also compared at the foot swab level (Table 3.8). The level of agreement was considerably lower at the foot swab level (kappa statistic = 0.096) as the *aprV2* allele was detected for 87% (363/417) of lesions swabs collected from clinically benign outbreaks. At the flock level, the DSe of the qPCR test was 100%, and the DSp was 31.3%. At the foot swab level, the DSe was 98.1%, and the DSp was 18.8%.

Table 3.7: Flock-level comparison of clinical diagnosis and the *aprV2/B2* qPCR (Frosth et al., 2015) using 758 lesion swabs collected from 40 Australian sheep flocks. Clinical diagnoses are given in Table 3.1. Data are the number of flocks in each category.

		Laboratory diagnosis (*aprV2/B2* qPCR)		
		Benign[1]	**Virulent**[2]	Total
Clinical diagnosis	**Benign**	5	11	16
	Virulent	0	24	24
	Total	5	35	40

[1]Flocks in which no swabs tested positive for the *aprV2* allele.

[2]Flocks in which ≥1 swab tested positive for the *aprV2* allele.

McNemar's X^2 = 11.0, P = 0.0009; kappa statistic = 0.353 (95% CI 0.105 – 0.601)

DSe = 100% (95% CI 87.5 – 100%); DSp = 31.3% (95% CI 11.0 – 58.6%)

Table 3.8: Comparison of clinical diagnosis and the *aprV2/B2* qPCR (Frosth et al., 2015) at the foot swab level. Genomic DNA was extracted directly from 758 foot swabs collected from 40 Australian sheep flocks. Clinical diagnosis was at the flock level (Table 3.1). Data are the number of foot swabs.

| | | Laboratory diagnosis (*aprV2/B2* qPCR) | | |
		Benign[1]	**Virulent**[2]	Total
Clinical diagnosis	**Benign**	84	363	447
	Virulent	6	305	311
	Total	90	668	758

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[1]Swabs that tested negative for the *aprV2* allele

[2]Swabs that tested positive for the *aprV2* allele

McNemar's X^2 = 345.39, $P < 0.0001$; kappa statistic = 0.096, 95% CI 0.064 – 0.128

DSe = 98.1% (95% CI 95.9 – 99.3%); DSp = 18.8% (95% CI 15.3 – 22.7%)

3.3.5.2 Sensitivity and specificity of the elastase test.

The elastase test was used to evaluate 469 *D. nodosus* isolates collected from 38 Australian sheep flocks (Table 3.9). The number of isolates obtained from each flock is indicated in Table 3.1. There was no significant difference ($P = 0.0833$) between the proportion of outbreaks classified as virulent by clinical diagnosis or by the elastase test. The level of agreement between clinical diagnosis and the elastase test beyond that expected by chance alone was almost perfect (kappa statistic = 0.822). At the flock-level, the DSe of the elastase test was 100%. Three outbreaks that were clinically benign were classified as virulent by the elastase test, thus DSp = 78.6%. Clinical diagnosis and the elastase test were also compared at the isolate-level (Table 3.10). There was a significant difference ($P < 0.0001$) between the proportion of isolates from clinically virulent outbreaks that were elastase-negative and those from clinically benign outbreaks that were elastase-positive. At the isolate-level, the level of agreement between clinical diagnosis and the elastase test beyond that expected by chance alone was moderate (kappa statistic = 0.431). DSe was 69.9% and DSp was 80.5%.

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Table 3.9: Flock-level comparison of clinical diagnosis and the elastase test using 469 *D. nodosus* isolates collected from 38 Australian sheep flocks. Lesion swabs were not collected for microbiological culture from flocks 39 and 40. Data are the number of flocks in each category.

		Laboratory diagnosis (elastase test)		
		Benign[1]	**Virulent[2]**	Total
Clinical diagnosis	**Benign**	11	3	14
	Virulent	0	24	24
	Total	11	27	38

[1]Flocks from which no *D. nodosus* isolates were elastase-positive at ≤12 days.

[2]Flocks from which ≥1 *D. nodosus* isolate was elastase-positive at ≤12 days.

McNemar's X^2 = 3.0, P = 0.0833; kappa statistic = 0.822, 95% CI 0.633 − 1.000

DSe = 100% (95% CI 85.8 − 100%); DSp = 78.6% (95% CI 49.2 − 95.3%)

Table 3.10: Isolate-level comparison of clinical diagnosis and the elastase test using 469 *D. nodosus* isolates collected from 38 Australian sheep flocks. Lesion swabs were not collected for microbiological culture from flocks 39 and 40.

		Laboratory diagnosis (elastase test)		
		Benign[1]	**Virulent[2]**	Total
Clinical diagnosis	**Benign**	107	26	133
	Virulent	101	235	336
	Total	208	261	469

[1]Isolates that were elastase-negative at ≤12 days.

[2]Isolates that were elastase-positive at ≤12 days.

McNemar's X^2 = 44.29, P < 0.0001; kappa statistic = 0.431, 95% CI 0.352 − 0.510

DSe = 69.9% (95% CI 64.7 − 74.8%); DSp = 80.5% (95% CI = 72.7 − 86.8%)

3.3.5.3 Specificity of the gelatin gel test.

The gelatin gel test was used to evaluate samples collected from six clinically benign outbreaks (Flocks 27, 28, 29, 30, 31, 32). Heat-stable (virulent) *D. nodosus* isolates were obtained from all six outbreaks. Isolate-level comparisons were also undertaken. Overall, 28 of 57 isolates were classified as virulent in this test.

3.3.6 Comparison of the *aprV2/B2* qPCR test with standard phenotypic virulence tests at the isolate level

Isolates cultured from lesion swabs obtained from 38 Australian sheep flocks were subjected to the elastase test and the *aprV2*/*B2* qPCR for laboratory diagnosis. Isolates collected from six flocks were also subjected to the gelatin gel test. For qPCR analyses, DNA was extracted from pure cultures of *D. nodosus* to ensure that the tests were being compared on the same isolates.

3.3.6.1 Comparison of the *aprV2/B2* qPCR test and the elastase test.

There was a significant difference (P <0.0001) between the results of the elastase test and the *aprV2/B2* qPCR: the qPCR was more likely to classify an isolate as virulent than was the elastase test. Only 52.7% (213/404) of *D. nodosus* isolates were classified as virulent by both tests (Table 3.11). There was a considerable discrepancy between the elastase test and the *aprV2/B2* qPCR for isolates classified as benign by the elastase test, as 73.2% (139/190) of isolates classified as benign by the elastase test were classified as virulent by the *aprV2/B2* qPCR. The level of agreement between the elastase test and the *aprV2/B2* qPCR beyond that expected by chance alone was only fair (kappa statistic = 0.275). Of the 139 isolates that were classified as benign by the elastase test and virulent by the qPCR test, 54.0% (75/139) were obtained from sheep in flocks with clinically virulent footrot, and 46.0% (64/139) were obtained from sheep in flocks with clinically benign footrot. A large proportion of these isolates (112/139) were elastase-positive after 16-28 days of incubation.

Table 3.11: Comparison of the elastase test and the *aprV2/B2* qPCR test (Frosth et al., 2015) using 404 *D. nodosus* isolates obtained from 38 Australian sheep flocks. Lesion swabs were not collected for microbiological culture from flocks 39 and 40. Data are the number of isolates classified as virulent by each test.

		Laboratory diagnosis (*aprV2/B2* qPCR)		
		Benign[1]	**Virulent**[2]	Total
Laboratory diagnosis	**Benign**	51	139	190
(elastase test)	**Virulent**	1	213	214
	Total	52	352	404

[1]Isolates that were negative for the *aprV2* allele

[2]Isolates that were positive for the *aprV2* allele.

McNemar's X^2 = 137.00, *P* < 0.0001; kappa statistic = 0.275, 95% CI 0.207 − 0.343

3.3.6.2 Comparison of the *aprV2/B2* qPCR test and the gelatin gel test

There was a significant difference ($P <0.0001$) between the results of the gelatin gel test and the *aprV2*/*B2* qPCR test, with 86.2% (25/29) of isolates classified as benign (unstable) by the gelatin gel test classified as virulent by the qPCR test (Table 3.12). The level of agreement between the gelatin gel test and the *aprV2*/*B2* qPCR test beyond that expected by chance alone was poor (kappa statistic = 0.101, 95% CI -0.024 – 0.244).

Table 3.12: Comparison of the *aprV2*/*B2* qPCR test (Frosth et al., 2015) and the gelatin gel test using 57 *D. nodosus* isolates obtained from Flocks 27, 28, 29, 30, 31, and 32, all of which presented with clinically benign footrot (see Table 3.1).

		Laboratory diagnosis (*aprV2*/*B2* qPCR)		
		Benign[1]	**Virulent**[2]	Total
Laboratory diagnosis	**Benign**	4	25	29
(gelatin gel test)	**Virulent**	1	27	28
	Total	5	52	57

[1]Isolates that were negative for the *aprV2* allele

[2]Isolates that were positive for the *aprV2* allele.

McNemar's X^2 = 23.04, $P < 0.0001$; kappa statistic = 0.101, 95% CI -0.024 – 0.244

3.3.6.3 Comparison of the elastase test and the gelatin gel test.

Paired elastase and gelatin gel test results were available for 56 *D. nodosus* isolates (Table 3.13). There was a significant difference between the results of the two tests (P < 0.0001), with 42.9% (21/49) of isolates classified as benign by the elastase test classified as virulent by the gelatin gel test. The level of agreement between the elastase test and the gelatin gel test beyond that expected by chance alone was slight (kappa statistic = 0.193).

Table 3.13: Comparison of the elastase test and the gelatin gel test using 56 *D. nodosus* isolates obtained from Flocks 27, 28, 29, 30, 31, and 32, all of which presented with clinically benign footrot (see Table 3.1).

| | | Laboratory diagnosis (elastase test) | | |
		Benign[1]	**Virulent**[2]	Total
Laboratory diagnosis	**Benign**	28	1	29
(gelatin gel test)	**Virulent**	21	6	27
	Total	49	7	56

[1]Isolates that were elastase-negative at the cut-off point of 12 days.

[2]Isolates that were elastase-positive at or before the cut-off point of 12 days.

McNemar's X^2 = 18.18, P < 0.0001; kappa statistic = 0.193, 95% CI 0.015 – 0.370

3.4 Discussion

In the present study, the qPCR tests developed by Stäuble et al. (2014a) and Frosth et al. (2015) were evaluated, and subjected the test developed by Frosth et al. (2015) to the initial steps of the validation pathway outlined in Chapter 1.1.6 of the *OIE Terrestrial Manual* (OIE, 2016).

The analytical performance of the two tests was first compared using a 10-fold dilution series of genomic DNA extracted from virulent and benign *D. nodosus* control strains, and genomic DNA extracted from 15 other bacterial species (Figures 3.2 and 3.3; Table 3.5). The analytical performance of the two tests was similar; however, when evaluating the two tests in parallel using a set of 430 samples collected from 18 Australian flocks, the test developed by Frosth et al. (2015) detected the *aprV2* allele in 48 samples and the *aprB2* allele in 26 samples that the test developed by Stäuble et al. (2014a) did not (Table 3.6). This discrepancy may have been a result of PCR inhibitors present in the DNA extract; Frosth et al. (2015) include BSA in their reaction mixture, which has been shown to reduce the effect of PCR inhibitors (Kreader, 1996). The lower sensitivity of the test developed by Stäuble et al. (2014a) may also be a consequence of variable primer binding, as there is known to be a single-nucleotide polymorphism (SNP) in the region of their forward primer in some *D. nodosus* strains (Myers et al., 2007). To avoid this SNP, Frosth et al. (2015) located their forward primer in an adjacent region that does not contain any known SNPs. The test developed by Frosth et al. (2015) only was evaluated during the remainder of the study to avoid potential false-negative results.

The qPCR test and current phenotypic virulence tests were compared with clinical diagnosis at both the flock- and foot swab-level. The qPCR test was also compared with the phenotypic virulence tests at the isolate-level. Lesion swabs were collected from 40 Australian flocks, including 16 outbreaks of clinically benign footrot and 24 outbreaks of clinically virulent footrot (Table 3.1). Flock history, breed, climatic conditions, and management practices were documented to support clinical diagnoses. The case definitions used to describe clinically virulent and clinically benign footrot were in line with those used by the New South Wales regulatory authority, which is based on the proportion of infected sheep that present with score 4 lesions (Egerton, 1989a). To further align with the dichotomous classification system used in New South Wales, which no longer recognises intermediate footrot, benign and clinically intermediate outbreaks were grouped together, such that outbreaks were classified as virulent if

score 4 lesions were observed in ≥10% of the flock or mob, or benign if score 4 lesions were observed in ≤10% of the flock or mob. Most of the outbreaks classified as clinically benign had few or no sheep with score 4 lesions.

The qPCR test was first compared with field diagnosis at the flock-level (Table 3.7), and there was only a moderate level of agreement (kappa statistic = 0.353) At the foot swab-level, this discrepancy was more marked (kappa = 0.096), as the *aprV2* allele was detected in 81.2% (363/447) of lesion swabs collected from outbreaks of clinically benign footrot (Table 3.8). As such, the DSp of the qPCR test was only 31.3% and 18.8% at the flock- and foot swab-level, respectively. In contrast, there was an almost perfect level of agreement between clinical diagnoses and the elastase test at the flock-level (kappa statistic = 0.822) (Table 3.9) and a moderate level of agreement at the isolate-level (kappa statistic = 0.431) (Table 3.10), despite both tests being based on the same virulence factor, resulting in DSp of 78.6% and 80.5% at the flock- and isolate-level, respectively. Isolate-level comparison of the qPCR test and elastase test indicated that there may be temporal and quantitative variations in the expression of AprV2 between genetically similar strains, as there was only slight agreement between the *aprV2/B2* qPCR and the elastase test (kappa statistic = 0.190); 73.2% (139/190) of isolates that were deemed benign by the elastase test were deemed virulent by the qPCR test (*aprV2*-positive) (Table 3.11). These isolates were classified as benign as they were elastase-negative at the cut-off point of 12 days; however, elastase activity was observed for 80.6% (112/139) of these isolates after 16-28 days of incubation. Of these 112 isolates, 61.6% (69/112) were from clinically virulent outbreaks, and 38.4% (43/112) were from clinically benign outbreaks. This finding suggests that identification of the *aprV2* allele alone may not be a reliable indicator of virulence. It is apparent that the elastase test should be used alongside the qPCR test to confirm that the isolate is phenotypically virulent, i.e. capable of expressing a functional protease.

Gelatin gel test results were available for 57 *D. nodosus* isolates collected from six flocks with clinically benign footrot. There was little agreement between the gelatin gel test and clinical diagnosis, as 49.1% (28/57) of isolates obtained from these flocks expressed a heat-stable protease. This discrepancy has been reported in previous studies: in a survey of NSW flocks, Cheetham et al. (2006) obtained isolates expressing a heat-stable or equivocal protease from 63.0% (233/370) of flocks with clinically benign footrot. Following a comparative evaluation of

the gelatin gel, Dhungyel et al. (2013b) concluded that false-positives may occur with the gelatin gel test.

These findings are in contrast with those of Stäuble et al. (2014a) and Frosth et al. (2015), who both report a high level of agreement between the qPCR test and clinical diagnosis. However, this may be a consequence of the case definitions in this study, which is based on the New South Wales system. When the case definitions used in Australia were applied to the limited validation data provided by these authors, similar discrepancies to those reported in the present study emerged. Stäuble et al. (2014a) validated their assay using 196 lesion swabs collected from sheep in Switzerland, Germany and France. The authors did not classify outbreaks as clinically virulent or benign; rather, flocks were classified as 'non-affected' (all feet assigned a score of 0) or 'affected' (one or more feet assigned a score ≥1). In total, one-hundred lesion swabs were collected from 16 'affected' flocks, 92 lesion swabs were collected from eight 'non-affected' flocks and four lesion swabs were collected from two flocks of an undetermined status. The number of lesion swabs collected from each flock ranged from one to 33, but typically fewer than 10 were collected per flock. Stäuble et al. (2014a) report that all 100 lesion swabs from 'affected' flocks were positive for the *aprV2* allele and negative for the *aprB2* allele, and that >80% of samples from the 'non-affected' flocks were positive for the *aprB2* allele. Based on these results, the authors concluded that there was a high level of agreement between clinical diagnosis and the qPCR test. The authors do not provide any data regarding the prevalence of each lesion score in the flocks that were included in the study, thus it is difficult to determine which outbreaks would be regarded as clinically virulent or benign according to the case definitions applied in this study. However, the authors state that for each sheep examined in an 'affected' flock, all four feet were inspected and scored, and a sample was collected from the foot with the highest lesion score. Based on the lesion scores provided in Supplemental Table 1, it is apparent that at least two of the 'affected' flocks could be described as clinically benign, as no score 4 lesions were observed in these flocks.

Similar discrepancies are apparent in the data provided by Frosth et al. (2015), who validated their assay using samples collected from twenty European flocks. Fifty sheep from each flock were examined and lesion scores assigned using the system described by Egerton and Roberts (1971). Thereafter, each flock was assigned to one of four categories: (i) predominantly score 0

with some score 1 lesions, (ii) many score 1 lesions but no scores >1, (iii) at least one animal with a score 2 lesion, and (iv) at least one animal with a score 3 lesion. It is evident that under the New South Wales classification system, categories one and two could equally describe a flock with ovine interdigital dermatitis (OID), benign footrot, or the early stages of virulent footrot. Similarly, categories three and four could equally describe an outbreak of benign footrot or the early stages of an outbreak virulent footrot. No category can be said to describe virulent footrot exclusively. The authors also report that all 20 flocks included in the study were examined when environmental conditions were favourable for expression of the disease; however score 3 lesions were only identified in 5/20 flocks, and score 4 lesions only identified in 1/20 flocks. The *aprV2* allele was only detected in samples collected from one flock with clinical footrot in Category 4, whilst the *aprB2* allele was identified in samples collected from twelve flocks: nine 'affected' and three 'unaffected'.

The present study has emphasised the detection of potentially virulent *D. nodosus* strains in flocks with clinically benign footrot because of the potential negative economic consequences of this finding. However, the identification of benign *D. nodosus* strains in flocks with clinically virulent footrot has also contributed to the poor level of agreement between clinical and laboratory diagnosis at the isolate-level. Less emphasis was placed on this finding as the detection of both virulent and benign strains of *D. nodosus* in strains with clinically virulent footrot is not unusual (Dhungyel et al., 2002; Egerton et al., 2002). Further, the identification of phenotypically benign *D. nodosus* strains in a flock with clinically virulent footrot would not have any regulatory significance, except in the event that only phenotypically benign *D. nodosus* strains were identified in a flock with clinically virulent footrot. However, this was not observed in the present study, and has not been reported elsewhere, except where benign *D. nodosus* strains have persisted following the elimination of virulent *D. nodosus* strains (Egerton and Parsonson, 1969).

This study demonstrates that *D. nodosus* strains classified as benign by the qPCR test (*aprB2*-positive) are capable inducing severe, underrun lesions in a small proportion of susceptible sheep (Table 3.1): 4/50 sheep examined in Flock 20, and 42/1716 sheep examined in Flock 33, presented with score 4 lesions despite all *D. nodosus* isolates obtained from these flocks being deemed benign by the qPCR test. Lesions of various grades were often present on all four feet of

sheep that presented with one or more score 4 lesions, whilst lesions were restricted to one or two feet of sheep that presented with milder lesions (data not shown). This observation appears to confirm that these sheep were more susceptible to the disease than other members of the flock, and this may explain why isolates that express AprB2 can cause severe lesions.

The identification of potentially virulent strains of *D. nodosus*, defined by the presence of the *aprV2* allele, in clinically healthy flocks provides further evidence that the *aprV2* may be an unreliable virulence marker. Previously, Stäuble et al. (2014a) reported that seven samples collected from 'non-affected' (clinically healthy) flocks were positive for the *aprV2* allele, alone or in combination with the *aprB2* allele; in Switzerland, Locher et al. (2015) evaluated the *aprV2/B2* qPCR of Stäuble et al. (2014a) as a potential screening tool for identifying virulent *D. nodosus* isolates in clinically healthy flocks, and reported that *aprV2*-positive isolates were identified in four flocks on one or more occasions, despite the flocks remaining clinically healthy for the duration of the study.

Clinical and laboratory diagnosis of footrot is further complicated by the existence of intermediate disease states and *D. nodosus* strains with intermediate phenotypes. In New South Wales, clinically intermediate and benign footrot are grouped together by the regulatory agency because the economic impacts of intermediate footrot are not thought to justify the cost of elimination. Furthermore, with the exception of the elastase test, laboratory virulence tests are unable to differentiate virulent and intermediate strains of *D. nodosus*. In the present study, three outbreaks classified as clinically benign met the criteria for intermediate footrot (Flocks 20, 29 and 33). Most *D. nodosus* isolates obtained from these flocks possessed the *aprV2* allele. This finding may indicate that virulent and intermediate *D. nodosus* strains may be genetically similar and are not distinguished by the *aprV2/B2* qPCR. Whole-genome sequencing of virulent and benign strains of *D. nodosus* supports this conclusion. Kennan et al (2014) reported that *D. nodosus* has a bimodal population structure and that intermediate genomic states most likely do not exist. The present study provides justification for further investigation of the factors, be they host, pathogen or environmental, that distinguish virulent, benign and intermediate footrot. The outcomes of this study indicate that the presence or absence of a single gene is insufficient to categorise a *D. nodosus* isolate as virulent or benign.

3.5 Conclusions

The qPCR test was reported to be capable of identifying *D. nodosus* and differentiating virulent and benign strains according to the presence or absence of the *aprV2* and *aprB2* alleles (Frosth et al., 2015; Stäuble et al., 2014a). The present study demonstrated that *aprV2*-positive *D. nodosus* isolates are frequently isolated from outbreaks of clinically benign footrot. Because of its poor diagnostic specificity, the qPCR test was not deemed fit for purpose, as there is a considerable risk of subjecting producers to unnecessary regulatory activity. A total of 139 *D. nodosus* isolates that possessed the *aprV2* allele did not appear to express a mature AprV2 protease in a form detectable by the elastase test, and therefore may not be capable of inducing virulent footrot *in vivo*. Further investigation of the molecular basis of virulence is required. This study also highlights the risk of relying exclusively on laboratory virulence tests for the diagnosis of virulent footrot.

Chapter 4

Detection and serogrouping of *Dichelobacter nodosus* infection using direct PCR from lesion swabs to support outbreak-specific vaccination for virulent footrot in sheep

4.1 Introduction

Footrot is an economic and animal welfare concern in most sheep-rearing countries, including Australia and the United Kingdom (Lane et al., 2015; Wassink et al., 2010). The clinical disease is the result of complex interactions between the essential causative agent, *Dichelobacter nodosus*, the host, and its environment (Beveridge, 1941). It is a highly infectious disease that manifests as painful separation of the horny epidermis of the hoof exposing the sensitive underlying dermal tissues. *D. nodosus* possesses an abundance of fine, filamentous appendages called fimbriae (Billington et al., 1996) which are the primary surface (K) antigen (Egerton, 1973). Isolates are divisible into ten serogroups (A-I, and M) by means of the slide agglutination test, which classifies strains according to the presence of major, group-specific fimbrial epitopes (Claxton, 1986b; Claxton et al., 1983; Ghimire et al., 1998) that are encoded by the *fimA* gene (Mattick et al., 1984). *D. nodosus* strains are further divisible into two classes based on the arrangement of the fimbrial gene region: Class I, which consists of serogroups A, B, C, E, F, G, I, and M, and Class II, which consists of serogroups D and H (Ghimire et al., 1998; Mattick et al., 1991).

Fimbriae are highly immunogenic and vaccines incorporating fimbrial proteins are protective (Dhungyel et al., 2013a; Egerton et al., 2002) but immunity is serogroup-specific, with little or no cross-protection between serogroups (Stewart et al., 1991). Up to 7 serogroups may be present in a flock of sheep (Claxton et al., 1983; Dhungyel et al., 2013a). Multivalent vaccines targeting up to nine serogroups have been trialled (Raadsma et al., 1994; Schwartzkoff et al., 1993) and remain commercially available in some countries, but provide only limited protection due to antigenic competition (Hunt et al., 1994). However, outbreak-specific mono- and bi-valent vaccines can be used successfully to treat, prevent, and eliminate virulent footrot (Dhungyel et al., 2013a; Egerton et al., 2002) as they avoid antigenic competition. Direct comparisons of multivalent vaccine and bivalent vaccine have not been reported.

Currently, in order to target the appropriate *D. nodosus* strain(s) with a mono- or bi-valent vaccine, the infecting *D. nodosus* strain(s) must be cultured from lesion material, and serogrouped using a slide agglutination test (Egerton, 1973) or multiplex *fimA* PCR (Dhungyel et al., 2002). These culture-based methods are slow, requiring up to six weeks for a result. This can delay the implementation and potentially the success of outbreak-specific vaccination programmes (Dhungyel et al., 2013a). Furthermore, culture-dependent testing is unlikely to detect all serogroups present in a flock unless there is an intensive sampling strategy (Hill et al., 2010). Consequently, there is a need for a more rapid and more sensitive testing procedure.

Strains of *D. nodosus* differ in virulence, a phenotype that is independent of serogroup, and this leads to forms of the disease that differ in severity. In Australia these are dichotomously classified as benign footrot and virulent footrot (Buller and Eamens, 2014). It is not usually economically justifiable to impose quarantine restrictions or use vaccine or other treatments unless the diagnosis is virulent footrot. Importantly, diagnosis is always made at flock-level. In some States (NSW, South Australia, Western Australia) quarantine and mandatory disease control is imposed but only in flocks with virulent footrot. While a clinical diagnosis of virulent footrot is often obvious, sometimes the environment is not conducive to disease expression, and a virulence test based on the protease activity of pure cultures of *D. nodosus* is used (Buller and Eamens, 2014). Such a test is mandatory in Western Australia and the results take priority over clinical diagnosis. Currently the only approved virulence tests in Australia require culture and assessment of protease activity, because real-time PCR tests for the protease gene *aprV2* lack specificity in Australian sheep flocks (McPherson et al., 2017).

While direct (culture-independent) tests for *D. nodosus* infection have long been sought (Rood and Yong, 1989) and more recently reported using both conventional and real-time PCR platforms targeting the 16S rRNA, *pnpA*, *rpoD*, and *aprV2/aprB2* genes (Belloy et al., 2007; Calvo-Bado et al., 2011; Frosth et al., 2015; Frosth et al., 2012; La Fontaine et al., 1993; Moore et al., 2005a; Stäuble et al., 2014a; Stäuble et al., 2014b), the diagnostic performances of these tests can vary (Dhungyel et al., 2013b; Frosth et al., 2012; McPherson et al., 2017; Moore et al., 2005a), and there are no reports of direct PCR-based serogrouping methods validated against a reference test at flock level. That these could be developed is suggested by reports of PCR amplification followed by cloning and sequencing (Cagatay and Hickford, 2005, 2006), or PCR-

single strand conformational polymorphism analysis (Cagatay and Hickford, 2011) of Class I and Class II-specific regions of the *fimA* gene. However, *fimA* has not been validated previously as a target for detection *per se* of *D. nodosus*.

The primary objectives of this study were to develop and validate a procedure for the direct detection and serogrouping of *D. nodosus* from foot lesion swabs and to determine whether this could be done accurately from the same swab that is cultured. Previously published conventional multiplex PCR assays targeting the single-copy *fimA* gene (Dhungyel et al., 2002) and the triple-copy 16S rRNA gene (La Fontaine et al., 1993) were compared to the reference tests of culture of *D. nodosus*, the slide agglutination test and serogroup-specific PCR on pure cultures.

4.2 Materials and methods

4.2.3 Collection of foot swabs

Twelve Merino sheep flocks with pre-existing diagnoses of virulent footrot located in south-eastern Australia (Tasmania, NSW) were included in this study. *D. nodosus* isolates obtained from each flock were previously subjected to the slide agglutination test and multiplex cPCR tests targeting serogroups A to I (Dhungyel et al., 2002) prior to the commencement of this study, and the flocks were known to be infected with one or more strains belonging to serogroups A to I only. Flock-level diagnosis of virulent footrot was based on the proportion of sheep with at least one severe (score 4) lesion (Egerton, 1989a). Each sheep in each flock was placed in dorsal recumbency and each foot was inspected. Foot swabs were collected for diagnostic purposes by the authors or an experienced veterinarian from sheep with footrot lesions. A score (0 to 4) was assigned to each foot of each sheep according to a scoring system (Egerton and Roberts, 1971). Foot swabs were collected from the active zone of the interdigital skin lesion or the active margin of a lesion beneath the horn of the hoof using a sterile, cotton-tipped swab (CLASSIQSwabs; Copan Italia, Brescia, Italy). Two swabs were collected from each foot lesion by operator one, who handed them to a second operator. Operator two, who had no knowledge of which swab was which, and in no particular order, placed one swab into a 5 mL serum vial (Techno Plas, St Marys, Australia) containing approximately 5 mL of modified mSTM (Amies, 1967) for microbiological culture and DNA preparation, and the other swab into a 1.5 mL screw-cap microcentrifuge tube (SSIBio, Lodi, U.S.A.) containing 500 uL of lysis buffer (LB) (Buffer

RLT; Qiagen, Hilden, Germany) for DNA preparation only. All swabs were transported to the laboratory on ice.

4.2.4 Isolation of *D. nodosus*

D. nodosus isolation was attempted from each lesion swab collected in mSTM, as described previously in Chapter 2.2.

4.2.5 Slide agglutination test

Each *D. nodosus* isolate was serogrouped using the slide agglutination test as described in Chapter 2.3

4.2.6 Elastase test

The virulence of a *D. nodosus* isolate was assessed using the elastase test, as described in Chapter 2.5.

4.2.7 DNA extraction.

DNA was extracted from a pure culture of a *D. nodosus* isolate by boiling and centrifugation, as described in Chapter 2.6.1. DNA was extracted from swabs by magnetic bead separation using the BioSprint 96 One-For-All Vet Kit (Qiagen, Hilden, Germany), as described in Chapter 2.6.3. DNA was stored at -20°C prior to testing.

4.2.8 Direct PCR testing of swabs collected in mSTM (PCR-mSTM) and LB (PCR-LB)

Modified STM is recommended for the transport of specimens of lesion material for microbiological culture by Australian animal health agencies (Buller and Eamens, 2014). It would be advantageous if a single swab collected in mSTM could be used for microbiological culture for virulence tests and then for direct PCR testing. Therefore, we compared microbiological culture and direct PCR using a single swab collected into mSTM. Immediately after being used to inoculate a HA plate (see above), each mSTM swab was transferred to a 1.5 mL screw-cap microcentrifuge tube (SSIBio, Lodi, U.S.A.) with 500 µL of LB and incubated at 4°C overnight. The microcentrifuge tube was vortexed at high speed for 30 s to separate material from the swab, and the swab was discarded. DNA was extracted from the lysate via magnetic bead separation. DNA was prepared from the lysate of the LB swabs in the same manner.

4.2.9 PCR detection of the *D. nodosus* 16S rRNA gene.

D. nodosus was detected by PCR amplification of a variable region of the 16S rRNA gene after optimising a published assay (La Fontaine et al., 1993). Primers and reactions conditions were as reported previously (La Fontaine et al., 1993); however, a customised touchdown thermal cycling programme was developed consisting of an initial denaturation step of 95°C for 3 mins, followed by two cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, two cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, 10 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and 15 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 4 mins. This was prompted by previous reports of poor sensitivity and non-specific PCR products using these primers with the cycling conditions reported by La Fontaine, et al. (1993), or modified cycling conditions (Frosth et al., 2012; Moore et al., 2005a). Amplification was performed in a Bio-Rad T100 thermal cycler (Bio-Rad, Gladesville, Australia). PCR product was visualised on a 2% agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, South Korea), and visualised under ultraviolet light. DNA prepared from virulent *D. nodosus* prototype strain A1001, and nuclease-free water, were included in each run as positive and negative controls, respectively. A successful PCR run was defined as one in which: (i) there was amplification of the positive control, indicated by the presence of an amplicon of the appropriate molecular weight on the 2% agarose gel, and (ii) there was no amplification of the negative control.

4.2.10 PCR detection of *D. nodosus fimA* gene

PCR serogrouping was undertaken by conventional PCR amplification of serogroup-specific variable regions of the serogroup A to I *fimA* genes, as described in Chapter 2.7. A serogroup M-specific PCR was not available.

4.2.11 Statistical analyses.

The level of agreement between microbiological culture and a PCR procedure was evaluated using Cohen's kappa statistic (Cohen, 1960) interpreted using previously proposed standards for strength of agreement (1977). McNemar's Chi-Square Test for Paired Observations (McNemar, 1947) was performed to test differences in the sensitivity between two tests. The influence of factors on the detection of *D. nodosus* by microbiological culture or 16S rRNA PCR was

evaluated using a Generalised Linear Mixed Model (GLMM). A binary outcome for the detection of *D. nodosus* via microbiological culture (1 = positive [one or more *D. nodosus* colonies were obtained], 0 = negative [no *D. nodosus* colonies were obtained]) or PCR (1 = positive [the *D. nodosus* 16S rRNA gene was amplified and a band of the appropriate size visualised via gel electrophoresis], 0 = negative [the *D. nodosus* 16S rRNA gene was not amplified]) was coded for analysis. The type of lesion (active or inactive), lesion score (0 to 4, as described by previously (Egerton and Roberts, 1971)), and the degree of soil/faecal contamination (low, moderate, or high) were accounted for in the fixed model including interactions between these three factors where computationally possible, while farm of origin was accounted for in the random model. Categories in which there were fewer than five observations were collapsed prior to analysing the data. All analyses were conducted in GenStat 16th Edition (© 2000-2016 VSN International Ltd, Hemel Hempstead, U.K.).

Table 4.1: Comparison of four different methods of serogrouping *D. nodosus* at the flock-level. Ambiguous slide agglutination test results are shown in parentheses. mSTM, modified Stewart's transport medium; LB, lysis buffer.

| | | | | Culture based serogrouping (pure cultures) | | | | Culture independent serogrouping (lesion swabs) | | | |
| | | | | Slide agglutination | | *fimA* PCR | | *fimA* PCR on mSTM swabs | | *fimA* PCR on LB swabs | |
Flock	No. sheep examined	No. sheep sampled	No. feet sampled	No. isolates tested	Serogroups detected	No. isolates tested	Serogroups detected	No. swabs tested	Serogroups detected	No. swabs tested	Serogroups detected
1	51	24	24	25	A, (D), (G)	25	A	24	A	24	A
2	52	42	50	46	A, B, H, I, (D), (F), (G)	36	A, B, H, I	50	A, B, G, H, I	50	A, B, G, H, I
3	42	20	20	36	C, D, E, G, I, (H), (M)	34	C, D, E, G, H, I	20	D, G, H	20	D, E, G, H
4	33	20	20	36	A, B, E, H, I, (C), (D)	36	A, B, E, H, I	20	A, B, E, G, H	20	A, B, E, G, H, I
5	34	20	20	33	A, B, D, G, H, (C), (M)	33	A, E, D, G, H, I	20	A, B, E, G, H	20	A, B, D, E, G, H
6	28	20	20	19	A, B, (D), (E), (H), (I)	19	B	20	A, B	20	A, B
7	13	13	13	13	A, B	13	B	13	A, B	13	A, B
8	27	20	20	20	E, G, H, (B)	20	E, G, H	20	G, H	20	E, G, H
9	20	20	20	14	A, B, E, G, H	14	A, B, E, H	20	A, B, E	20	A, B, E, G
10	12	12	12	11	G	10	G	0	Not tested	12	E, F, G, H
11	26	25	25	22	C, H, (B), (D), (E), (F)	21	H	0	Not tested	25	A, C, D, E, G, H
12	26	25	25	15	E, (C), (G)	14	E	0	Not tested	25	E
Total	**364**	**261**	**269**	**290**		**275**		**207**		**269**	

4.3 Results

A total of 269 foot swabs were collected into LB and modified mSTM (Amies, 1967) from 269 foot lesions from 261 sheep in 12 Merino flocks in south-eastern Australia between September and December, 2014 (Table 4.1). Swabs were collected from one foot of each sheep, except for Flock 2 in which two feet were sampled from each of eight sheep and one foot from the other 34 sheep. Sub-cultures of 290 *D. nodosus* isolates obtained from culture of the mSTM swabs were analysed by the slide agglutination test and sub-cultures of 275 of these were also analysed by *fimA* PCR. Fifteen sub-cultures that failed to grow were not tested by *fimA* PCR. A total of 62 foot swabs collected into mSTM from three flocks (Flocks 10 to 12) were not retained after being used to inoculate HA plates, therefore direct PCR results for foot swabs collected into mSTM were available for 207 foot swabs collected from Flocks 1 to 9. DNA extracts from each of the foot swabs were analysed in the various PCR assays which are named below according to the type of swab and the gene target.

4.3.1 Comparison of sample collection methods for culture-independent serogrouping.

In order to undertake culture-independent serogrouping directly from foot swabs using PCR, we first needed to ensure that extraction and detection methodologies were satisfactory for the detection *per se* of *D. nodosus*. This is because microbiological mSTM is commonly used for sample collection, and as culture may be required by regulatory authorities for virulence testing, it would be advantageous to only need to collect one swab for both tests, and because *fimA* has never been used before as the sole test for the presence of *D. nodosus* and has unknown diagnostic specificity. Therefore assays were conducted on swabs placed into the transport medium mSTM after they had been used to inoculate HA plates, and on swabs collected into LB. Then culture and PCR for the *D. nodosus* 16S rRNA gene were used as reference tests.

16S rRNA PCR testing of 207 swabs collected into mSTM was more sensitive than culture of these swabs (McNemar's $X^2 = 7.04$, $P = 0.0082$); there were 32 feet/swabs on which *D. nodosus* was detected by 16S rRNA PCR only, and 14 feet/swabs on which *D. nodosus* was detected by culture only. *D. nodosus* was detected by PCR on approximately 86% of feet/swabs compared to 77% by culture. The level of agreement between the two methods was fair (kappa = 0.282).

A similar analysis was done using 269 foot swabs collected into LB. 16S rRNA PCR testing of swabs in LB was more sensitive than culture of mSTM swabs (McNemar's X^2 = 34.32, P < 0.0001); there were 52 feet/swabs on which *D. nodosus* was detected by PCR only, and seven feet/swabs on which *D. nodosus* was detected by culture only. *D. nodosus* was detected on approximately 95% of feet/swabs by PCR and 78% by culture. The level of agreement between the two method was poor (kappa = 0.118).

A comparison of 16S rRNA PCR results for foot swabs collected from 207 feet into both mSTM and LB confirmed that use of LB swabs was superior (McNemar's X^2 = 10.67, P = 0.0011) (Table 4.4). Thus, detection of *D. nodosus* using 16S rRNA PCR of swabs collected into LB was a significantly more sensitive procedure than culture.

The direct *fimA* PCR on LB swabs was compared with culture and 16S rRNA PCR on LB swabs for the detection of *D. nodosus*. The *fimA* PCR was more sensitive than culture (McNemar's X^2 = 9.94; P = 0.0017) to the extent that the level of agreement between the two tests was poor (kappa = 0.092; 95% CI -0.038 – 0.221). The 16S rRNA PCR on LB swabs procedure was more sensitive than the *fimA* PCR on LB swabs (McNemars X^2 = 10.53, P = 0.0012) leading to poor agreement between these tests (kappa = 0.070; 95% CI 0.070 – 0.211). Nevertheless, there was agreement between the *fimA* PCR and 16S rRNA PCR for 84.5% of the LB swabs tested.

4.3.2 Impact of lesion characteristics on the detection of *D. nodosus*.

The impact of three foot lesion characteristics (foot score, lesion type, and faecal/soil contamination) on the detection of *D. nodosus* by culture and direct PCR was evaluated. The proportion of sheep assigned to each category in each flock is provided in Table 4.2. A relatively small number of inactive lesions were sampled, as we were attempting to sample mostly active lesions, however inactive lesions were found to have a significant effect on the detection of *D. nodosus*, in that they were less likely to yield a positive test outcome. Lesion scores were assigned to each foot using a previously described scoring system (Egerton and Roberts, 1971). Score 4 lesions were distributed across all 12 flocks, which was consistent with a clinical diagnosis of virulent footrot. Contamination of foot lesions with soil and faecal material was present in all flocks. The frequencies of positive test outcomes, arranged according to each lesion characteristic, are provided in Table 4.3 and reveal that for each test the lesion type and the

degree of contamination had a marked impact on the likelihood of test positivity, and that foot score also appeared to be important for culture and PCR off mSTM swabs but not LB swabs. These features were confirmed with statistical analysis using a generalised linear mixed model (GLMM). Using microbiological culture, the odds of detecting *D. nodosus* in a score 2, score 3 and score 4 lesion were 1.72, 7.32 and 1.90 times that of a score 1 lesion, respectively, with the significant difference ($P = 0.021$) being due to the comparison of score 3 and the reference category score 1. The odds of detecting *D. nodosus* in an inactive lesion was 0.26 that of an active lesion ($P = 0.027$) while the odds of detecting *D. nodosus* in a lesion with moderate and low degree of contamination were 4.99 and 11.21 times that of a lesion with a high degree of contamination, respectively ($P < 0.001$) (see Appendix A, Table A.1). Interactions between these factors were not significant ($P > 0.05$).

Using 16S rRNA PCR on mSTM swabs, the odds of detecting *D. nodosus* in a score 2, score 3 and score 4 lesion were 40.13, 25.25 and 12.74 times that of a score 1 lesion, respectively. The odds of detecting *D. nodosus* in an inactive lesion was 0.09 that of an active lesion while the odds of detecting *D. nodosus* in a lesion with a moderate and low degree of contamination were 14.32 and 25.15 times that of a lesion with a high degree of contamination, respectively. These results were statistically significant ($P < 0.001$) (see Appendix A, Table A.2). Interactions between these factors were not significant ($P > 0.05$).

Using 16S rRNA PCR on LB swabs, the odds of detecting *D. nodosus* in an inactive lesion was 0.10 times that of an active lesion ($P = 0.008$). The odds of detecting *D. nodosus* in a lesion with low/moderate soil and faecal contamination was 8.50 times that of a lesion with a high degree of contamination ($P = 0.007$) while lesion score, which had been collapsed into two categories due to sample size (score 1 and 2; score 3 and 4) was not significant ($P = 0.422$) (see Appendix A, Table A.3). The model did not converge with interactions included so they were omitted. Examination of the results for each foot score category (Table 4.3) suggest little difference in positive rate between foot scores, and substantial differences due to lesion type and contamination, consistent with the results of the GLMM.

Table 4.2: Frequency of observations for each lesion characteristic in each flock at the time of collection of swabs. Foot scores were assigned using a scoring system (Egerton and Roberts, 1971). Contamination refers to the presence of soil and faeces on the foot lesion.

						Flock no.						
Factor	1	2	3	4	5	6	7	8	9	10	11	12
Foot score												
1	11	1	2	0	2	0	2	1	1	0	1	2
2	2	2	2	0	2	1	2	4	10	0	9	14
3	0	7	11	2	11	9	2	8	4	7	4	6
4	11	40	5	18	5	10	7	7	5	5	11	3
Total	**24**	**50**	**20**	**20**	**20**	**20**	**13**	**20**	**20**	**12**	**25**	**25**
Lesion type												
Active	18	46	18	20	20	20	13	19	20	12	25	22
Inactive	6	4	2	0	0	0	0	1	0	0	0	3
Total	**24**	**50**	**20**	**20**	**20**	**20**	**13**	**20**	**20**	**12**	**25**	**25**
Contamination												
Low	5	21	14	19	14	15	3	6	0	10	4	8
Moderate	19	17	6	1	5	5	7	11	11	2	14	17
High	0	12	0	0	1	1	3	3	9	0	7	0
Total	**24**	**50**	**20**	**20**	**20**	**20**	**13**	**20**	**20**	**12**	**25**	**25**

Table 4.3: Frequency of positive microbiological culture and direct 16S rRNA PCR results for the detection of *D. nodosus*. Data are arranged according to the fixed terms in the GLMM. Foot scores were assigned according to a scoring system (Egerton and Roberts, 1971). Contamination refers to the presence of soil and faeces on the foot lesion.

Variable	Microbiological culture		16S rRNA PCR on mSTM swabs		16S rRNA PCR on LB swabs	
	% Swabs positive	Total	% Swabs positive	Total	% Swabs positive	Total
Foot score						
1	45.7	25	45.5	22	84.0	25
2	75.0	44	95.2	21	97.7	44
3	90.7	75	98.3	58	100	75
4	75.0	125	84.8	106	92.7	125
Lesion type						
Active	79.8	253	88.1	194	96.0	253
Inactive	50.0	16	46.2	13	75.0	16
Contamination						
Low	89.8	118	94.8	96	99.2	118
Moderate	75.7	115	80.5	82	93.0	115
High	47.2	36	69.0	29	86.1	36

4.3.3 Culture-dependent serogrouping.

Sub-cultures of 290 *D. nodosus* isolates were tested with the slide agglutination test, and sub-cultures of 275 of the same isolates were tested with the multiplex *fimA* PCR (Dhungyel et al., 2002). The number of *D. nodosus* serogroups detected in each flock using a combination of the slide agglutination test and *fimA* PCR testing of pure cultures ranged from one to seven (Table 4.1). The number of *D. nodosus* serogroups detected on a single swab/foot using a combination of the slide agglutination test and the *fimA* PCR ranged from one to three. There were five instances in which a serogroup was detected at flock level by the slide agglutination test only, and two instances in which a serogroup was detected at flock level by *fimA* PCR only.

Ambiguous slide-agglutination test outcomes were observed for 158 isolates originating from nine of the 12 flocks. These are shown in parentheses in Table 4.1. In contrast, the serogrouping outcomes from the multiplex *fimA* PCR assays were unambiguous. That is, there was a clear serogroup result for every culture that was tested in the PCR.

The slide agglutination test and the *fimA* PCR were compared at the foot level for the detection of *D nodosus* serogroups. Given that there was only one sampling event per foot, this comparison is also a swab-level comparison. There was complete agreement between these tests for 67.4% of swabs/feet tested. In the remaining 32.6% of swabs/feet tested, one or more different serogroups were detected in one or other of the tests.

Table 4.4: Comparison of 16S rRNA PCR conducted on DNA extracts from mSTM swabs and LB swabs for the detection of *D. nodosus*. Analyses are at swab/foot level.

		PCR-mSTM		
		Negative	**Positive**	Total
PCR-LB	**Negative**	10	4	14
	Positive	20	173	193
	Total	30	177	207

McNemar's $X^2 = 10.67$, $P = 0.0011$; kappa = 0.399, 95% CI 0.201 – 0.588

4.3.4 Elastase test.

A total of 162 of the 269 *D. nodosus* isolates, distributed across all 12 flocks, were subjected to the elastase test. Approximately 78% of the isolates tested were elastase-positive. Even though 107 isolates were not tested, in most flocks there were elastase-positive isolates within each of the serogroups detected (data not shown).

4.3.5 Direct serogrouping.

DNA prepared from 269 lesion swabs collected into LB from sheep in 12 flocks, and 207 swabs collected into mSTM from sheep in nine flocks, were analysed by multiplex *fimA* PCR (Dhungyel et al., 2002) (Table 4.1). The number of serogroups detected in each flock by *fimA* PCR of mSTM swabs ranged from one to five. The number of serogroups detected in each flock by *fimA* PCR of LB swabs ranged from one to six. Up to four additional serogroups were identified in each of these flocks by direct *fimA* PCR on swabs collected into LB compared with those identified by slide agglutination and *fimA* PCR testing of pure cultures, but only one additional serogroup was detected in two flocks by direct *fimA* PCR testing of swabs collected into mSTM. In five of nine flocks, more serogroups were detected by direct *fimA* PCR testing of swabs collected into LB than of swabs collected into mSTM. Up to four serogroups were detected on one foot by *fimA* PCR of mSTM swabs, and up to five serogroups were detected on one foot by *fimA* PCR of LB swabs.

Culture from mSTM swabs and direct *fimA* PCR from LB swabs were compared at the swab/foot level for the detection of *D. nodosus* serogroups. There was complete agreement between culture and the direct *fimA* PCR for 33.8% of swabs/feet tested. In the remaining 66.2% of swabs/feet one or more different serogroups were detected by one or other of the tests.

4.5 Discussion

The success of outbreak-specific vaccination programmes for virulent footrot is underpinned by accurate and timely detection and serogrouping of the infecting *D. nodosus* strain(s) (Dhungyel et al., 2013a; Dhungyel et al., 2008; Egerton et al., 2002). We developed a sensitive procedure for direct detection and serogrouping of *D. nodosus* in specimens of lesion material on cotton swabs, based on existing conventional PCR assays targeting the 16S rRNA and *fimA* genes (Dhungyel et al., 2002; La Fontaine et al., 1993). This study is the first report of direct PCR-based serogrouping (Dhungyel et al., 2002) of *D. nodosus* with validation against a reference test at flock level, and the first extensive comparison of the slide agglutination test and serogroup-specific PCR testing (Dhungyel et al., 2002) of *D. nodosus* field isolates.

Procedures for sample collection, sample transport, and DNA preparation were optimised first for a PCR assay targeting the 16S rRNA gene of *D. nodosus*. Previously reported primers (La Fontaine et al., 1993) were used; however, the assay was modified and a customised touchdown thermal cycling program was developed to enhance its sensitivity and specificity; this was prompted by reports of poor sensitivity and non-specific PCR products in previous studies (Frosth et al., 2012; Moore et al., 2005a).

Two procedures for handling lesions swabs were compared, one of which enabled prior culture of the swab (mSTM swabs). 16S rRNA PCR conducted on DNA prepared from both types of swabs resulted in more sensitive detection of *D. nodosus* than culture. Notably, *D. nodosus* was detected by direct 16S rRNA PCR in 66 to 88% of lesions that were culture-negative, which could be explained by the detection of both viable and non-viable organisms by the PCR. DNA extracts from mSTM swabs were less likely to yield positive PCR results than those from LB swabs, possibly because lesion material had been dislodged when the former were used to inoculate culture plates. If culture is not required, swabs collected in LB are recommended for direct PCR detection of *D. nodosus.* If culture is required, duplicate swabs should be collected, one in mSTM for culture and the other in LB for PCR.

With respect to the detection of *D. nodosus per se*, based on these results we undertook a three way comparison of the direct *fimA* PCR, the 16S rRNA PCR (both using DNA extracts from swabs collected into LB) and culture of swabs collected into mSTM. The direct *fimA* PCR was

more sensitive than culture, but less sensitive than the 16S rRNA PCR. This is not surprising, since there are three copies of the 16S rRNA gene in the *D. nodosus* genome (La Fontaine and Rood, 1996), but only one copy of the *fimA* gene (Mattick et al., 1991). Logically, the 16S rRNA PCR would be run in tandem with the *fimA* PCR to signal possible false negative test outcomes in the latter. However, given that there was agreement between the two tests for 84% of samples tested, and because multiple samples need to be tested for accurate flock level diagnosis (Hill et al., 2010), the direct *fimA* PCR alone may be sufficient for detection of *D. nodosus* as well as serogrouping at flock level.

The rates of detection (86% to 95%) achieved using the direct conventional PCR procedures that were optimised in this study were higher than those reported in previous studies using conventional (Moore et al., 2005a) or real-time (Frosth et al., 2012) PCR amplification of the *D. nodosus* 16S rRNA gene. Although real-time PCR is generally regarded as having a greater analytical sensitive than conventional PCR, these results demonstrate that high rates of detection, along with high analytical specificity, can be achieved with a conventional PCR assay with an appropriate procedure for sample collection and DNA preparation. The difference between culture and direct PCR detection of the 16S rRNA gene reported here is less marked than has been reported before (Frosth et al., 2012; Moore et al., 2005a); however, the rates of detection by culture reported in those studies (27% and 43%, respectively) were much lower than was achieved in this study (76.8% to 78.0%). We opted to use a conventional 16S rRNA PCR assay (La Fontaine et al., 1993) as the objective of this study was to develop a direct testing procedure to use with the conventional *fimA* PCR (Dhungyel et al., 2002), and the products of the two reactions could be visualised conveniently on the same agarose gel. Although the conventional 16S rRNA PCR assay was reported to be capable of testing specimens of lesion material directly (La Fontaine et al., 1993), the authors were unable to detect *D. nodosus* in samples collected from score 2 interdigital lesions, and speculated that the *D. nodosus* load may have been too low. Recent studies have shown that *D. nodosus* load is highest on feet with interdigital lesions (Maboni et al., 2016; Moore et al., 2005a; Witcomb et al., 2014; Witcomb et al., 2015), so the failures to detect D. nodosus in score 2 lesions were probably due to other factors. Subsequently the analytical sensitivity of the 16S rRNA PCR test was also reported to be low when applied to direct testing of lesion material, and the reaction conditions were modified (Moore et al., 2005a); this increased analytical sensitivity by up to 17% compared to microbiological culture, and the

false-negative rate (culture-positive/PCR-negative) was only 0.8% (2/263) of feet tested. However there were concerns about the analytical specificity of this modified form of the test, due to non-specific PCR products (Frosth et al., 2012). Consequently, we introduced further modifications to the 16S rRNA PCR test to enhance the analytical sensitivity and specificity.

We undertook an extensive comparison of the slide agglutination test and the serogroup-specific PCR (Table 4.1). Ambiguous slide agglutination test outcomes, defined as a delayed or fine agglutination reaction, were frequently observed (reported in parentheses in Table 4.1). Interpreting such results can be challenging because there are several possible causes. Fine (low-titre) agglutination reactions have been ascribed to reactions between antisera and non-fimbrial antigens (Thorley, 1976), minor cross-reactivity between closely-related serogroups that share common fimbrial epitopes can occur (Claxton, 1986b; Claxton et al., 1983), and the strength of an agglutination reaction is known to vary according to the degree of fimbriation of an isolate (Stewart et al., 1986).

PCR test results from putative pure cultures are typically given priority when slide agglutination test results are ambiguous (Dhungyel et al., 2013a); this strategy is intended to enhance the specificity of the serogrouping procedure. The slide agglutination and PCR tests are typically performed on different sub-cultures of the same *D. nodosus* primary culture, and multiple serogroups may be present on a single foot, so the results of the two tests do not always align (Hill et al., 2010). There were five flocks in this study in which a serogroup was detected by the slide agglutination test but not by PCR testing of pure cultures (Table 4.1), reinforcing the limitations of culture-dependent testing that were highlighted in a previous study in which even the most intensive sampling strategies failed to identify all serogroups present in a flock (Hill et al., 2010). Substantially increasing the number of sheep examined and the number of isolates tested would increase the accuracy of current serogrouping procedures (Claxton et al., 1983; Hill et al., 2010), but intensive sampling is not practical and the cost would be prohibitive. Direct PCR testing of lesion swabs increased the number of serogroups detected in a flock compared to culture. Additional serogroups (range one to four) were detected in four flocks (Table 4.1).

The direct serogrouping procedure developed in this study, which is based upon the cPCRs published by Dhungyel et al. (2002), which are capable of detecting serogroups A to I only. It is apparent that if a flock were infected with serogroup M only, the direct testing procedure

developed in this study would be unable to detect the organism. As such, there is a clear need to develop a serogroup M-specific cPCR test.

Using the most sensitive method of testing swabs collected into LB, *D. nodosus* was most likely to be detected in active lesions, and on feet with only minor faecal or soil contamination, but lesion score was not significant (Tables 4.3 and Appendix A, Table A.3), even though *D. nodosus* load is known to be highest in interdigital lesions (Maboni et al., 2016; Witcomb et al., 2014; Witcomb et al., 2015). Interestingly, *D. nodosus* was most likely to be detected by culture on feet with score 3 lesions (Appendix A, Table A.1). This may be due to the physical characteristics of score 3 lesions rather than *D. nodosus* load: at the score 3 stage the horn of the epidermis has only recently begun to separate, forming a small cavity that is relatively free of debris and necrotic material from which a non-contaminated specimen can be collected. Although *D. nodosus* load was reported to be higher on feet with interdigital lesions than on feet with severe, underrun lesions (Maboni et al., 2017; Maboni et al., 2016; Witcomb et al., 2014), in those studies the specimens were collected from the interdigital skin rather than from the active margin of the lesion.

4.6 Conclusions

Serogroup-specific vaccines can be used to treat and control virulent footrot, but their success is contingent upon detecting and serogrouping the infecting *D. nodosus* strain(s) that are present in the flock. Current culture-dependent methods are unlikely to detect all serogroups present in a flock. We have developed and validated a sensitive, culture-independent procedure for the detection and serogrouping of *D. nodosus* directly from lesion swabs collected into a lysis buffer. A duplicate swab for microbial culture should be collected if a culture-based virulence test is also required. Samples should be collected from active lesions that are relatively free from contamination with soil and faeces. This procedure will enhance the detection and serogrouping of *D. nodosus* and expedite the administration of serogroup-specific vaccines.

Chapter 5

Development of a conventional polymerase chain reaction test for the detection of *Dichelobacter nodosus* serogroup M

5.1 Introduction

Footrot is prevalent in most sheep-producing countries, including Australia, the U.K. and Sweden (Greber et al., 2016; Lane et al., 2015; Wassink et al., 2003). The essential causative agent, *D. nodosus*, is a slow-growing anaerobic bacterium, and an obligate parasite of the ruminant hoof (Beveridge, 1941). *D. nodosus* possesses fine, filamentous appendages called fimbriae or pili, which are responsible for the bacterium's characteristic twitching motility (Mattick, 2002), and which are essential for protease expression, virulence, and natural competence (Kennan et al., 2001). Fimbriae are composed of a single, repeating subunit protein which is encoded by the *fimA* gene (Mattick et al., 1984). The subunit protein consists of a highly conserved amino-terminal domain and a highly variable carboxy-terminal domain, the latter of which constitutes approximately 70% of the mature subunit protein (Dalrymple and Mattick, 1987).

Strains of *D. nodosus* are divisible into ten immunologically distinct serogroups (A to I, and M) according to the variable carboxy-terminal domain of the fimbrial subunit protein (Claxton, 1986b; Claxton et al., 1983; Ghimire et al., 1998; Mattick et al., 1991), and the *fimA* gene (Dhungyel et al., 2002). Each of ten serogroups is further divisible into a number of sub-groups (serotypes) (Claxton, 1986b; Claxton et al., 1983). The ten *D. nodosus* serogroups are also divisible into two distinct classes according to differences in the structure and organisation of the fimbrial gene region: Class I, which consists of serogroups A, B, C, E, F, G, I and M; and Class II, which consists of serogroups D and H (Ghimire et al., 1998; Hobbs et al., 1991; Mattick et al., 1991)

Fimbriae are highly immunogenic and vaccines incorporating fimbrial proteins are protective (Dhungyel et al., 2013a; Egerton et al., 2002) but immunity is serogroup-specific, with little or no cross-protection between serogroups (Stewart et al., 1991). Multiple serogroups (up to seven) may be present in a flock (Claxton et al., 1983; Dhungyel et al., 2013a), and up to four serogroups have been detected on a single foot (Claxton et al., 1983). Multivalent fimbrial vaccines targeting up to nine serogroups have been evaluated (Raadsma et al., 1994;

Schwartzkoff et al., 1993) but provide only limited protection due to antigenic competition (Hunt et al., 1994). However, outbreak-specific mono- and bivalent vaccines can be used to treat, prevent, and eliminate virulent footrot (Dhungyel et al., 2013a; Egerton et al., 2002) as they avoid antigenic competition. It is not necessary to differentiate sub-types within a common serogroup for the purposes of immunisation, however, as there is sufficient cross-protective immunity between sub-types within a common serogroup (Elleman et al., 1990; Stewart et al., 1991).

Currently, in order to target the appropriate *D. nodosus* strain(s) with a mono- or bivalent vaccine, the infecting *D. nodosus* strain(s) must be isolated from specimens of lesion material and serogrouped (Dhungyel et al., 2013a). This is a costly and laborious process that many laboratories are not equipped to undertake. Strains of *D. nodosus* are assigned to serogroups by means of the slide agglutination test, which differentiates serogroups according to the presence of major, serogroup-specific fimbrial antigens (Egerton, 1973), or PCR amplification of serogroup-specific regions of the *fimA* gene (Dhungyel et al., 2002).

Serological cross-reactivity between closely-related serogroups has been reported using the slide agglutination test, particularly with unabsorbed antisera (Chetwin et al., 1991; Claxton, 1986b; Claxton et al., 1983; Ghimire et al., 1998). For instance, in a retrospective study of Australian and N.Z. archival strains, cross-reactivity was reported between serogroup M isolates and serogroup F antisera (Chetwin et al., 1991). In response, stock cultures of Australian and N.Z. *D. nodosus* isolates previously assigned to serogroup F were re-typed using serogroup M antisera. In total, 31% of Australian and 50% of N.Z. isolates initially assigned to serogroup F were found to belong to serogroup M. Cross-reactivity between serogroup F and serogroup M-antisera was not reported (Chetwin et al., 1991)

To improve the specificity of the serogrouping procedure, a multiplex conventional PCR (cPCR) targeting the *fimA* gene was developed that is capable of detecting nine *D. nodosus* serogroups (A to I) (Dhungyel et al., 2002). The multiplex *fimA* PCR has been used extensively to support successful serogroup-specific vaccine programmes, alone or in combination with the slide agglutination test (Dhungyel et al., 2013a; Dhungyel et al., 2008). However, a serogroup M-specific PCR has not been published. The absence of a PCR test for the detection of serogroup M contributed to the failure of a serogroup-specific vaccine programme due to the unexpected

presence of serogroup M in two sheep flocks on King Island, Tasmania, in south-eastern Australia (Dhungyel et al., 2015). Serogroups A and E were initially detected in the first flock, and serogroups A and B in the second flock. In the first round of vaccination, bivalent vaccines targeting A+E and A+B were administered to the first and second flock, respectively. However, after the completion of the first-round of vaccination, disease prevalence remained high in both flocks, and a large proportion of *D. nodosus* isolates collected from both flocks were untypable with antisera for serogroups A to I. This led the authors to suspect that a novel serogroup may be present in the flock. The isolates were tested with the slide agglutination test using antisera raised against Nepalese and N.Z. serogroup M strains, which revealed that the isolates most likely belonged to serogroup M. Sequencing of the *fimA* gene of the untypable isolates verified the slide agglutination test outcomes.

Phylogenetic analysis of the *fimA* gene of the Australian serogroup M strains, along with those from N.Z. and Nepal, revealed that the Australian strains were most closely related to the N.Z. strains (Dhungyel et al., 2015). Interestingly, the Australian and N.Z. serogroup M strains were more closely related to the N.Z. serogroup F strain than the Nepalese serogroup M strains, which formed a sister separate clade (Dhungyel et al., 2015). The similarity of the *fimA* genes of the serogroup M and F explains the reported cross-reactivity between the two serogroups with the slide agglutination test (Chetwin et al., 1991).

Serogroup M has been reported in Australia, N.Z., Nepal, Norway and the U.K. (Chetwin et al., 1991; Day et al., 1986; Dhungyel et al., 2015; Ghimire et al., 1998; Gilhuus et al., 2013). In Australia, the prevalence of serogroup M is unknown, but it appears to be uncommon and to date it has only been detected on King Island (Dhungyel et al., 2015) and mainland Tasmania. Given that serogroup M could be more widespread, and the potential for misclassification using the slide agglutination test, there is a need for a serogroup M-specific PCR test to accompany the multiplex *fimA* PCR developed by Dhungyel et al. (2002). The aim of this study was to develop a cPCR test for the detection of *D. nodosus* serogroup M.

5.2 Materials and Methods

5.2.1 Bacterial strains

Prototype strains representing all ten *D. nodosus* serogroups were obtained from the Farm Animal Health culture collection. These were used to assess the analytical sensitivity and specificity of the PCR test (Table 5.1). Freeze-dried cultures of each *D. nodosus* strain were reconstituted in 200 µL of sterile PBS, pH 7.4, (Astral Scientific, Taren Point, Australia), spread plated onto 4% HA plates (Thomas, 1958), and incubated anaerobically, as described in Chapter 2.2. If other contaminating bacterial species were present, putative *D. nodosus* colonies, identified by colony morphology (Buller and Eamens, 2014), were picked with a sterile inoculation loop and sub-cultured onto a second 4% HA plate and incubated under the conditions described in Chapter 2.2. The serogroup M PCR was also evaluated using DNA previously prepared from 27 *D. nodosus* field isolates identified as serogroup M by means of the slide agglutination test, including 26 isolates from Tasmania, Australia, and one isolate from Norway.

Table 5.1: Prototype *D. nodosus* strains representing all 10 serogroups used to evaluate the analytical sensitivity and specificity of the serogroup M-specific PCR test.

Strain No.	Serogroup (serotype)	Country of origin	Year isolated
VCS1001	A (A1)	Australia	1974
VCS1006	B (B1)	Australia	1974
VCS1008	C (C1)	Australia	1974
VCS1172	D	Australia	1978
VCS1137	E (E1)	Australia	1978
VCS1070	F (F1)	Australia	1975
VCS1220	G (G1)	Australia	1979
VCS1687	H (H1)	Australia	1978
VCS1623	I	Australia	1985
NEP116	M	Nepal	1996
SVC 08/258/1-3	M	Australia	2008
SVC 10/058/4-1	M	Australia	2010

5.2.2 Phylogenetic analysis of *fimA* gene sequences

Phylogenetic analyses of 27 *fimA* sequences representing all ten *D. nodosus* serogroups was undertaken to determine which of serogroups A to I had the highest level of sequence similarity to serogroup M. This was necessary when designing the oligonucleotide primers to ensure that the PCR had a high level of analytical specificity. Sequences representing serogroups A to I were obtained from Cox (1992). Sequences were obtained from Genbank for Australian (accession no. HM486325.1), N.Z. (accession no. AY835839.1) and Nepalese (accession no. AF038920.1) serogroup M isolates. Sequences were aligned in Clustal Omega (Sievers and Higgins, 2014). Molecular phylogenetic analyses were undertaken in MEGA6 (Tamura et al., 2013) using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993; Tamura et al., 2013).

5.2.3 Design and synthesis of oligonucleotide primers

An initial attempt was made at designing the oligonucleotide primers with the Primer Express software (Applied Biosystems, North Ryde, Australia). However, evaluation of the primers generated by the software with the basic local alignment search tool (BLAST) (Altschul et al., 1990) revealed a high degree of sequence similarity to regions of the *fimA* genes of serogroups E and F. As such, I concluded that the primers generated by the software would probably have an unsatisfactory specificity. The oligonucleotide primers were therefore designed manually using the *fimA* sequence alignment generated with Clustal Omega (Sievers and Higgins, 2014) with the following criteria: 18-30 base pairs in length; 40-60% G/C content; a G/C clamp at the 3' end of each primer; T_m of 55-60°C; T_m of forward and reverse primer within 5°C of one another; must not span long homopolymer runs or dinucleotide repeats (Dieffenbach et al., 1993). Forward and reverse primers were designed, producing a 94 bp amplicon (Table 5.2). Oligonucleotide primers were ordered from Sigma-Aldrich, Castle Hill, Australia.

Table 5.2: Details of the primers designed for the conventional serogroup M-specific PCR test.

Primer ID	Direction	Sequence (5' to 3')	Product size (bp)
C1618	F	AGCWGTAATCAGTGGTACTTAT	94
C1670	R	TGATCCATAAGTAATAGTTACGAC	

5.2.4 PCR reaction conditions

Each 20 µL reaction mixture consisted of 10x PCR buffer, 0.2 mM of each dNTP, 1.9 mM of MgCl$_2$, 500 nM of each primer, 5 U/µL of Taq polymerase, and 2.0 µL of DNA template. Amplification was performed in a Bio-Rad T100 thermocycler (Bio-Rad, Gladesville, Australia). Cycling conditions consisted of an initial denaturation step of 95°C for 10 mins, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min, followed by a final extension phase of 72°C for 5 mins. PCR product was visualised on a 2% agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, South Korea) and visualised under ultraviolet light.

5.2.5 Analytical sensitivity and specificity

The analytical sensitivity of the serogroup M PCR was evaluated using a serial dilution of genomic DNA prepared from the Australian serogroup M strain 08/251/4.1. DNA template concentrations ranged from 16.0 ng to 0.0004 ng of genomic DNA per reaction. The equivalent number of *D. nodosus* genome copies was estimated using the following formula:

$$\text{Number of copies} = \frac{\text{DNA weight (in ng) x } 6.022\text{*E10}^{23}}{\text{Genome size x } 1.0\text{*E10}^{9} \text{ x } 650}$$

Where:

6.022*E10^{23} = Avogadro's number

1.0*E10^{9} = conversion to nanograms

650 = average weight of a bp (in Daltons)

Genome size = size of the *D. nodosus* genome (1,389,350 bp (Myers et al., 2007))

The analytical specificity of the serogroup M PCR was evaluated using DNA prepared from prototype *D. nodosus* strains representing all ten serogroups, along with DNA previously prepared from 15 additional bacterial species, the details of which are provided in Table 5.3.

5.2.6 cPCR amplification of the serogroup A to I *fimA* genes

The *fimA* gene of serogroups A to I were amplified using the multiplex cPCR published by Dhungyel et al. (2002), as described in Chapter 2.7.

5.2.7 Statistical analyses

The level of agreement between the serogroup M PCR and the slide agglutination test was evaluated using Cohen's kappa statistic (Cohen, 1960), interpreted using previously proposed standards for strength of agreement (Landis and Koch, 1977). McNemar's chi-square test for paired observations (McNemar, 1947) was performed to test for differences in the sensitivity of the serogroup M PCR and the slide agglutination test.

Table 5.3: Bacterial species used to test the specificity of the serogroup M PCR test.

Species	Location	Host	ID no.
Cardiobacterium hominis	NSW, Australia	Human	FD-3235
Corynebacterium ovis	NSW, Australia	Ovine	FD-2798
Dermatophilus congolensis	NSW, Australia	Ovine	FD-2839
Enterococcus uberis	NSW, Australia	Bovine	NA
Erysipelothrix rhusiopathiae	QLD, Australia	Ovine	FD-2825
Escherichia coli	NSW, Australia	Ovine	FD-2669
Fusobacterium necrophorum	NSW, Australia	Ovine	FD-2842
Klebsiella spp.	NSW, Australia	Bovine	NA
Moraxella bovis	NSW, Australia	Bovine	FD-2574
Nocardia spp.	NSW, Australia	Bovine	15-166
Pseudomonas auruginosa	NSW, Australia	Ovine	FD-2696
Salmonella Typhimurium	VIC, Australia	Porcine	NA
Staphylococcus aureus	NSW, Australia	Bovine	2793
Streptococcus B	NSW, Australia	Ovine	2438
Suttonella indologenes	NSW, Australia	Human	FD-3234

5.3 Results

5.3.1 Phylogenetic analyses

Molecular phylogenetic analysis was undertaken using 27 *D. nodosus fimA* sequences representing serogroups A to I, and M (Figure 5.1). Serogroup M was most closely related to serogroups F, followed by serogroups E and A. The Australian and N.Z. serogroup M strains are closely related to one another, as shown in the phylogenetic tree in which they occupy a single clade, sister to the Nepalese serogroup M strain.

5.3.2 Analytical sensitivity and specificity

The serogroup M PCR had an analytical sensitivity of 0.0004 ng of *D. nodosus* genomic DNA (Figure 5.2), which is equivalent to approximately 250 copies of the *D. nodosus* genome. The assay was 100% specific for serogroup M, with no amplification of serogroups A to I or the 15 other bacterial species tested (Figure 5.3).

Table 5.4: Representative *D. nodosus fimA* sequences included in the phylogenetic analyses (Figure 5.1). Sequences representing serogroups A to I were obtained from Cox (1992). Sequences were obtained from Genbank for Australian (accession no. HM486325.1), N.Z. (accession no. AY835839.1) and Nepalese (accession no. AF038920.1) serogroup M isolates.

Serogroup	Isolate ID	Country of isolation
A	A-1001/198	Australia
A	A2-286-TE	Australia
B	B1-215	Australia
B	B1-1006	Australia
B	B2-183-TE	Australia
B	B3-112-TE	Australia
B	B3-127	Australia
B	B3-235	Australia
B	B4-54	Australia
B	B4-1125	Australia
C	C1-1008	Australia
C	C1-1617	Australia
D	D-340-TE	Australia
D	D-1172	Australia
E	E1-1137	Australia
E	E2-1114	Australia
F	F1-1017	Australia
G	G1-1220	Australia
G	G-238-TE	Australia
H	H-1057	Australia
H	H1-1215	Australia
H	H2-351-TE	Australia
I	I-1613	Australia

Serogroup	Isolate ID	Country of isolation
I	I-1636	Australia
M	M-KI	Australia
M	M-NEP	Nepal
M	M-NZ	N.Z.
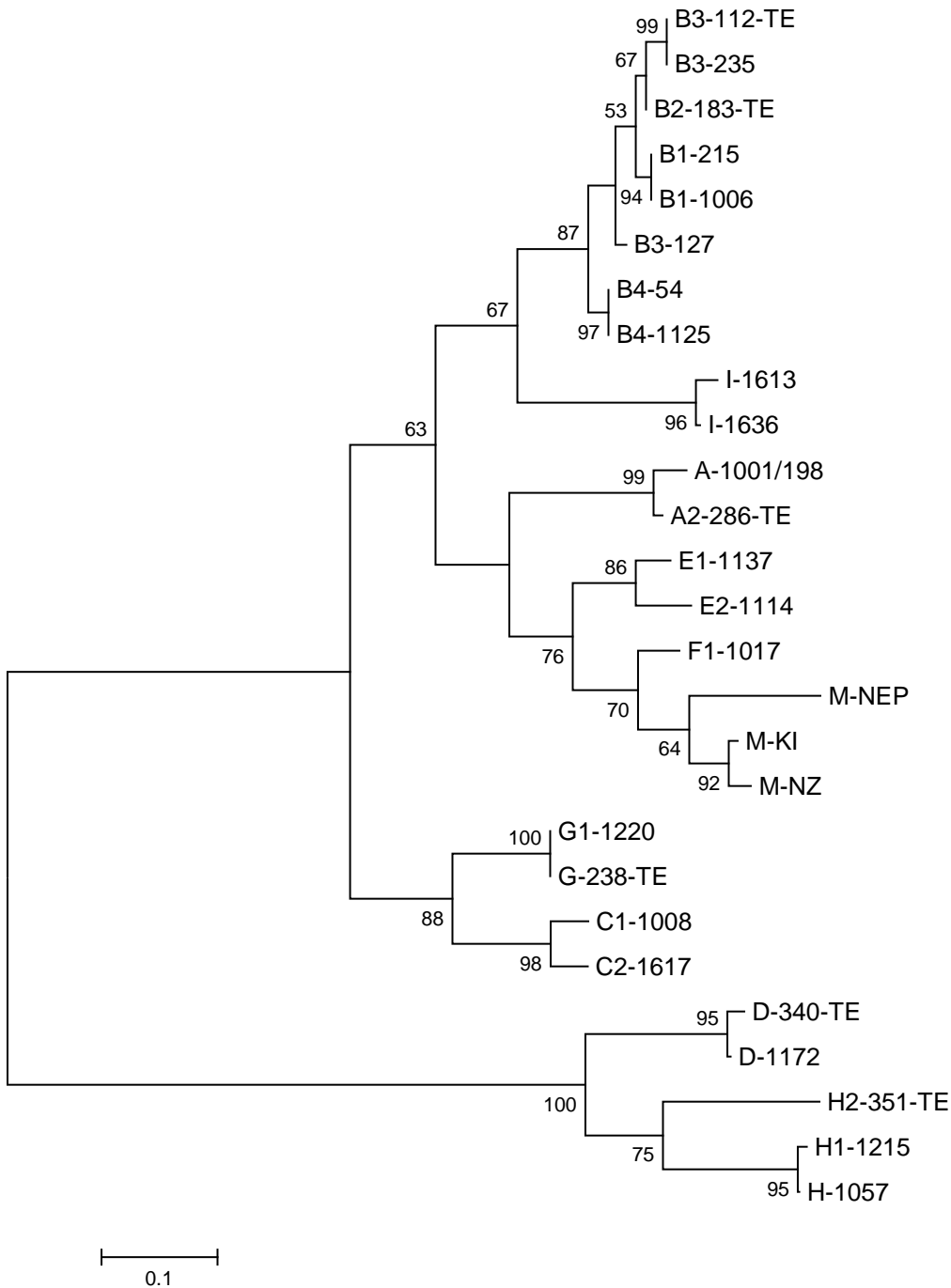
Figure 5.1: Molecular phylogenetic analysis by the Maximum likelihood method. The phylogenetic relationship of all 10 serogroups was determined using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Details of each *fimA* sequence are provided in Table 5.4. The tree with the highest log likelihood (-2457.4058) is shown. The percentage of trees in which associated taxa clustered together is shown next to the branches. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).
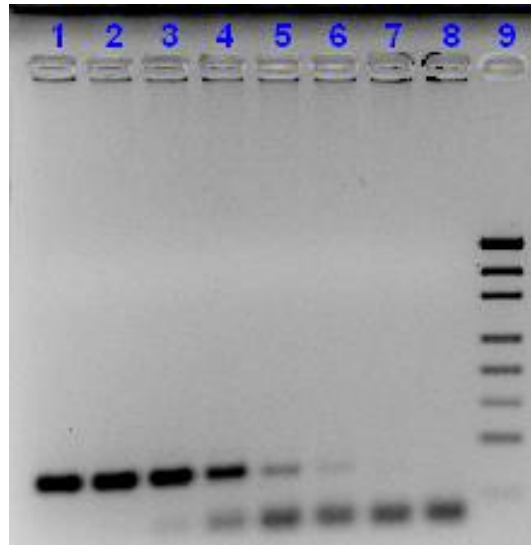
Figure 5.2: Results of sensitivity testing using genomic DNA prepared from Australian serogroup M strain 08/251/4.1. DNA concentrations ranged from 16.0 ng per reaction (Lane 1) to 0.0004 ng per reaction (Lane 8).
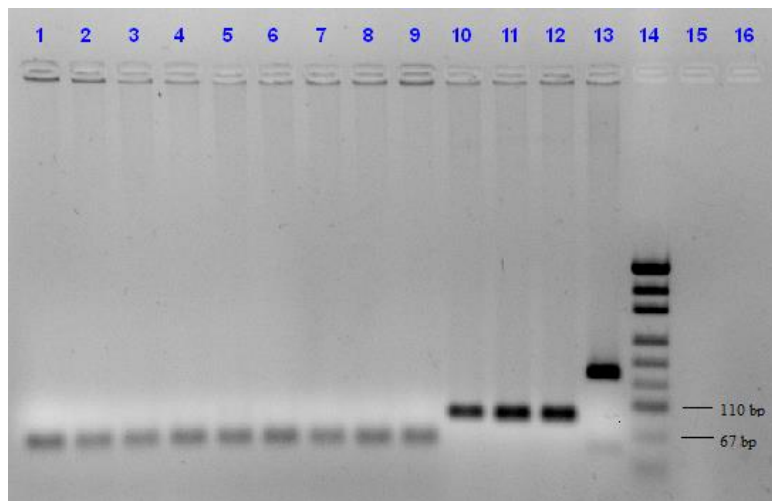
Figure 5.3: Results of specificity testing using DNA prepared from *D. nodosus* prototype strains representing serogroups A (Lane 1), B (Lane 2), C (Lane 3), D (Lane 4), E (Lane 5), F (Lane 6), G (Lane 7), H (Lane 8), I (Lane 9), Nepalese serogroup M strain NEP116 (Lane 10), Australian serogroup M strains 08/251/4.1 (Lane 11) and 10/058/1.3 (Lane 12). The common forward primer and serogroup A-specific reverse primer reported by Dhungyel et al. (2002) were used as a positive control (Lane 13). A molecular marker (HpaII) is included in Lane 14.

5.3.3 Evaluation of field isolates

The serogroup M PCR was evaluated using 27 *D. nodosus* field isolates, 26 of which were isolated from sheep in four flocks in Tasmania, and one of which was isolated from a sheep in a Norwegian sheep flock (Table 5.5). All isolates were previously assigned to serogroup M using the slide agglutination test. The Norwegian isolate was identified as serogroup M, as were 85% of the Tasmanian isolates. Three Tasmanian isolates were negative in the serogroup M PCR, but were identified as serogroup B with the multiplex *fimA* cPCR (Dhungyel et al., 2002). There was no significant difference between the results of the two tests ($P = 0.0833$).

Table 5.5: Comparison of the serogroup M PCR and the slide agglutination test.

		Slide agglutination test		
		Positive	**Negative**	Total
Serogroup M PCR	**Positive**	24	0	24
	Negative	3	0	3
	Total	27	0	27

McNemar's $X^2 = 3.0$, $P = 0.0833$

5.4 Discussion

Strains of *D. nodosus* are divisible into 10 immunologically distinct serogroups (A to I, and M) due to the antigenic variability of the fimbrial subunit protein, which is encoded by the *fimA* gene (Mattick et al., 1991). Strains are assigned to serogroups by means of the slide agglutination test (Claxton et al., 1983), or PCR amplification of serogroup-specific regions of the *fimA* gene (Dhungyel et al., 2002). The former requires microbiological culture of *D. nodosus* from specimens of lesion material collected with cotton swabs, and serological cross-reactivity has been reported between closely related serogroups using this test (Chetwin et al., 1991; Claxton et al., 1983; Ghimire et al., 1998). The multiplex *fimA* cPCR, which is capable to detecting serogroups A to I in three triplex reactions, was developed to enhance the diagnostic specificity of the serogrouping procedure (Dhungyel et al., 2002). The multiplex *fimA* PCR can be used to test DNA prepared from pure cultures or directly from cotton swabs (McPherson et al., 2018). To date, a serogroup M-specific PCR test has not been published, despite the identification of serogroup M has been reported in the U.K., Nepal, N.Z., Norway, and Australia (Chetwin et al., 1991; Day et al., 1986; Dhungyel et al., 2015; Ghimire et al., 1998; Gilhuus et al., 2013). This serogroup has not yet been detected in flocks on mainland Australia, but previous studies indicate that serogroup M is common in Tasmania (Dhungyel et al., 2015). Therefore, there is a need for a serogroup M-specific PCR test to support serogroup-specific vaccination programmes. The aim of this study was to develop a serogroup M-specific cPCR to accompany the serogroup A to I multiplex *fimA* PCRs published by Dhungyel et al. (2002).

Phylogenetic analysis of 27 *fimA* genes representing all ten serogroups confirmed that the Australian and N.Z. serogroup M strains were closely related and occupied the same phylogenetic clade, with the Nepalese serogroup M strain occupying a sister clade (Figure 5.1). In a previous study, the N.Z. serogroup M strain was found to be more closely related to the N.Z. serogroup F strain than the Nepalese serogroup M strain (Dhungyel et al., 2015). However, in the present study the Australian and N.Z. serogroup M strains were more closely related to the Nepalese serogroup M strain than the serogroup F strain included in the phylogenetic analyses, which was of Australian origin. This suggests that the degree of antigenic cross-reactivity between serogroup M and serogroup F antisera might differ according to the serogroup F sub-type used to raise the antisera, and the number of fimbrial epitopes common to the two serogroups.

The analytical sensitivity of the serogroup M PCR was 0.0004 ng of genomic DNA. This is approximately equivalent to 250 copies of the *D. nodosus* genome, which is 1,389,250 bp in length (Myers et al., 2007). This is lower than the sensitivity of the serogroup A to I multiplex *fimA* PCRs, which are reported to be capable of detecting 50-100 *D. nodosus* cells depending on the serogroup(s) present (Dhungyel et al., 2002), but not substantially different. The lower sensitivity is most likely due to the characteristics of the serogroup M-specific primers designed in this study and their binding ability. The *fimA* gene of the Australian serogroup M strain shares up to 95% homology with serogroup F (Dhungyel et al., 2015); consequently, there were very few primer binding sites that would enable amplification of serogroup M and not serogroup F. Furthermore, the *fimA* genes of the Australian and Nepalese serogroup M strains share only 89% homology, therefore a number of potential primer binding sites had to be excluded because of the presence of two or more single-nucleotide polymorphisms in one of the serogroup M sub-types. The region of the *fimA* gene selected for the forward primer containing on SNP, however the use of a degenerate base in the forward primer enabled amplification of both the Australian and Nepalese serogroup M *fimA* genes. Both primers were within the recommended length of 18-30 bp, and did not span any long homopolymer runs or dinucleotide repeats; however, there was no G/C clamp at the 3' end of the forward primer. Additionally, because the selected primer binding sites were G/C poor, the G/C content of the primers was 36% and 33%, respectively, which is less than the recommended 40% to 60% (Dieffenbach et al., 1993).

The serogroup M PCR was 100% specific to serogroup M, with no amplification of serogroups A to I or the 15 other bacterial species tested. The specificity of the serogroup M PCR was compared with that of the slide agglutination test using 27 serogroup M field isolates (Table 5.5). Twenty-six of the isolates were obtained from four sheep flocks in Tasmania, and one was obtained from one sheep flock Norway. All of these isolates previously returned a positive slide agglutination test result for serogroup M. There was agreement between the two tests for 89% of the isolates tested; however, three Tasmanian isolates, which were not identified as serogroup M by the PCR test, were shown to belong to serogroup B with the multiplex *fimA* PCR (Dhungyel et al., 2002). Cross-reactivity between serogroup B and serogroup M antisera has not been reported previously, and the phylogenetic tree presented in Figure 5.1 indicates that serogroup B and serogroup M are not closely related, so it is unclear why the false-positive slide agglutination

test outcomes occurred. Fine (low-titre) agglutination reactions have been ascribed to reactions between antisera and non-fimbrial antigens (Thorley, 1976), which may explain this discrepancy.

The serogroup M PCR developed in this study is more specific than the slide agglutination test when applied to pure cultures of *D. nodosus*. The next step is to evaluate the serogroup M cPCR developed in this study with the direct serogrouping procedure developed in Chapter 4, which was beyond the scope of this study.

5.5 Conclusion

The serogroup M-specific PCR test developed in this study provides diagnosticians with a rapid test for the detection of *D. nodosus* serogroup M in sheep with footrot. The PCR test is more specific than the slide agglutination test, and will enhance the implementation of serogroup-specific vaccine programmes. The next step is to evaluate the analytical characteristics of the PCR when coupled with a direct testing procedure, particularly in mixed infections where serogroup M is not a dominant serogroup.

Chapter 6

Characterisation of the microbiome of the footrot lesion in Merino sheep

6.1 Introduction

Footrot is prevalent in most sheep-producing countries, including Australia, N.Z., U.K., U.S.A., and Sweden (Gradin et al., 1993; Greber et al., 2016; Greer, 2005; Lane et al., 2015; Nieuwhof and Bishop, 2005; Wassink et al., 2010). The disease is responsible for a decrease in productivity, including reduced fleece weights and quality, and poor reproductive performance (Marshall et al., 1991b). The essential transmitting agent, *D. nodosus* (formerly *Bacteroides nodosus*, *Fusiformis nodosus*) (Beveridge, 1941; Dewhirst et al., 1990; Mraz, 1963), is a fastidious anaerobe and an obligate parasite of the ruminant foot (Beveridge, 1941). *D. nodosus* infection manifests as a spectrum of clinical entities, from an indiscernible infection of the epidermis, through to a moderate or severe interdigital dermatitis, and may progress to complete or partial separation of the horny epidermis from the underlying dermal tissues, resulting in profound lameness (Beveridge, 1941).

The clinical manifestations of footrot are the result of complex synergistic interactions between *D. nodosus* and the bacterial community of the foot (Beveridge, 1941; Egerton et al., 1969; Hine, 1983; Roberts and Egerton, 1969). The severity of these manifestations differs according to the virulence of the infecting *D. nodosus* strain(s) (Stewart et al., 1986), the susceptibility of the host (Egerton and Raadsma, 1991; Egerton et al., 1983; Emery et al., 1984), and environmental conditions, with mild ambient temperatures and high, evenly distributed rainfall favouring disease progression (Graham and Egerton, 1968).

The bacterial community of the ovine hoof is diverse (Calvo-Bado et al., 2011; Egerton et al., 1969; Hine, 1983; Maboni et al., 2017); establishing which taxa contribute to the disease process and the extent to which they contribute is challenging. The interactions that occur between *D. nodosus* and the bacterial community of the hoof are poorly understood, with much of the existing literature focussed on interactions between *D. nodosus*, *F. necrophorum*, and *C. pyogenes* (Egerton et al., 1969; Roberts, 1967a; Roberts, 1967b; Roberts and Egerton, 1969). *F. necrophorum* is a constituent of the normal gastrointestinal flora and is introduced to the foot through exposure to faeces (Nagajara et al., 2005; Roberts and Egerton, 1969). *F. necrophorum*

contributes to both the early and latter stages of the disease process; the bacterium expresses a leukocytic exotoxin that prevents leukocytes from accessing the site of infection, thereby enabling itself and other bacterial taxa to invade the epidermis and proliferate, provided there is sufficient environmental predisposition (Egerton et al., 1969; Roberts and Egerton, 1969). *F. necrophorum* also enhances the severity of foot lesions by inducing a severe inflammatory response (Egerton et al., 1969; Roberts and Egerton, 1969). In fact, prior to the identification of *D. nodosus*, *F. necrophorum* was thought to be the primary causative agent of footrot due to the organism's ubiquity in footrot lesions and its role in other mixed bacterial diseases of the epidermis in sheep and cattle, such as OID and BDD (Egerton and Laing, 1978; Parsonson et al., 1967). However, experimental trials have since demonstrated that experimental challenge with *F. necrophorum* alone does not induce underrun lesions (Beveridge, 1941; Egerton, 1969). Recently, there has been some debate as to whether colonisation of the epidermis by *F. necrophorum* is essential for initiation of the disease process, or if the bacterium is simply an opportunistic secondary invader (Maboni et al., 2016; Witcomb et al., 2014; Witcomb et al., 2015). A recent study reported that *F. necrophorum* was more likely to be detected in specimens collected from severe, underrun lesions than in those collected from mild interdigital lesions, and that whilst there was an association between increasing *D. nodosus* load and the development of an interdigital lesion, there was no association between *F. necrophorum* load and the development of an interdigital lesion (Witcomb et al., 2014). Furthermore, although *F. necrophorum* was more likely to be detected in severe, underrun lesions, the bacterium was not associated with the development of such lesions (Witcomb et al., 2014). A subsequent study reported that *D. nodosus* load was significantly higher on feet with interdigital lesions and severe, underrun lesions than on healthy feet, but that *F. necrophorum* load was significantly higher in feet with severe, underrun lesions only (Witcomb et al., 2015). These findings are at odds with earlier studies, which have demonstrated that without exposure to faeces, and therefore *F. necrophorum*, experimental challenge with *D. nodosus* fails to induce foot lesions, even with environmental predisposition (Roberts and Egerton, 1969). This does not exclude the possibility that other organisms present in faeces, and possibly in soil or pasture, contribute to the disease process in addition to *F. necrophorum*, although histological examination of tissue sections from footrot lesions indicates that only two organisms invade fresh epidermal tissues: *D. nodosus* and *F. necrophorum* (Egerton et al., 1969; Hine, 1983).

Corynebacterium is part of the normal bacterial community of the ovine foot and is abundant on both healthy and diseased feet (Calvo-Bado et al., 2011; Egerton et al., 1969; Maboni et al., 2017). The bacterium is thought to contribute to the disease process indirectly by enhancing the infectivity of *F. necrophorum* through the provision of a growth factor (Roberts, 1967a; Roberts, 1967b; Smith et al., 1991). There do not appear to be any direct synergistic interactions between *C. pyogenes* and *D. nodosus* (Roberts and Egerton, 1969). It is not clear whether *C. pyogenes* is essential to the disease process, as several other bacterial taxa have been shown to enhance the infectivity of *F. necrophorum* in a similar manner (Brook and Walker, 1986; Smith et al., 1991; Smith et al., 1989; Timoney et al., 1988). It is possible that the genus contributes directly to the disease process: *C. pyogenes* has previously been implicated as a cause of acute lameness in sheep (Gardner, 1961), and another member of the genus, *C. minutissimum*, was found to abundant in OID lesions (Pepin and Littlejohn, 1964). *C. pyogenes* has also been implicated as a causative agent in diseases of keratinised tissues in humans (Aly and Maibach, 1978; Tilgen, 1979). For instance, in an ultrastructural study of pitted keratolysis, a disease of the plantar skin surface, an organism belonging to the *Corybacterium* genus was identified in the upper layers of the *stratum corneum*, and was suspected of inducing keratolysis, either alone or synergistically with other bacterial taxa (Tilgen, 1979).

Much of what is currently known about the bacterial aetiology of ovine footrot was ascertained using classical microbiological techniques and examination of histological sections (Beveridge, 1941; Egerton et al., 1969; Hine, 1983; Roberts and Egerton, 1969). Such studies are inherently limited to bacterial taxa that can be cultured on common growth media, or those with distinctive morphologies that are readily identifiable in smears or histological sections. More recently, molecular techniques such as the PCR and fluorescent *in-situ* hybridisation (FISH) have furthered our understanding of the disease process and the interactions between *D. nodosus* and *F. necrophorum* (Maboni et al., 2016; Witcomb et al., 2014; Witcomb et al., 2015), but these techniques only target specific taxa.

The advent of new technologies such as NGS and amplicon-based metagenomics has enabled bacterial communities to be examined in greater detail (Clarridge, 2004; Hugenholtz et al., 1998; McCabe et al., 1999; O'Sullivan, 1999). These technologies have previously been used to characterise the bacterial communities of the ovine and bovine foot (Calvo-Bado et al., 2011;

Krull et al., 2014; Maboni et al., 2017). The first study to attempt to characterise the bacterial communities on the feet of clinically healthy sheep and those with footrot reported that *Staphylococcus*, *Macrococcus* and *Micrococcus* were the most abundant genera associated with severe, underrun foot lesions, *Corynebacterium* was the most abundant genus associated with interdigital lesions, and *Peptostreptococcus* was the most abundant genus associated with healthy feet (Calvo-Bado et al., 2011). A subsequent study that employed newer sequencing technologies and bioinformatic tools also reported that *Corynebacterium* was one of the most abundant genera in interdigital lesions, but that *Corynebacterium* was also abundant on healthy feet along with *Psychrobacter* and *Acinetobacter*, and that the bacterial communities present on feet with severe, underrun lesions were dominated by *Mycoplasma*, *Corynebacterium*, *Psychrobacter*, and *Treponema* (Maboni et al., 2017). With the exception of *Corynebacterium*, which is known to contribute to the disease process (Roberts and Egerton, 1969), the significance of these genera to the aetiopathogenesis of ovine footrot has not been demonstrated experimentally.

The Merino is the main sheep breed in Australia (Meat and Livestock Australia, 2017). The breed is particularly susceptible to footrot, but the basis of this susceptibility is contentious (Beveridge, 1941; Egerton and Morgan, 1972; Emery et al., 1984; Skerman et al., 1982). The interdigital skin of the foot of the Merino sheep appears to be less resistant to bacterial invasion than that of European sheep breeds (Emery et al., 1984), and there is evidence that European sheep breeds are able to mount a more effective humoral immune response to *D. nodosus* infection than the Merino (Skerman et al., 1982). Breed-specific differences in the bacterial community of the foot may also be of significance; however, to my knowledge the bacterial community of the Merino foot has not yet been characterised using NGS. A previous study indicated that there may be breed-specific differences in the bacterial communities of the ovine foot, including both healthy sheep and sheep with footrot (Calvo-Bado et al., 2011). Given that the feet of Merino sheep appear to be more susceptible to bacterial invasion than the feet of European breeds (Emery et al., 1984), it is possible that the foot of the Merino is colonised by a greater number or diversity of opportunistic pathogens following environmental predisposition. Consequently, the bacterial taxa of interest to the disease process may differ to some extent between Merinos and European sheep breeds.

The aims of this study were (i) to characterise the bacterial communities present on the feet of a group of healthy Merino sheep and two groups of Merino sheep with footrot using 16S ribosomal ribonucleic acid (rRNA) amplicon-based metagenomics, (ii) identify biomarker taxa that are preferentially abundant in footrot lesions and that might therefore contribute to the disease process, and (iii) to describe how the composition of the bacterial community changes over time as footrot lesions progress.

6.2 Materials and Methods

6.2.1 Sheep

Two groups of sheep were sourced from two Merino flocks with footrot in NSW, Australia ($n = 10$ sheep per mob). The sheep were selected by the author from a proportion of the parent flock that had been drafted-off by the farmer, on the basis that they all had two or more feet with footrot lesions. Group 1 was selected from a flock located near Crookwell, on the southern tablelands of NSW. Group 2 was selected from a flock located near Bombala, in south-eastern NSW. The sheep were transported separately to The University of Sydney facilities at Camden, NSW two weeks prior to the commencement of the study. Three Merino sheep with clinically healthy feet were sourced from a flock with no history of footrot located at the Camden campus as representative healthy sheep. Each group of sheep was maintained in a separate ~500 m^2 paddock. These paddocks were separated from one another by a buffer zone approximately 10 m wide. The pasture consisted of native and introduced species, and was dominated by kikuyu (*Pennisetum clandestinum*), paspalum (*Paspalum dilatatum*), pigeon grass (*Setaria geniculata*), and Rhodes grass (*Chloris guyana*). The pasture composition was similar throughout the paddocks, and was approximately 150-250 mm in length at the commencement of the study.

6.2.2 Examination of parent flocks

Each of the parent flocks had been examined on one or more occasions by the author during a previous study (McPherson et al., 2017). During the previous examination(s), each foot of each sheep was inspected, and a score was assigned to each foot using a previously described scoring system, as described in Chapter 2.

6.2.3 Study design and sample collection

In each of the two mobs with footrot, active foot lesions ($\geq$score 1) that were suitable for sampling were identified when the sheep were first examined in March 2016. Foot swabs were collected from the same feet on a monthly basis for 10 months (March to December, 2016). Foot swabs were collected from the interdigital skin (in the case of score 1 and 2 lesions) or the active margin of the underrun lesion (in the case of score 3 and 4 lesions) by scraping the surface with a sterile, flocculated nylon swab (FLOQswabs; Copan Italia, Brescia, Italy). Each foot swab was transported dry in an individual plastic tube provided with the swab. All foot swabs were stored at -80°C prior to DNA preparation. Foot swabs were also collected from each lesion at each time point with a sterile, cotton-tipped swab (CLASSIQSwabs; Copan Italia, Brescia, Italy) into mSTM (Amies, 1967) for microbiological culture of *D. nodosus*.

6.2.4 Isolation of *D. nodosus*

D. nodosus was isolated from each lesion swab collected in mSTM, as described in Chapter 2.2.

6.2.5 Elastase test

The virulence of each *D. nodosus* isolate was assessed using the elastase test, as described in Chapter 2.5.

6.2.6 DNA preparation

DNA was prepared from each flocculated nylon swab using the QIAamp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany), following a modified version of the protocol provided by the manufacturer for the preparation of microbial DNA from swabs, with an additional mechanical pre-lysis step. Briefly, the head of the swab was cut off and placed into a 1.5 mL DNA LoBind microcentrifuge tube (Eppendorf, Hamburg, Germany) with 650 µL of Buffer ATL. The tube was pulse-mixed on a vortex mixer for 10 s, and incubated for 10 mins at 56°C in a dry heat block. The contents of the tube were mixed by gentle inversion at 1 min intervals and placed back into the dry heat block. The tube was centrifuged at 6000 x g for 5 s to remove any drops of liquid from the lid of the tube, and the entire 650 µL of Buffer ATL was aspirated and transferred to a 2 mL tube (Eppendorf, Hamburg, Germany) containing 0.5 g of 0.1 mm silica-zirconia beads (BioSpec, Bartlesville, USA) Mechanical lysis was undertaken with a TissueLyser II (Qiagen, Hilden, Germany) for two periods of 45 s at 30 Hz. The bead tubes were centrifuged at 6000 x g

for 5 s to remove any drops of liquid from the lid, and the supernatant was transferred to a new 1.5 mL DNA LoBind microcentrifuge tube. Forty-microliters of proteinase K was added to each tube, mixed on a vortex mixer for 10 s, and incubated for 10 mins at 56°C in a dry heat block. Two-hundred-microlitres of Buffer APL2 was added to each tube, mixed on a vortex mixer for 30 s, and incubated for 10 mins at 70°C. Three-hundred-microliters of 100% ethanol was added to each tube, and mixed on a vortex mixer for 30 s. Six-hundred-microlitres of the mixture was transferred to a QIAamp UCP mini column in a 2 mL collection tube, centrifuged at 6000 $x g$ for 1 min, and the flow-through discarded. This process was repeated for the remaining mixture. Six-hundred-microliters of Buffer APW1 was added to each mini column, centrifuged at 6000 $x g$ for 1 min, and the flow-through discarded. Seven-hundred-and-fifty-microliters of Buffer APW2 was added to each tube, vortexed at 20,000 $x g$ for 3 mins, and the flow-through discarded. The mini column was then vortexed at 20,000 x g for 3 mins to remove any remaining buffer from the column, and the flow-through discarded. The mini-column was placed in a new collection tube, and incubated at 56°C for 2 mins in a dry heat block, with the lid open, to dry the membrane. The mini column was then placed into a 1.5 mL DNA LoBind microcentrifuge tube, and 50 μL of Buffer AVE was deposited directly onto the membrane. The mini column was incubated at room temperature for 2 mins, and centrifuged at 20,000 $x g$ to elute the DNA. The eluted DNA was stored at -20°C.

DNA was prepared from each pure culture of *D. nodosus* using the Wizard® Genomic DNA Purification Kit (Promega, Madison, U.S.A) in accordance with the protocol for the purification of genomic DNA from Gram-negative bacteria.

6.2.7 Evaluation of DNA quality

The quantity of double-stranded genomic DNA (dsDNA) prepared from each foot swab was analysed with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, U.S.A.) using the Qubit dsDNA BR (Broad-Range) Assay Kit (Invitrogen, Carlsbad, U.S.A.). Briefly, the Qubit working solution and dsDNA Standards 1 and 2 were prepared according to the manufacturer's instructions. Samples were prepared by mixing 195 μL of the working solution with 5 μL of DNA extract, mixed with the vortex mixer for 3 s, and incubated at room temperature for 2 mins. The Qubit was calibrated using the two dsDNA standards prior to testing the samples. The purity of each dsDNA extract was analysed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, North Ryde,

Australia), following the manufacturer's instructions. dsDNA extracts that had a 260 nm/280 nm absorbance ratio ≥1.8 and a 260 nm/230 nm absorbance ratio ≥1.5 were deemed acceptable. dsDNA extracts that were below these thresholds were purified using a DNA clean-up and concentrator kit (DNA Clean-Up and Concentrator -5; Zymo Research, Irvine, U.S.A.), following the manufacturer's instructions.

6.2.8 Real-time PCR detection of *D. nodosus*

D. nodosus was identified by real-time PCR amplification of the *D. nodosus* 16S rRNA gene, as described in Chapter 2.8.

6.2.9 Sequencing of the bacterial 16S rRNA gene

The V3-V4 region of the bacterial 16S rRNA gene was amplified using the universal primers 319F and 806R (Caporaso et al., 2011; Muyzer et al., 1993), and the products were sequenced on an Illumina MiSeq (Illumina, San Diego, U.S.A), by the Brisbane node of the Australian Genome Research Facility Ltd. (AGRF).

6.2.10 Analysis of bacterial community structure and diversity

The quality of the raw data set was assessed with FastQC v0.11.3 (Andrews, 2010). Paired-end reads were merged with USEARCH v10.0 (Edgar, 2010) using the default parameters: maximum of five mismatches in alignment; minimum 90% identity; and a minimum overlap of 16 bases. Quality filtering and diversity analyses were undertaken with Quantitative Insights into Microbial Ecology (QIIME) v2.7.10 (Caporaso et al., 2010b), available through The University of Sydney's High Performance Computing (HPC) service. Quality filtering was undertaken in accordance with previously recommended parameters (Bokulich et al., 2013): minimum Phred scores of 3; a minimum of 75% consecutive high-quality bases per read; a maximum of three consecutive low-quality bases; and no ambiguous (N) characters. *De novo* and reference-based chimera detection was performed with USEARCH v6.1 (Edgar, 2010), as implemented in QIIME, against the GreenGenes 13_8 97% OTU database (McDonald et al., 2012). *De novo* operational OTUs were generated with a minimum sequence similarity of 97% using the 'UCLUST' algorithm (Edgar, 2010) and taxonomically classified against the GreenGenes 13_8 97% OTU database (McDonald et al., 2012). OTUs that represented <0.005% of the total data set were discarded to avoid artificial inflation of diversity estimates due to the presence of spurious

OTUs (Bokulich et al., 2013). A representative set of sequences was generated and aligned with PyNAST (Caporaso et al., 2010a). The alignment was filtered to remove gaps and variable positions with Lane masking (Lane, 1991) and a phylogenetic tree was constructed with FastTree (Price et al., 2009).

Prior to undertaking within-sample (alpha) diversity analyses, the data were rarefied with minimum and maximum sampling depths of 10 and 40,000 reads per sample, respectively, in steps of 2,000 reads, with 10 iterations per step. Alpha diversity was assessed, as defined by: the number of observed OTUs; the Chao1 index, which estimates the true species diversity (Chao, 1984); and Shannon's diversity index, which considers both the abundance and evenness of taxonomic groups (Shannon, 1948). Alpha diversities were compared between categories of interest (group, lesion score, time point) using a non-parametric t-test with 10,000 Monte Carlo permutations and Bonferroni corrected P-values. Between-sample (beta) diversity was assessed with a dataset rarefied to 20,000 reads using weighted and unweighted UniFrac distances (Lozupone et al., 2006; Lozupone and Knight, 2005; Lozupone et al., 2011), which measure the phylogenetic distance between taxa in a phylogenetic tree. Weighted UniFrac considers the presence or absence of taxonomic groups and their relative abundances, but unweighted UniFrac considers presence or absence only (Lozupone et al., 2006; Lozupone and Knight, 2005; Lozupone et al., 2011). Principle coordinate analysis (PCoA) was performed on the resultant UniFrac matrices and the results were plotted with EMPeror (Vazquez-Baeza et al., 2013), as implemented in QIIME. Changes in community structure between categories of interest (disease status, lesion score, time point) were compared using the non-parametric analysis of similarity (AnoSim) and ADONIS tests with 10,000 permutations.

6.2.11 Identification of metagenomic biomarkers

Linear discriminant analysis effect size (LEfSe) was used to identify metagenomics biomarkers, which are defined as taxonomic groups that are preferentially abundant in a biological class of interest (Segata et al., 2011). Taxonomic count data generated in QIIME were analysed using LEfSE v1.0, available on the Galaxy server (http://huttenhower.sph.harvard.edu/galaxy/), using the default parameters. LEfSe uses the non-parametric factorial Kruskal-Wallis sum-rank test (Kruskal and Wallis, 1952) to identify taxonomic groups with significant differential abundance between two or more biological categories of interest. Thereafter, biological consistency is

examined using the Wilcoxon rank-sum test (Wilcoxon, 1945), and the effect size of each differentially abundant taxonomic group is estimated using linear discriminant analysis (LDA) (Fisher, 1936). It is important to note that taxonomic groups which are identified as biomarkers in a class of interest may be present in other classes, but inconsistently or at a lower abundance.

6.3 Results

6.3.1 Collection of foot swabs

Foot swabs were collected from a group of healthy Merino sheep ($n = 2$ sheep, 14 swabs) and from two groups of sheep with footrot ($n = 10$ sheep, 40 swabs per mob) (Table 6.1). The foot swabs were collected on a monthly basis for a period of 10 months (March to December, 2016). Ten feet/lesions were selected for sampling, including four feet from each of the two groups with footrot, and two from the healthy group (Table 6.1). The same 10 feet were sampled at each time point. Usually one foot per sheep was sampled, but two feet were sampled from two sheep (Tag no. 318 and 389) that presented with footrot lesions at the commencement of the trial. The intention was to collect a total of 20 specimens from clinically healthy sheep; however, the sheep in this group were removed from the trial prematurely in September, thus only 14 swabs were collected from this group. The number of foot swabs collected from sheep in Group 1 and Group 2 was greater than the number of foot swabs collected from sheep in the healthy group; however, more foot swabs were collected from sheep in Groups 1 and 2 across the trial period in an attempt to obtain a similar number of foot swabs from lesions of each respective score.

6.3.2 Temperature and rainfall

Average daily air temperatures and daily rainfall for the trial period are presented in Figure 6.1. With the exception of a brief period of high rainfall in early June, rainfall was low but consistent throughout the trial period. The topography of the paddock in which the healthy group and Group 2 were maintained was more variable; consequently, the paddock was drier throughout the trial period than the paddock in which Group 1 were maintained.
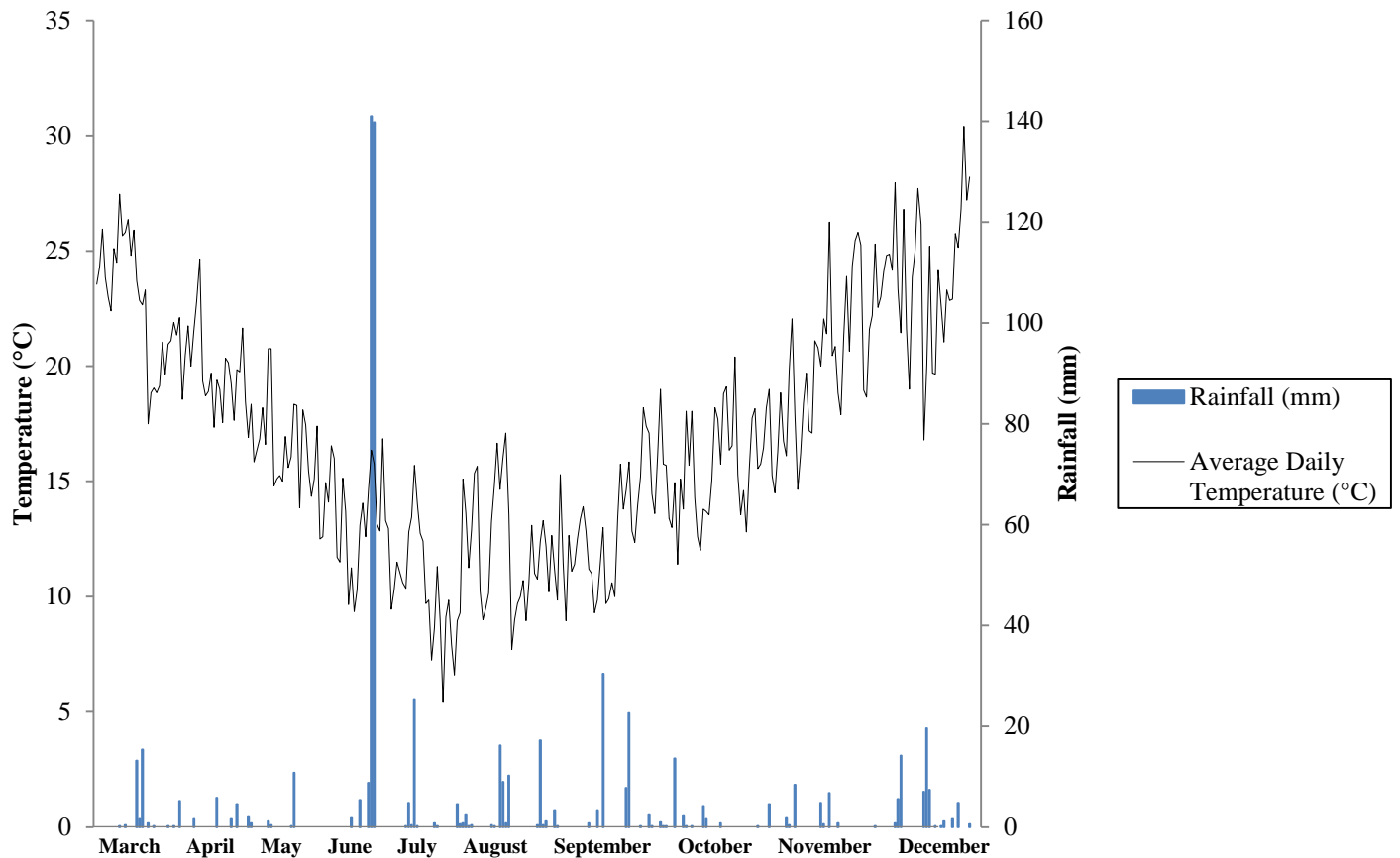
Figure 6.1: Average air temperature (°C) and rainfall (mm) for the trial period (March to December, 2016). Weather data were drawn from the Australian Bureau of Meteorology (BOM) weather station located at Camden Airport (Station No. 068192), which is located approximately two kilometres from The University of Sydney, Camden.

6.3.3 Lesion scores

The sheep in the healthy group remained free of *D. nodosus* infection for the duration of the trial and had foot scores of 0. Lesion severity varied across the trial period for sheep with footrot in Group 1 and Group 2, as shown in Table 6.1. Clinically, the disease was more severe in Group 1 than in Group 2 from June to October. However, with the advent of spring the lesions became more severe in Group 2 than in Group 1.

Table 6.1: Foot scores for each foot sampled during this study. Scores were assigned to each foot using the scoring system devised by Egerton and Roberts (1971). LH = left hind, RF = right fore, RH = right hind.

Group	Tag No. (Foot)	Foot score									
		Mar	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
Healthy	130 (LH)	0	0	0	0	0	0	0	*	*	*
	287 (RF)	0	0	0	0	0	0	0	*	*	*
1	317 (RH)	1	1	1	1	1	0	1	1	2	2
	318 (RF)	2	2	2	3	1	2	3	2	2	2
	318 (RH)	2	2	3	2	2	2	2	3	2	2
	319 (RH)	2	2	1	1	1	1	3	2	2	2
2	389 (RF)	2	2	3	2	1	1	2	2	4	3
	389 (RH)	2	2	3	2	1	2	2	2	2	2
	390 (RH)	1	0	0	1	1	1	2	1	3	3
	475 (RH)	2	2	0	1	1	1	2	2	2	2

6.3.4 Detection of *D. nodosus*

Cotton swabs were collected for microbiological culture at six time points during the trial (Appendix B, Table B.1). The number of swabs collected from Groups 1 and 2 at each time point ranged from two to five. The number of sheep sampled in Groups 1 and 2 at each time point ranged from two to five. One or two swabs were also collected from sheep in the healthy group. *D. nodosus* was not detected in the healthy group at any of these time points. The number of putative *D. nodosus* isolates obtained from Groups 1 and 2 at each time point ranged from six to ten, and four to eight, respectively. qPCR amplification of the 16S rRNA gene (Frosth et al., 2012) confirmed that each isolate was *D. nodosus*. Elastase-positive and elastase-negative *D. nodosus* isolates were recovered from swabs collected from each of the groups with footrot at each of the six time points.

6.3.5 General description of data set

16S rRNA sequences obtained from 80 swabs collected from the feet of two groups of sheep infected with *D. nodosus* were analysed, however two swabs collected from sheep in Group 1 (Samples 24 and 36) were excluded from all diversity analyses due to the low number of reads that remained after quality filtering of the data set. The raw data set consisted of 8,179,973 paired-end reads with a length of 300 bp. After merging and quality filtering, the dataset consisted of 5,594,991 reads with an average length of 450 bp. The mean number of reads per sample was 59,521 (range 14,241 to 100,148). Sample metadata are reported in Appendix B (Table B.2).

6.3.6 Bacterial diversity

Within-sample (alpha) diversity was assessed after rarefaction, defined by the number of observed OTUs, Chao-1 and Shannon's diversity index. The rarefaction curves depicted in Figures 6.2 to 6.4 demonstrate that the number of observed/expected taxa plateaued with increasing rarefaction depth, which suggests that the sequencing depth and diversity coverage achieved were satisfactory to accurately describe the diversity of the bacterial communities.

Alpha diversity was significantly greater in samples collected from Groups 1 and 2 than in those collected from the healthy group, as defined by the number of OTUs observed (P = 0.003) (Figure 6.2), Chao1 (P = 0.003) (Figure 6.3) and Shannon's diversity index (P = 0.003) (Figure 6.4). The mean number of OTUs identified on the feet of sheep in the healthy group was 227 (range 148 – 308), and the mean number of OTUs identified on the feet of sheep in Groups 1 and 2 were 528 (range 255 – 659) and 566 (range 299 – 719), respectively. Alpha diversity was not significantly different between the samples collected from sheep in Groups 1 and 2, as defined by the number of observed OTUs (P = 0.255) or Chao-1 (P = 0.201). However, Shannon's diversity index was significantly different when comparing alpha diversity between Groups 1 and 2 (P = 0.036).

(a)   (b)
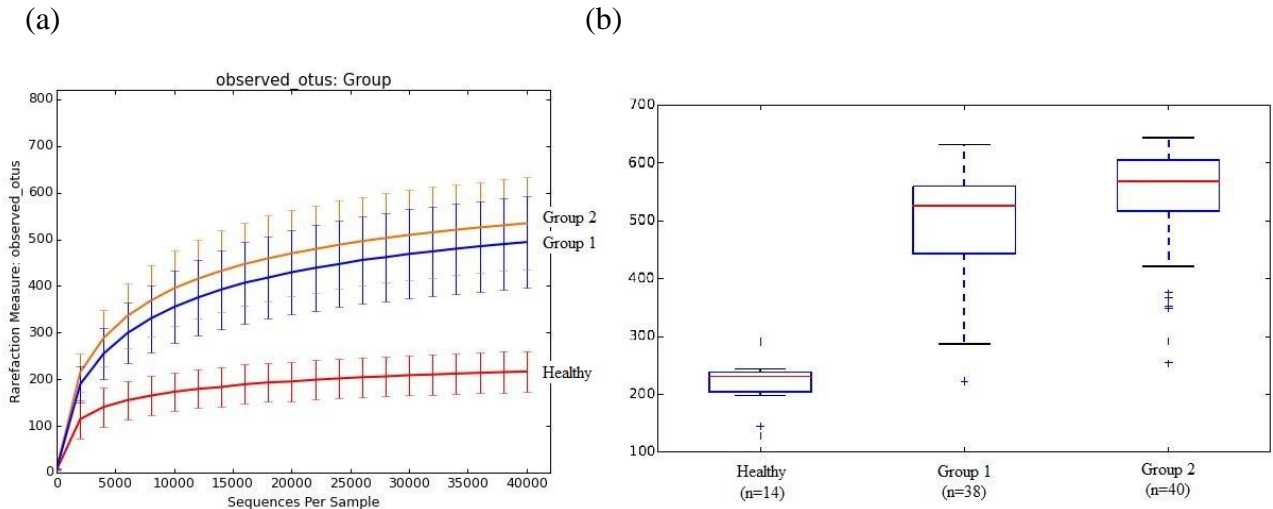
Figure 6.2: Alpha diversity, as defined by the number of observed OTUs: (a) alpha rarefaction curves indicating the relationship between rarefaction depth and the number of taxa observed. Each point represents the average number of OTUs identified, and error bars indicate the standard error. The number of expected taxa plateaus with increasing rarefaction depth, indicating that the sequencing depth and diversity coverage was satisfactory; (b) boxplots illustrating alpha diversity estimates for the bacterial communities of the feet Groups 1 and 2 and the healthy sheep.
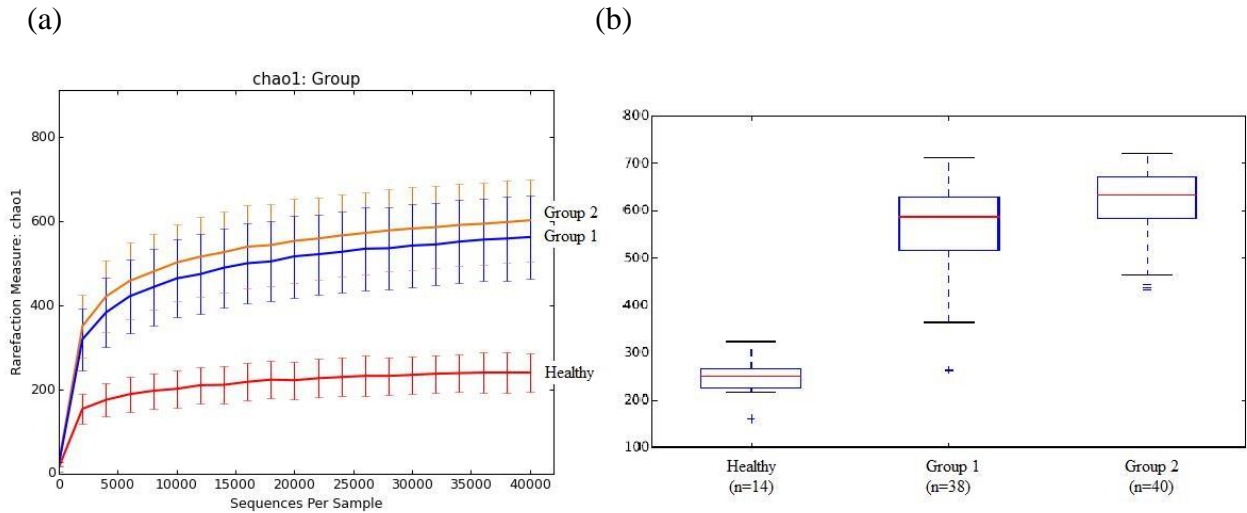
Figure 6.3: Alpha diversity, as defined by Chao-1: (a) alpha rarefaction curves indicating the relationship between rarefaction depth and the number of expected taxa. Each point represents the average number of OTUs identified, and error bars indicate the standard error. The number of expected taxa plateaus with increasing rarefaction depth, indicating that the sequencing depth and diversity coverage was satisfactory; (b) boxplots illustrating alpha diversity estimates for the bacterial communities of the feet of sheep in Groups 1 and 2 and the healthy group.
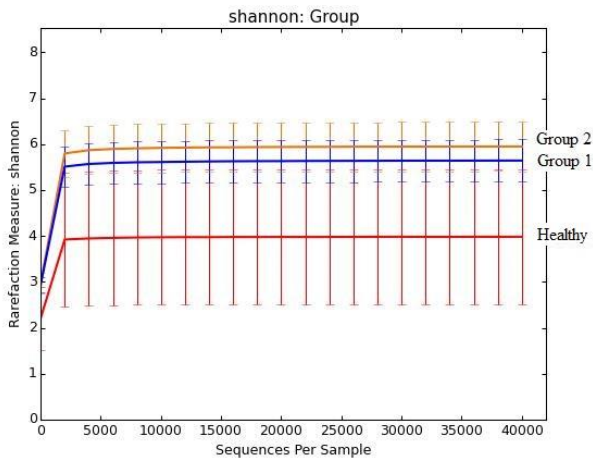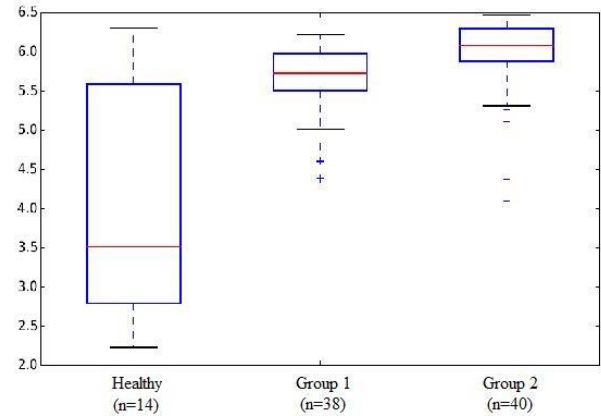
Figure 6.4: Alpha diversity, as defined by Shannon's diversity index: (a) alpha rarefaction curves indicating the relationship between rarefaction depth and the evenness of the bacterial communities. Each point represents the average number of OTUs identified, and error bars indicate the standard error. The number taxa plateaus with increasing rarefaction depth, indicating that the sequencing depth and diversity coverage was satisfactory; (b) boxplots illustrating alpha diversity estimates for the bacterial communities of the feet of sheep in Groups 1 and 2 and the healthy group.

Beta diversity was assessed after rarefaction using weighted and unweighted UniFrac distances. The PCoA plots generated from both the unweighted and weighted UniFrac matrics indicated a clear separation of the bacterial communities present on the feet of sheep in the healthy group and those present on the feet of sheep in Groups 1 and 2 (Figure 6.9). The PCoA plots generated from the unweighted UniFrac distance matrices (Figure 6.9), which considers the presence or absence of taxa only, demonstrated a slight separation of the bacterial communities present on the feet of sheep in Groups 1 and 2. However, there was no apparent separation of the bacterial communities present on the feet of sheep in Groups 1 and 2 in the PCoA plots generated from the weighted UniFrac matrices, which considers the presence or absence of taxa, as well as their relative abundances (Figure 6.9). Non-parametric statistical tests were used to evaluate the UniFrac distance matrices. Sheep in the healthy group were compared to sheep infected with *D. nodosus*, which included all sheep in Groups 1 and 2, rather than considering sheep in Group 1 and 2 as two distinct groups, given that there wasn't a clear separation of the two groups in the PCoA plot generated from the weighted UniFrac distance matrix. There was a significant difference in the diversity of bacterial communities on the feet of the healthy and footrot-affected sheep for both the weighted (AnoSim, R = 0.94, $P < 0.001$; ADONIS, $R^2 = 0.34$, $P < 0.001$) and unweighted (AnoSim, 0.97, $P < 0.001$; ADONIS, $R^2 = 0.32$, $P < 0.001$) UniFrac distances.
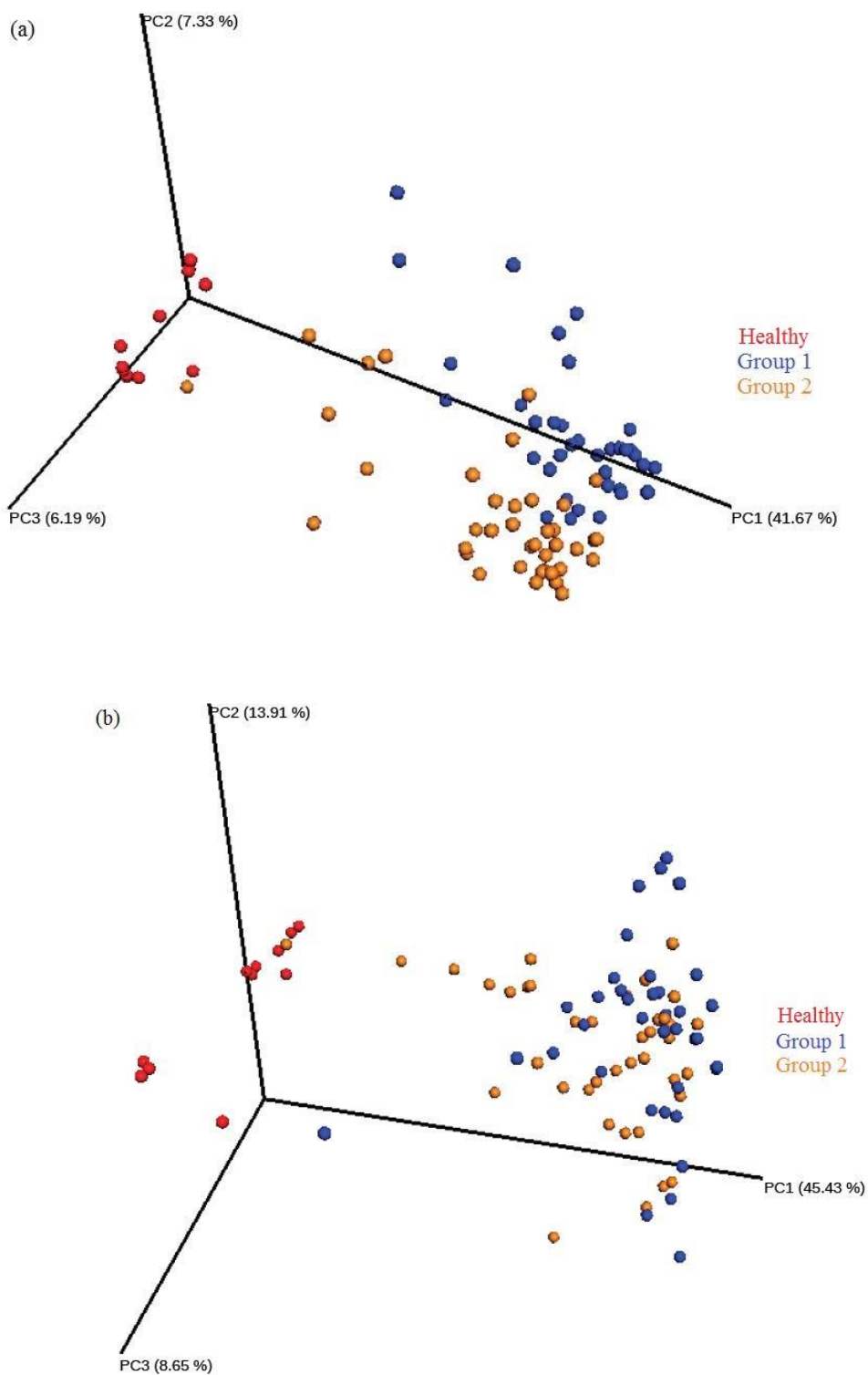
Figure 6.5: PCoA plots for group based on (a) unweighted and (b) weighted UniFrac distance matrices.

The dominant bacterial families on the feet of healthy sheep were *Corynebacteriaceae* (35.9%), *Staphylococcaceae* (20.7%), *Moraxellaceae* (11.0%), *Sphingomonadaceae* (4.3%), and *Microbacteriaceae* (4.3%) (Figure 6.6). The dominant bacterial families detected on the feet of sheep in Group 1 were *Tissierellaceae* (33.6%), *Porphyromonadaceae* (15.0%), *Mycoplasmataceae* (10.5%), *Corynebacteriaceae* (7.0%), and *Fusobacteriaceae* (4.5%) (Figure 6.7). The dominant bacterial families detected on the feet of sheep in Group 2 were *Tissierellaceae* (33.2%), *Porphyromonadaceae* (15.1%), *Corynebacteriaceae* (13.9%), *Peptostreptococcaceae* (4.4%) and *Mycoplasmataceae* (3.7%) (Figure 6.8). These families were also detected on the feet of the healthy sheep but at a lower relative abundance (Figure 6.6). *Corynebacteriaceae* was one of the dominant bacterial families in all three groups (Figures 6.6 – 6.8), but was less abundant than on the feet of the healthy sheep (Figure 6.6). *Staphlyococcaceae* was detected on the feet of sheep in Groups 1 and 2 but was not one of the dominant families.
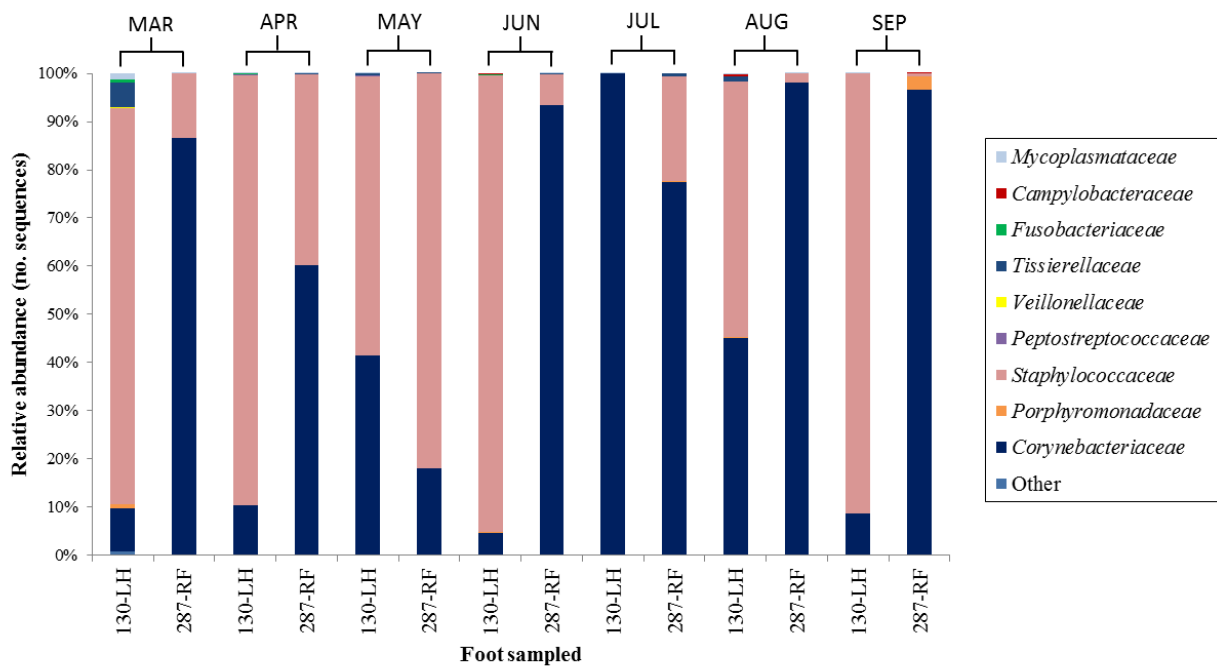
Figure 6.6: Relative abundance of the dominant bacterial families on the feet of the healthy sheep based on the number of bacterial 16S rRNA sequences. Bacterial families that represent at least one percent of the total data set are depicted. The dominant families are *Staphylococcaceae* and *Corynebacteriaceae*, but all of those shown in the legend are present at low relative abundance.
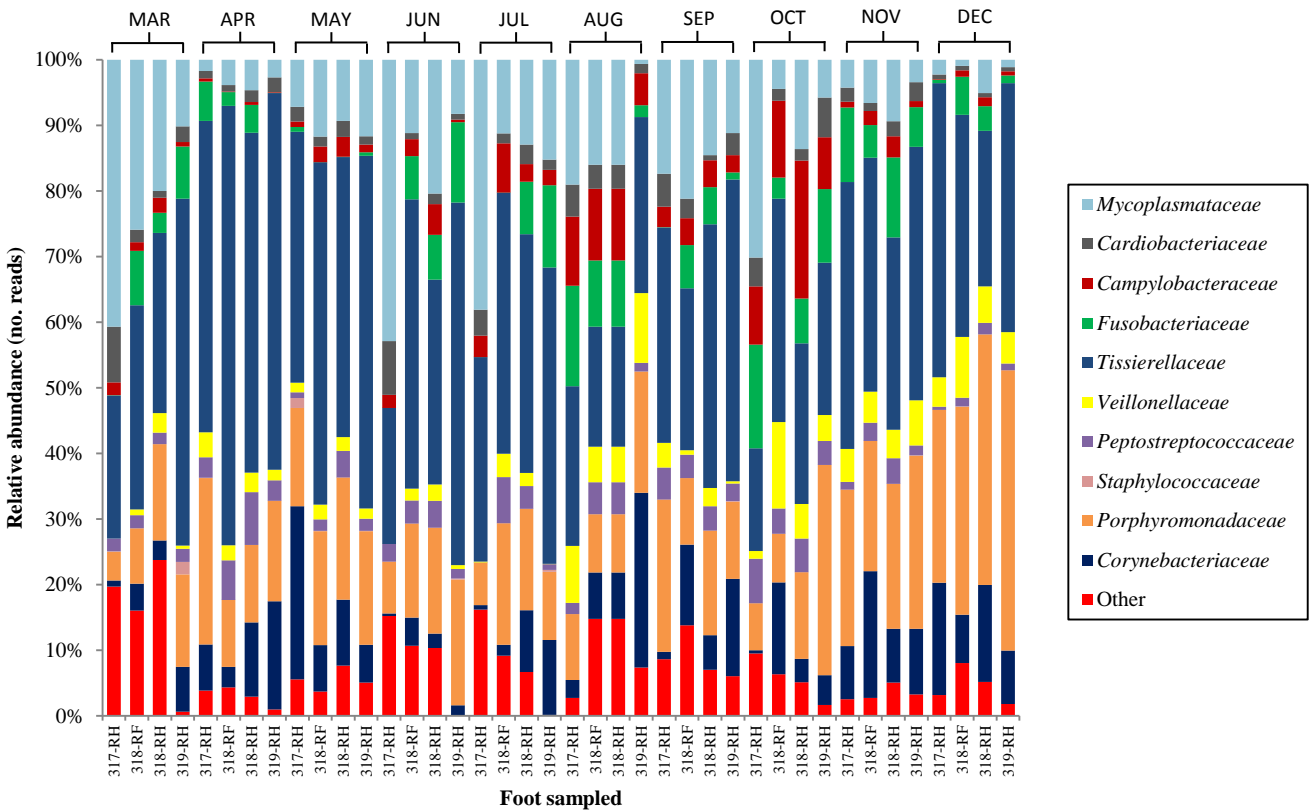
Figure 6.7: Relative abundance of the dominant bacterial families on the feet of sheep in Group 1 based on number of bacterial 16S rRNA sequences. Bacterial families that represent at least one percent of the total data set are depicted.
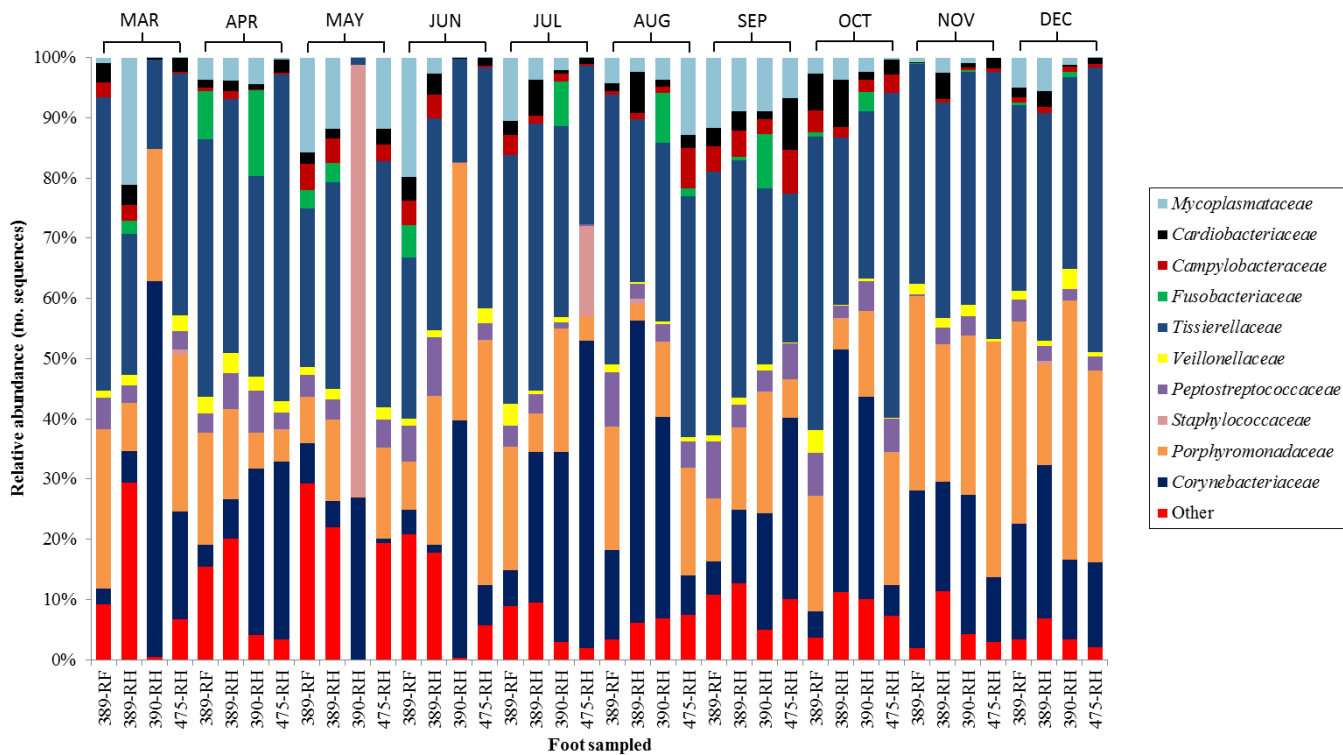
Figure 6.8: Relative abundance of the dominant bacterial families on the feet of sheep in Group 2 based on number of bacterial 16S rRNA sequence reads. Bacterial families that represent at least one percent of the total data set are depicted.

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The composition of the bacterial communities on feet assigned a score of 0 appeared to differ considerably between healthy and footrot-affected sheep. As such, alpha and beta diversity metrics were compared for feet assigned a score of 0 in the healthy sheep and footrot-affected sheep. Alpha diversity was significantly greater in the score 0 lesions of footrot-affected sheep than in those of the healthy sheep, as defined by the number of observed OTUs ($P = 0.003$) and Chao1 ($P = 0.003$). Shannon's diversity index was not significant ($P = 0.13$). Beta diversity differed significantly between the two groups according to both unweighted (AnoSim, $R^2 = 0.35$, $P = 0.002$; ADONIS, $R^2 = 0.71$, $P = 0.004$) and weighted (AnoSim, $R^2 = 0.56$, $P = 0.008$; ADONIS, $R^{2 =} 0.26$, $P = 0.006$) UniFrac distances. Beta diversity was also assessed between all scores in the footrot-affected group.

The compositions of the bacterial communities on the feet of footrot-affected sheep were compared between scores. Samples collected from Score 4 lesions were excluded from these analyses as only two samples were collected from such lesions. Within the group of footrot-affected Merino sheep, alpha diversity did not differ significantly between lesion scores, as defined by the number of observed OTUs, Chao1, and Shannon's diversity index ($P > 0.05$). However, beta diversity differed significantly between scores within the group of footrot-affected Merino sheep according to unweighted (ADONIS, $R^2 = 0.038$, $P = 0.002$) and weighted (ADONIS, $R^2 = 0.050$, $P = 0.004$) UniFrac distances.

To examine the stability of the bacterial communities across time, alpha diversity was also assessed according to time point (month), as defined by the number of observed OTUs, Chao-1 and Shannon's diversity index. There was no significant difference ($P > 0.05$) between time points for any of the three groups according each of the three alpha diversity metrics.

Beta diversity was also assessed according to lesion score. The PCoA plots generated from the unweighted and weighted UniFrac matrices demonstrated a clear separation of the bacterial populations present on most feet assigned a score of zero, from those on feet assigned a score of 1 to 3 (Figure 6.10), however some of the samples from score zero in Groups 1 and 2 lesions did cluster with the samples from active lesions. There was no obvious separation of samples collected from lesions assigned a score of 1 to 3 (Figure 6.10).

(a)

(b)

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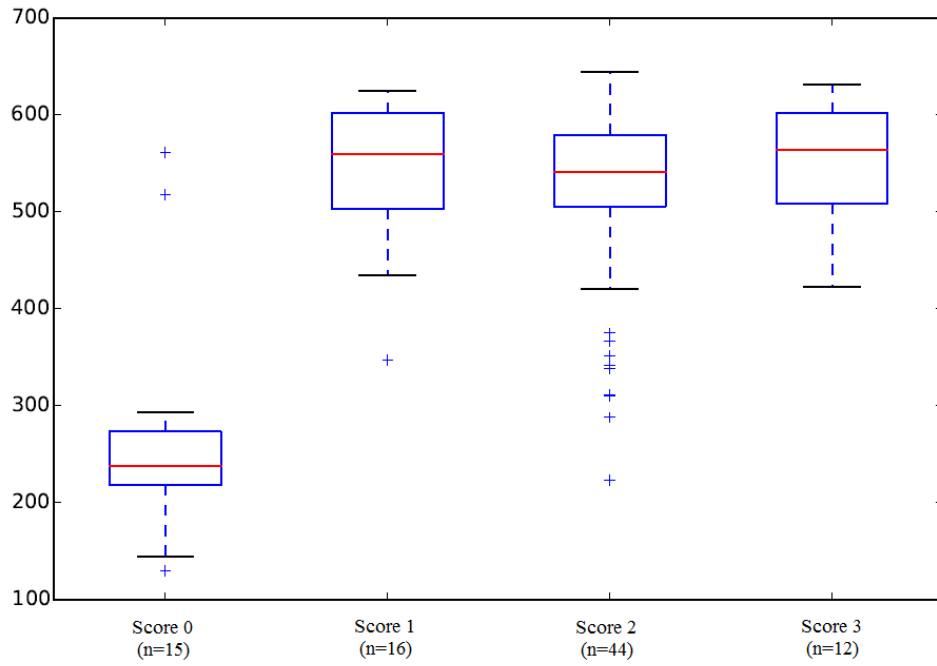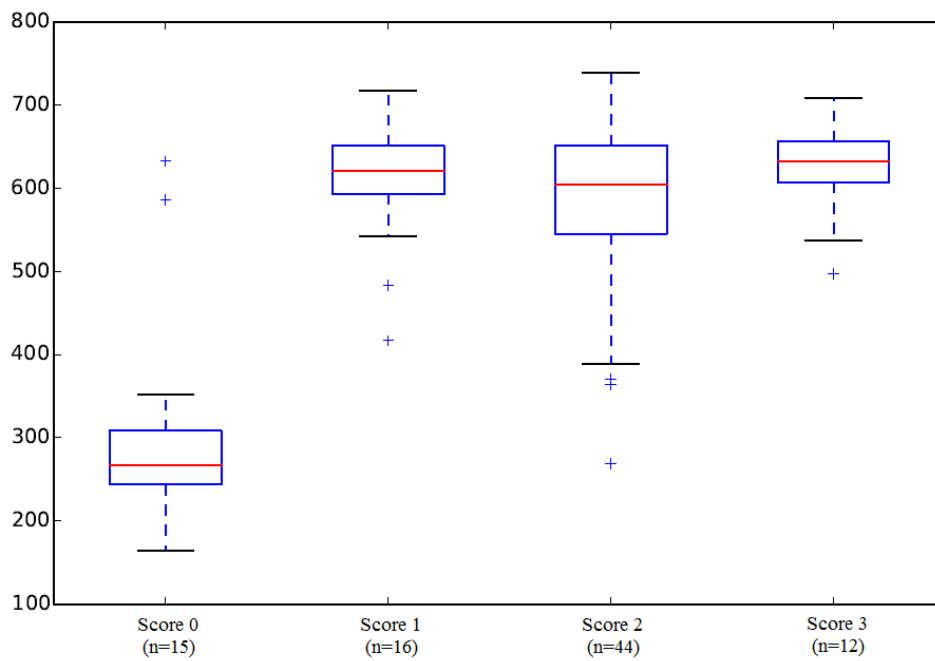
(c)
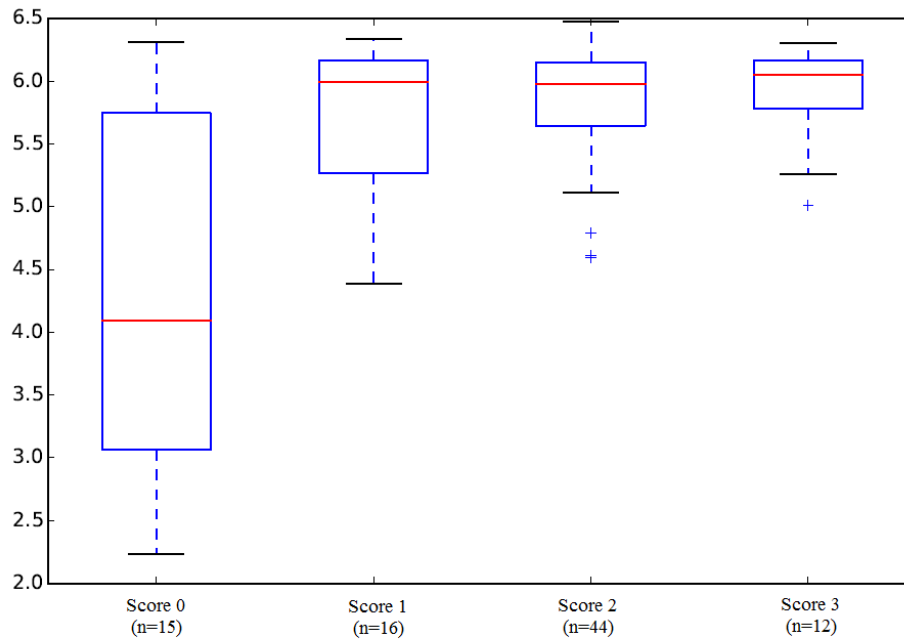
Figure 6.9: Alpha diversity boxplots for each lesion score, defined by (a) number of observed OTUs, (b) Chao-1 and (c) Shannon's diversity index.

(a)
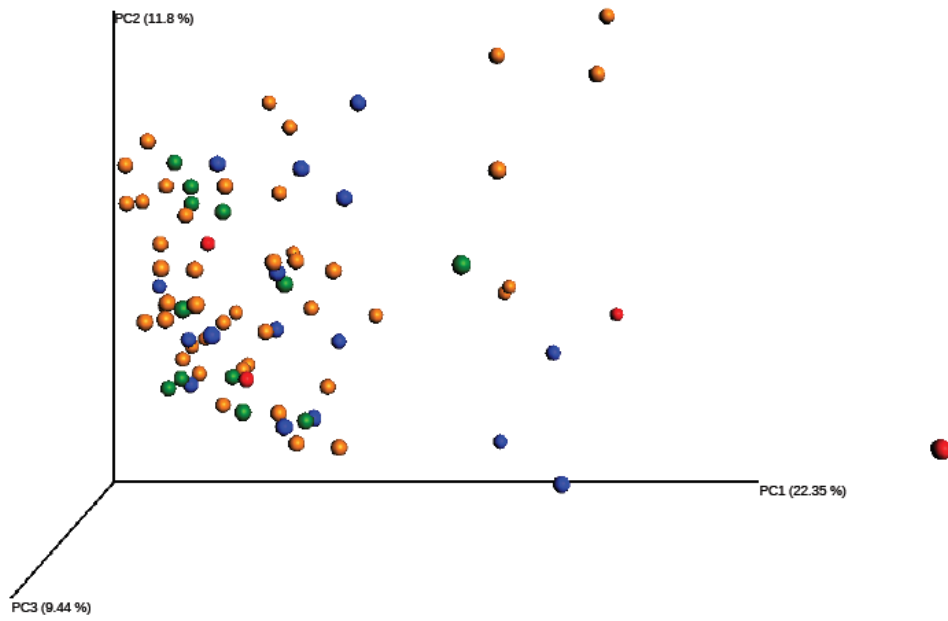
(b)
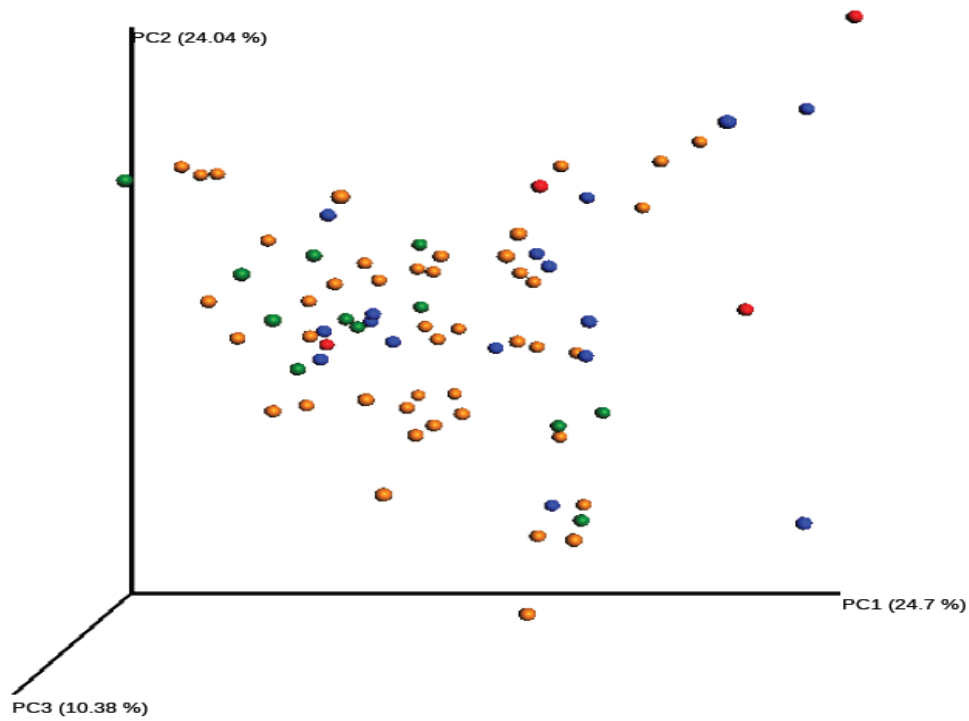
Figure 6.10: PCoA plots comparing bacterial communities from different lesion scores in the footrot-affected group, based on (a) unweighted and (b) weighted UniFrac distance matrices.

6.3.7 Identification of biomarker species (LEfSe)

No taxonomic groups were differentially abundant in samples collected from the feet of sheep in Groups 1 and 2; as such, sheep in the healthy group were compared to footrot-affected sheep, which included all sheep in Groups 1 and 2, rather than consider sheep in Group 1 and 2 as two distinct groups. Fifteen genera were significantly more abundant on the feet of footrot-affected sheep than on the feet of sheep in the healthy group (Table 6.2). Six genera were significantly more abundant on the feet of sheep in the healthy group than on the feet of footrot-affected sheep (Table 6.2).

Table 6.2: Biomarker genera identified using LEfSe. All taxa were significantly more abundant in the class of interest (Kruskal-Wallis $P <0.05$) and had an LDA score >2. Phylum, Class, Order, and Family are also listed for each of biomarker genus.

Group	Taxonomic group	LDA Score
Healthy	p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Staphylococcus	4.19
Healthy	p__Proteobacteria.c__Alphaproteobacteria.o__Sphingomonadales.f__Sphingomonadaceae.g__Sphingomonas	3.66
Healthy	p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Macrococcus	3.63
Healthy	p__Actinobacteria.c__Actinobacteria.o__Actinomycetales.f__Microbacteriaceae.g__Pseudoclavibacter	3.37
Healthy	p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales.f__Moraxellaceae.g__Acinetobacter	3.37
Healthy	p__Bacteroidetes.c__Flavobacteriia.o__Flavobacteriales.f___Weeksellaceae_.g__Wautersiella	3.10
Footrot	p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae.g__Porphyromonas	4.17
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f___Tissierellaceae_.g__Peptoniphilus	3.98
Footrot	p__Tenericutes.c__Mollicutes.o__Mycoplasmatales.f__Mycoplasmataceae.g__Mycoplasma	3.83
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f___Tissierellaceae_.g__1_68	3.69
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f___Tissierellaceae_.g__Anaerococcus	3.50
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f___Tissierellaceae_.g__GW_34	3.50
Footrot	p__Fusobacteria.c__Fusobacteriia.o__Fusobacteriales.f__Fusobacteriaceae.g__Fusobacterium	3.48
Footrot	p__Proteobacteria.c__Epsilonproteobacteria.o__Campylobacterales.f_Campylobacteraceae.g__Campylobacter	3.42
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f___Tissierellaceae_.g__Tepidimicrobium	3.36
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae.g__Dialister	3.32
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f___Tissierellaceae_.g__Gallicola	3.28
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f___Tissierellaceae_.g__Helcococcus	3.27
Footrot	p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae.g__Clostridium	3.26
Footrot	p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Alcaligenaceae.g__Oligella	3.17
Footrot	p__Proteobacteria.c__Gammaproteobacteria.o__Cardiobacteriales.f__Cardiobacteriaceae.g__Dichelobacter	3.00
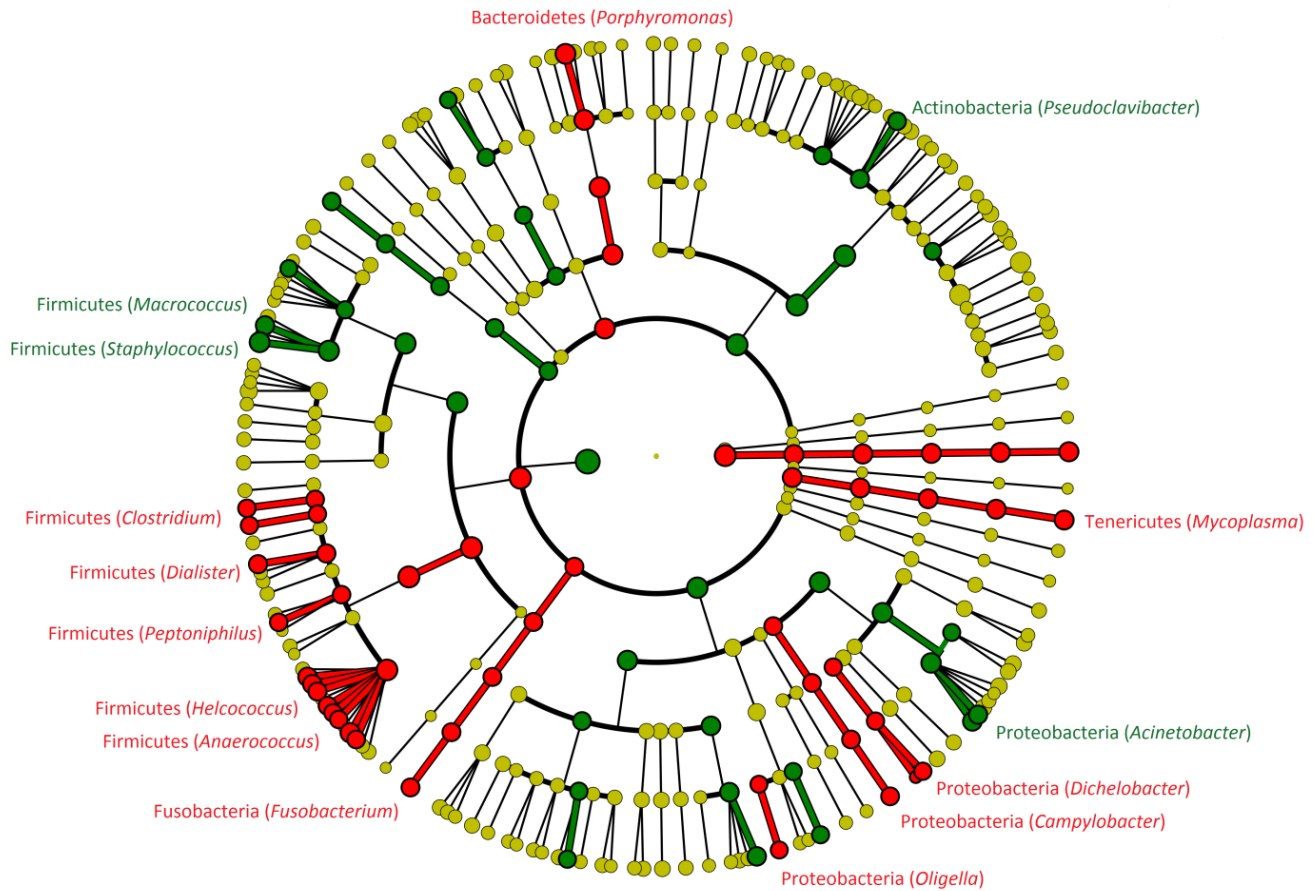
Figure 6.11: Cladogram of the bacterial taxa that had a significant Kruskal-Wallis test result (P < 0.05) and an LDA score ≥2.0 as determined by LEfSe. Bacterial phyla are represented by the central ring of the cladogram, and each subsequent ring represents the next taxonomic level. Taxa that were significantly more abundant on the feet of sheep in the healthy group or Footrot group are represented in green and red, respectively, and non-significant taxa are represented in yellow. The labels indicate significant phyla, with genera listed in parentheses.

6.4 Discussion

The first aim of this study was to characterise the bacterial communities on the feet of Merino sheep with footrot, and to compare these communities to those on the feet of Merino sheep with clinically healthy feet. In doing so, the objective was to identify bacterial taxa that are preferentially associated with footrot lesions. The second aim of this study was to characterise and compare the bacterial communities in footrot lesions of different scores, to determine whether there is any association between specific bacterial taxa and each stage of disease. The third aim of this study was to examine the bacterial communities in each group longitudinally to assess the stability of the bacterial community over time.

The bacterial communities on the feet of sheep in Groups 1 and 2, both of which were footrot-affected, were more taxonomically diverse than those on the feet of healthy sheep (Figures 6.2 – 6.4). A qualitative shift occurred in the bacterial communities on the feet of footrot-affected sheep in Groups 1 and 2 in which several bacterial families within the phyla Firmicutes and Proteobacteria came to dominate the bacterial community (Figures 6.6 – 6.8). This was reflected in the PCoA plots which demonstrated a clear separation of the samples collected from the healthy group and those collected from Groups 1 and 2, using both weighted and unweighted UniFrac distances (Figure 6.9). The unweighted PCoA plots demonstrated a separation of Groups 1 and 2 (Figure 6.9a), which suggested that the bacterial communities of each group may have differed significantly; however, there was no apparent separation of these groups in the weighted PCoA plots (Figure 6.9b), which suggested that the bacterial communities on the feet of both groups are dominated by the same bacterial taxa, and primarily differ by the presence or absence of rare taxa.

LEfSe was used to identify metagenomic biomarkers, which are defined as preferentially abundant bacterial taxa in a biological class of interest (Segata et al., 2011). Fifteen bacterial genera including *Porphyromonas*, *Mycoplasma*, *Fusobacterium*, *Peptoniphilus* and *Anaerococcus* were significantly more abundant on the feet of footrot-affected sheep (Table 6.2), all of which include pathogens of significance to human and veterinary medicine. Genera with the greatest effect size, as defined by LDA score (Table 6.2), are discussed further.

Porphyromonas is a member of the normal gastrointestinal flora of ruminants and is excreted into the environment in faeces, so detection of the genus in foot lesions is not surprising. Indeed, *Porphyromonas* was previously found to be abundant in the lesions of sheep and goats with footrot (Jimenez et al., 2003; Maboni et al., 2017; Piriz Duran et al., 1990a) and in the lesions of cattle with BDD (Collighan and Woodward, 1997; Moe et al., 2010; Santos et al., 2012; Sweeney et al., 2009). The extent to which *Porphyromonas* contributes to the disease process is unknown, however previous studies indicate that the organism may enhance the severity of foot lesions. For instance, in a previous studies the severity experimentally induced BDD lesions was greater when cattle were injected subdermally with a mixed culture of *F. necrophorum* and *Porphyromonas levii* (formerly *Bacteroides levii*) than when injected with *F. necrophorum* only (Walter and Morck, 2002). In a more recent study, *Porphyromonas* was found to be associated with high levels of inflammation in the interdigital skin of sheep with footrot (Maboni et al., 2017). The role of *Porphyromonas* in the aetiopathogenesis of ovine footrot has not been closely examined, but *Porphyromonas* may be capable of initiating the disease process in a similar manner to *F. necrophorum*, as experimental challenge of penned sheep with a mixed culture of *D. nodosus* and *Porphyromonas levii* was shown to induce foot lesions of a similar severity to those induced by challenge with *D. nodosus* and *F. necrophorum* (Nattermann et al., 1993). Previous studies describing the role of *Porphyromonas* in periodontal disease provide some support for this hypothesis. *P. gingivalis*, a member of the normal human oral bacterial community, is able to manipulate the host's immune response and inhibit leukocyte-mediated killing mechanisms, leading to unrestrained proliferation of other members of the bacterial community (How et al., 2016; Wang et al., 2010). This triggers in a qualitative shift in the bacterial community of the periodontal space in which some bacterial taxa become dominant and others can no longer be detected (Darveau et al., 2012). This is remarkably similar to the qualitative shift in the bacterial community in footrot lesions observed in the present study, and those reported in previous studies of ovine footrot lesions (Calvo-Bado et al., 2011; Maboni et al., 2017). *Porphyromonas* is also suspected to play an important role in the development of BDD lesions. As such, it is curious that the role of *Porphyromonas* in the aetiopathogenesis of ovine footrot has not received greater attention.

Two bacterial genera within the *Tissierellaceae* family, *Peptoniphilus* and *Anaerococcus* (both of which were formerly in the genus *Peptostreptococcus* (Ezaki et al., 2001)), were significantly

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more abundant on the feet of footrot-affected sheep than those of healthy sheep (Table 6.2). Gram-positive anaerobic cocci (GPAC) such as *Peptoniphilus* and *Anaerococcus* constitute a major proportion of the normal mucosal and skin flora of humans and animals, and are known to be opportunistic pathogens (Murdoch, 1998; Murphy and Frick, 2013). In humans, *Peptoniphilus* is reported to be prevalent in chronic wounds such as ulcers (Dowd et al., 2008; Smith et al., 2010; Wolcott et al., 2009), bone and joint infections (Walter et al., 2014), skin infections (Song et al., 2007), and postoperative surgical wounds (Murdoch, 1998). In animals, *Peptoniphilus* has been isolated from cattle with mastitis (Spittel and Hoedemaker, 2012), and organisms identified as *Peptostreptococcus* sp. were reported to be abundant in the foot lesions of sheep and goats infected with *D. nodosus* (Moore et al., 2005b; Piriz Duran et al., 1990b). *Anaerococcus* was previously reported to be abundant in footrot lesions but was not associated with high levels of inflammation (Maboni et al., 2017). Give that these organisms are all known to be opportunistic pathogens, they may contribute to the disease process, but a causative role has yet to be been demonstrated experimentally.

Organisms belonging to the genus *Mycoplasma* were abundant on the feet of footrot-affected sheep (Figure 6.7 – 6.8). *Mycoplasma* was previously found to be abundant in footrot lesions of sheep (Maboni et al., 2017) and BDD lesions of cattle (Collighan and Woodward, 1997; Krull et al., 2014; Nielsen et al., 2016; Santos et al., 2012; Wyss et al., 2005), it is unclear if the organism contributes to the disease process. *Mycoplasma* spp. are most commonly associated with mastitis, joint infections, and pleuropneumonia in small ruminants (Damassa et al., 1992; Johnstone and King, 2003), rather than diseases of the epidermis, but *Mycoplasma* spp. have been associated with necrotic lesions of the mucosa and skin in children (Ferrandiz-Pulido and Garcia-Patos, 2013).

The genus *Corynebacterium* was abundant on the feet of both the healthy and footrot-affected sheep, which is consistent with previous studies (Beveridge, 1941; Calvo-Bado et al., 2011; Egerton et al., 1969; Maboni et al., 2017), therefore the genus was not found to be preferentially abundant in either group. However, *C. pyogenes* is suspected of contributing to the disease process indirectly by enhancing the infectivity of *F. necrophorum* through the provision of nutrient factors (Roberts, 1967a). The proliferation of *C. pyogenes* and other aerobic bacteria in the superficial layers of the epidermis is also thought to aid the disease process by removing

oxygen and eliminating hydrogen peroxide, thus creating a favourable environment for strict anaerobes such as *D. nodosus* and *F. necrophorum* to establish and proliferate (Parsonson et al., 1967). The abundance of *Corynebacterium* in active lesions suggests that the bacterium may continue to contribute to the process as the disease progresses; however, it is possible that the lesion simply provides a favourable environment for the bacterium to persist. The abundance of *Corynebacteriaceae* was greater in samples collected from the feet of sheep in Group 2 (Figure 6.8) than in samples collected from the feet of sheep in Group 1 (Figure 6.7). Clinically, the disease was less severe in Group 2 from June to October because the topography of the paddock they were maintained in was varied and consequently the pasture and soil remained drier throughout much of trial period. This may explain why the abundance of *Corynebacteriaceae* was higher in this group. This may also reflect group-specific differences in the relative abundance of particular bacterial genera. The lesions in Groups 1 and 2 began to resolve during the winter months where environmental conditions were not amenable to disease progression (cold, dry), with the exception of a brief period of high rainfall (Figure 6.1). Interestingly, in May and July, respectively, the bacterial communities in samples collected from 390-RH and 475-RH in the group with VFR were dominated by *Corynebacteriaceae* and *Staphylococcaceae* (Figure 6.8). At these points in time, the lesions on these feet had partially resolved due to cold, dry environmental conditions (Table 6.1). The lesions recrudesced with the advent of spring and the bacterial communities returned to a state of dysbiosis.

The genus *Staphylococcus* was significantly more abundant on the feet of healthy sheep than on the feet of footrot-affected sheep (Table 6.2). This is at odds with an earlier metagenomic study in which *Staphylococcus* was most abundant on feet with footrot lesions (Calvo-Bado et al., 2011). However, *Staphylococcus* was not abundant in footrot lesions in a more recent study (Maboni et al., 2017), and is not widely regarded as significant to the aetiopathogenesis of ovine footrot. Despite being abundant on healthy feet, the genus was not abundant on footrot-affected feet in the present study, which suggest that organisms belonging to this genus had been out-competed by other members of the bacterial community. *Staphylococcus* was previously shown to enhance the infectivity of *F. necrophorum* (Smith et al., 1991), so the genus may be of significance to the very early stages of the disease process. Further investigation is required to exclude a contributory role in footrot.

The genera *Sphingomonas*, *Macrococcus*, *Pseudoclavibacter*, and *Acinetobacter*, all of which are known to be opportunistic pathogens, were significantly more abundant on the feet of healthy sheep. Organisms in the genus *Sphingomonas* are opportunistic pathogens that take of advantage of underlying disease, and have been implicated in cases of septicaemia and bacteraemia caused by the intravenous administration of contaminated solutions (Ryan and Adley, 2010). Organisms belonging to the genus *Sphingomonas* are also frequently isolated from nosocomial infections, and have been isolated from water and soil (Ryan and Adley, 2010). As such, the detection of *Sphingomonas* spp. on the feet of healthy sheep can probably be attributed to the presence of soil. The genus is not known to contribute to the aetiopathogenesis of footrot, but has been isolated from the feet of lame cattle (Cengiz et al., 2015). The genus *Macrococcus* was previously found to be abundant on the feet of healthy sheep (Calvo-Bado et al., 2011) and various skin sites of dogs (Brawand et al., 2017). Organisms belonging to the genus *Macrococcus* are also known to be opportunistic pathogens and have been isolated from abscesses in lambs (Delafuente et al., 1992). The genus *Acinetobacter* was previously found to be significantly more abundant on the feet of healthy sheep than on the feet of sheep with footrot (Maboni et al., 2017).

The bacterial communities on the feet of footrot-affected sheep were compared according to lesion score and sampling point using alpha and beta diversity metrics. There was no significant difference in diversity of bacterial communities between samples collected from active lesions of different grades (scores 1 to 3), which suggests that the composition of the bacterial community shifts early in the disease process and that this state of dybiosis persists whilst the disease is active. Surprisingly, there was no significant difference between the bacterial communities in active lesions (scores 1 to 3) and those in healed lesions (score 0) in the footrot affected group. It is possible that this persistent state of dybiosis could enhance or accelerate the recrudescence of lesions with the advent of favourable pasture conditions. There was no significant difference in the composition of the bacterial communities between sampling points in any of the three groups, which suggests that the bacterial communities of both healthy and diseased feet vary little across time.

It is difficult to determine whether the dominant taxa identified in footrot lesions contribute to the disease process, or whether the lesion simply provides a favourable environment for these

taxa to proliferate. It is likely that both processes occur simultaneously. It is interesting to note that the qualitative shift from predominantly Gram-positive, aerobic taxa on clinically healthy feet, to predominantly Gram-negative, anaerobic taxa on diseased feet observed in this study is generally characteristic of a transition from a state of health to a state of disease for skin or mucosal surfaces in general (Haffajee et al., 2005; Yang et al., 2009). Furthermore, an increase in taxonomic diversity is also associated with a transition from a state of health to a state of disease (Kennedy et al., 2016).

The dominant bacterial genera identified on the feet of healthy and footrot-affected Merino sheep in this study differ to those reported on the feet of European sheep breeds in previous studies which were conducted in the U.K, which suggests that there may be either breed or geographic influences on the results, or both. Specimens were collected from three sheep flocks by Calvo-Bado et al. (2011): the first flock consisted of Badger Faced Welsh Mountain sheep; the second consisted of Wiltshire Horn sheep; and the third consisted of Suffolk cross mule sheep. The authors report that the composition of the bacterial communities differed between flocks, but concede that this may have also been a consequence of geographic location, severity of disease, or a combination of all three factors. The authors reported that the genera *Staphlococcus*, *Macrococcus* and *Micrococcus* were most abundant on the feet of sheep with footrot, while organisms belonging to the genus *Peptostreptococcus* were most abundant on the feet of healthy feet. These results differ entirely to those of the present study, in which *Staphlococcus* and *Macrococcus* were abundant on healthy feet, and organisms formerly assigned to the genus *Peptostreptococcus* were most abundant on footrot-affected feet. The results presented here are similar to those of a more recent study, in which *Mycoplasma*, *Porphyromonas*, and *Anaerococcus* were shown to be abundant on footrot-affected feet, and *Corynebacterium* and *Acinetobacter* to be abundant on healthy feet (Maboni et al., 2017). Some potential breed-specific differences were identified in the present study, as organisms belonging to the genera *Peptoniphilus*, *Fusobacterium*, *Campylobacter*, *Dialister*, *Gallicolla*, *Clostridium*, and *Oligella* were found to be abundant on the feet of Merino sheep with footrot.

The three groups of Merino sheep included in this study were all obtained from different flocks in different geographic locations, which may have contributed to the observed variation in the composition of the bacterial communities. There are several tiers of genetic susceptibility to

footrot, even within a breed (Egerton and Raadsma, 1991; Egerton et al., 1983). Variation between strains within a breed, between bloodlines within a strain, and between sire lines within a bloodline have been reported (Egerton and Raadsma, 1991). Interestingly, there was no significant difference in the composition of the bacterial communities on the feet of sheep in Groups 1 and 2, despite the sheep originating from different flocks. Future investigations would benefit from the use of healthy and footrot-affected sheep sourced from the same flock.

The results of amplicon-based metagenomic studies need to be considered in light of the limitations of the methods used. Each of the methods used in this study has inherent biases, from sample collection through to DNA extraction and data analysis. I chose to analyse material collected from each foot with swabs rather than collect post-slaughter interdigital skin biopsies, as has been reported in previous studies (Calvo-Bado et al., 2011; Krull et al., 2014; Maboni et al., 2017; Nielsen et al., 2016). This enabled the same feet to be sampled repeatedly over a period of several months. However, there were concerns that the amount of genetic material obtained from each swab would be insufficient to capture the true diversity of the bacterial community on the foot, particularly on those collected from the interdigital skin of healthy feet. However, the use of swabs to collect specimens from the epidermis for NGS has previously been reported (Cheng et al., 2015; Klymiuk et al., 2016), and I obtained a sufficient quantity (>10.0 ng/µL) of DNA from each swab for amplification of the 16S rRNA gene. I chose to amplify the V3-V4 region of the bacterial 16S rRNA gene, as this region was previously shown to be suitable for discriminating between clinically important genera (Chakravorty et al., 2007), and to enable comparison with previous studies that have chosen to target this region (Maboni et al., 2017). The bacterial 16S rRNA gene is approximately 1500 bp in length, however the maximum read length achieved by most common NGS platforms is much shorter; for instance, the Illumina MiSeq platform can accommodate a maximum read length of 300 bp. Sequences approximately 400 to 500 bp in length can be generated by merging paired-end reads, depending the region of the 16S rRNA gene that was sequenced. Nevertheless, most amplicon-based metagenomics studies must infer taxonomy using only partial 16S rRNA sequences.

6.5 Conclusion

The aim of this study was to characterise and compare the bacterial communities on the feet of healthy and footrot-affected Merino sheep. I identified 15 bacterial genera that were preferentially abundant on feet with footrot lesions. These genera may be of significance to the disease process, however further experimental investigation is required to establish their respective roles. The composition of the bacterial communities on the feet of both healthy and footrot-affected Merino feet were different to those reported on the feet of European sheep breeds in the U.K. (Calvo-Bado et al., 2011; Maboni et al., 2017). This may indicate that there are breed-specific differences in the aetiology of ovine footrot; however geographical variation is probably a contributing factor and more investigation is required. The composition of the bacterial communities on feet with active foot lesions ($\geq$1) did not differ between scores, which suggests that a state of dybiosis emerges early in the disease process and that this state persists as the disease progresses, irrespective of environmental conditions. The results of this study provide a foundation for future, targeted investigation of the complex bacterial aetiology of ovine footrot.

Chapter 7

A pasture-based experimental model for the induction of footrot in sheep

7.1 Introduction

Footrot is a highly contagious mixed bacterial disease of sheep and other ruminants (Beveridge, 1941; Egerton, 1989b; Stewart, 1989). The disease is economically significant to the sheep meat and wool industries in most countries where sheep are reared (Greer, 2005; Lane et al., 2015; Nieuwhof and Bishop, 2005; Wassink et al., 2010). The essential causative agent, *Dichelobacter nodosus*, is a fastidious, Gram negative, anaerobic bacterium and an obligate parasite of the ruminant hoof (Beveridge, 1941). The bacterium is easily transmitted between animals by means of contaminated pasture, soil, and other surfaces (Beveridge, 1941; Egerton et al., 1969; Stewart, 1989; Whittington, 1995), but is not thought to survive in the environment for longer than 14 days under natural conditions (Stewart and Claxton, 1993), except in hoof clippings where the bacterium can remain viable for up to six weeks (Beveridge, 1941).

Footrot presents as a continuum of clinical entities, ranging from a mild interdigital dermatitis, through to severe underrunning of the hoof capsule, exposing the sensitive underlying dermal tissues (Beveridge, 1941; Egerton, 1989a; Stewart and Claxton, 1993; Stewart et al., 1986). However, for descriptive and disease control purposes outbreaks are generally consigned to discrete categories using objective measures, such as foot scoring systems (Egerton, 1989a; Egerton and Roberts, 1971). Two clinical forms of the disease are generally recognised in the literature: virulent footrot and benign footrot (Buller and Eamens, 2014; Stewart and Claxton, 1993). In Australia, virulent and benign footrot are differentiated by the prevalence of severe, underrun (score 4 lesions) (Egerton, 1989a; Egerton and Roberts, 1971).

The extent to which the disease progresses is contingent upon the virulence of the infecting *D. nodosus* strain(s) (Stewart et al., 1986), the susceptibility of the host (Emery et al., 1984), and environmental conditions, with mild air temperatures and moisture required for full disease expression (Glynn, 1993; Graham and Egerton, 1968). Despite the invasiveness of *D. nodosus*, the bacterium is unable to invade the epidermis unless it is compromised in some manner (Beveridge, 1941; Egerton et al., 1969). Water maceration, which follows prolonged exposure to moist pasture and soil, is regarded as the most common predisposing factor (Beveridge, 1941;

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Thomas, 1962a). However, water maceration alone is not sufficient (Roberts and Egerton, 1969) Mechanical damage from stones, crop stubble, and rough pasture may also compromise the interdigital skin (Beveridge, 1934, 1941; Glynn, 1993; Graham and Egerton, 1968; Shahan, 1942; Whittington, 1995).

Strains of *D. nodosus* are classified as virulent or benign according to their *in vitro* phenotypic and genotypic characteristics (Frosth et al., 2015; Palmer, 1993; Stäuble et al., 2014a; Stewart, 1979). However, current *in vitro* virulence tests lack sensitivity and specificity (Dhungyel et al., 2013b; McPherson et al., 2017; Palmer, 1993). Experimental challenge of penned sheep has also been suggested as an indicator of virulence (Stewart et al., 1986).

The clinical manifestations of footrot are the result of synergistic interactions between *D. nodosus* and the bacterial community of the foot following invasion of the epidermis (Egerton and Parsonson, 1969; Egerton et al., 1969; Hine, 1983; Roberts, 1967b; Roberts and Egerton, 1969). These interactions remain poorly understood, but *F. necrophorum*, a normal inhabitant of the gastrointestinal tract that is introduced to the foot through exposure to faeces (Nagajara et al., 2005), is thought to be essential for initiation and progression of the disease process (Egerton et al., 1969; Roberts and Egerton, 1969). The inflammatory activity triggered by invasion of the epidermis by *F. necrophorum* results in hyperkeratosis, producing fissures in which *D. nodosus,* a strict anaerobe, can establish and proliferate (Egerton et al., 1969; Hine, 1983). A leukocytic toxin expressed by *F. necrophorum* promotes bacterial invasion of the epidermis by preventing leukocytes from accessing the site of infection (Roberts and Egerton, 1969). Once *D. nodosus* has established in the superficial layers of the epidermis, this bacterium leads the invasion of fresh epidermal tissues, closely followed by *F. necrophorum* (Egerton et al., 1969). This cycle is repeated as the disease progresses, resulting in localised destruction of the epidermal matrix (Egerton et al., 1969). Consequently, exposure to faeces is essential for initiation and progression of the disease (Roberts and Egerton, 1969).

Experimental models have been developed to study the aetiopathogenesis of footrot (Beveridge, 1941; Depiazzi and Richards, 1985; Egerton et al., 1969; Graham and Egerton, 1968; Han et al., 2008; Jelinek et al., 2000; Kennan et al., 2001; Kennan et al., 2010; Roberts and Egerton, 1969), to assess the efficacy of prophylactic and therapeutic measures such as vaccines (Hunt et al., 1994), to evaluate diagnostic tests (Dhungyel et al., 2001; Stewart, 1979), and to establish the

virulence phenotype of *D. nodosus* strains (Han et al., 2008; Kennan et al., 2010). Historically, investigators have employed simple models in which sheep are maintained on irrigated or boggy pastures, which facilitates softening and maceration of the interdigital skin, and challenged by the introduction of sheep naturally infected with *D. nodosus* or by transfer to contaminated paddocks (Emery et al., 1984; Jelinek et al., 2000; Marsh and Tunnicliff, 1934). The application of infective material collected from the feet of sheep naturally infected with *D. nodosus* has also been used as a challenge method (Mohler and Washburn, 1904), as has mechanical disruption of the interdigital skin to promote bacterial invasion of the epidermis, for instance by scarifying the interdigital skin with a scalpel blade (Beveridge, 1941; Emery et al., 1984).

Presently, indoor, pen-based experimental systems are favoured as they enable investigators to manipulate environmental factors such as temperature, moisture, and exposure to soil or faeces. Secure, indoor quarantine-approved facilities are also required for experimental challenge with genetically modified organisms (GMOs) (Han et al., 2008; Kennan et al., 2001; Kennan et al., 2010). Pen-based systems may mimic the natural environmental conditions in which footrot is naturally transmitted and expressed; for instance, to mimic wet pasture, pens may be lined with wet hessian sacking or foam rubber matting (Egerton et al., 1969). Faeces are allowed to accumulate to some extent, but exposure to soil and organic material is not permitted.

The pen-based model developed by Egerton et al. (1969) appears to be the preferred method of experimental footrot transmission, having been used extensively in footrot research (Bhardwaj et al., 2014; Depiazzi and Richards, 1985; Depiazzi et al., 1991; Depiazzi et al., 1998; Egerton, 1974; Egerton and Merritt, 1973; Egerton and Roberts, 1971; Emery et al., 1984; Ghimire et al., 1999; Han et al., 2008; Hunt et al., 1994; Jelinek and Depiazzi, 2003; Jelinek et al., 2000; Kennan et al., 2001; Kennan et al., 2010; Marshall et al., 1991a; Skerman et al., 1982; Stewart et al., 1991). Briefly, sheep are maintained on wet foam rubber matting for four days to facilitate softening and maceration of the interdigital skin, and invasion of the epidermis by *F. necrophorum*. Thereafter, a pure culture of *D. nodosus* on 4% HA is placed between the digits against the interdigital skin and secured with cotton gauze bandages, which are kept on for five days to ensure *D. nodosus* is transmitted. The sheep are kept in the pen or transferred onto pasture for the remainder of the trial as required. This method is similar to the method used earlier by Mohler and Washburn (1904): the inoculum, in this case infective material collected

from a sheep with virulent footrot, was applied directly to the scarified interdigital skin of a non-infected sheep, and secured with cotton bandaging. Alternative challenge methods have been used, coupled with the indoor system of Egerton et al. (1969), for example the introduction of naturally infected sheep to the pen following predisposition (Hunt et al., 1994).

Pen-based experimental models are effective for inducing footrot lesions, and provide investigators with control over several factors including moisture, exposure to faecal material, and time and dose of bacterial challenge, but they are not representative of the environment in which footrot is naturally transmitted and expressed. Furthermore, challenge methods that involve bandaging the foot are problematic because the bandages themselves can cause mechanical damage of the interdigital skin, enhancing the process of bacterial invasion. This is of particular significance to pathogenicity testing, as virulent and benign strains of *D. nodosus* are distinguished primarily by their capacity to invade the epidermis (Egerton and Parsonson, 1969; Egerton et al., 1969), but is also relevant to the evaluation of virulence tests and studies of the aetiopathogenesis of the disease.

Pen-based experimental models are also problematic with regards to animal welfare. Sheep need to be maintained in pens for several weeks, which prevents them from exhibiting normal behaviours such as grazing. Furthermore, challenge methods such as bandaging of the feet require high levels of intervention. Because of the complex, multifactorial nature of the disease, the use of live sheep cannot be avoided in most circumstances, but it is clear that experimental techniques can be further refined in order to minimise stress and other adverse impacts on animal welfare. Previous studies have attempted to minimise the number of affected feet through the use of rubber boots (Knappe-Poindecker et al., 2014; Morck et al., 1994). For example, Knappe-Poindecker et al. (2014) describe a method in which a rubber boot is placed over one foot, water is poured into the boot to facilitate softening and maceration of the interdigital skin, and kept in place for four days. Thereafter, the boot is removed and a new boot is applied containing a liquid suspension of *D. nodosus*, which is kept on for several days to facilitate transmission. Whilst this method reduces the number of feet with lesions, it requires a high level of intervention, and does not replicate the natural environment and transmission process. Most importantly, the foot is not exposed to faecal material, which is essential for initiation and progression of the disease process

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(Roberts and Egerton, 1969). As such, it is not surprising that this method was reported to be ineffective (Knappe-Poindecker et al., 2014).

There is a need for a low-intervention, pasture-based experimental model that replicates the environment in which footrot is naturally transmitted and expressed. The aim of this study was to develop a pasture-based experimental model and challenge method that more closely resembles the means and environment in which *D. nodosus* is naturally transmitted, that minimises intervention and improves animal welfare, in accordance with the principles outlined in the National Health and Medical Research Council (NHMRC) *Australian code for the care and use of animals for scientific purposes* (NHMRC, 2013).

7.2 Materials and methods

7.2.1 Site

Trials were conducted at the farms of The University of Sydney located at Camden, NSW, Australia. The Camden area has a mean annual rainfall of 767 mm, which is approximately evenly distributed across all four seasons (30% in summer, 28% in autumn, 23% in spring, and 18% in winter). The pasture was composed of native and introduced species, and was dominated by kikuyu (*Pennisetum clandestinum*), paspalum (*Paspalum dilatatum*), pigeon grass (*Setaria geniculata*), and Rhodes grass (*Chloris guyana*). The pasture composition was similar throughout the paddock, and was approximately 100-200 mm long at the commencement of each trial.

7.2.2 Experimental animals

Trial animals were Merino wethers aged 1.5 to 2 years. All sheep were sourced from a single farm located at Bredbo, NSW, Australia. The flock was deemed free of footrot based on examination, microbiological culture and direct 16S rRNA PCR testing of foot swabs collected from 10 randomly selected animals.

7.2.3 Experimental design

Four trials were conducted between January 2015 and February 2016. Prior to the commencement of each trial, all sheep were predisposed to infection in a single irrigated paddock, approximately 60 m^2, for a period of four days. Sixteen sheep were included in Trials 2-4 trial, with four sheep randomly allocated to each treatment group by means of a random

number list. In Trial 1, four sheep were allocated to each group, with the exception of Group 4, which consisted of 10 sheep involved in a concurrent vaccine trial, contributing to a total of 22 sheep. All sheep were maintained in pasture-based pens, with the exception of Group 4 in Trial 1, which were maintained in a 12 m^2 indoor pen. The indoor pen was used to compare the efficacy of the bandaging method of challenge (described below) in an indoor system, as described in previous studies (for example, Roberts and Egerton (1969)) with the pasture-based system developed in this study. The indoor pen was constructed from steel grating overlayed with rubber matting, on top of which was an additional layer of foam rubber matting approximately 3 cm thick. The foam rubber matting was kept moist by hosing once or twice per day as required, and was roughly cleaned by raking accumulated faeces from the surface of the matting on a daily basis (Figure 7.1).

Figure 7.1: Indoor pens used in Trial 1 for Group 4.

Pasture-based pens were constructed from steel fence panels. The pens were constructed along a boundary fence, and separated from each other by a buffer zone of three meters (Figure 7.2). The slope of the paddock prevented run-off from pens entering adjacent pens. The pasture-based pens were each 15 m^2. Partial shade was provided for each pen by a strip of polyethylene shade cloth approximately 4.5 m^2 (Figure 7.3).
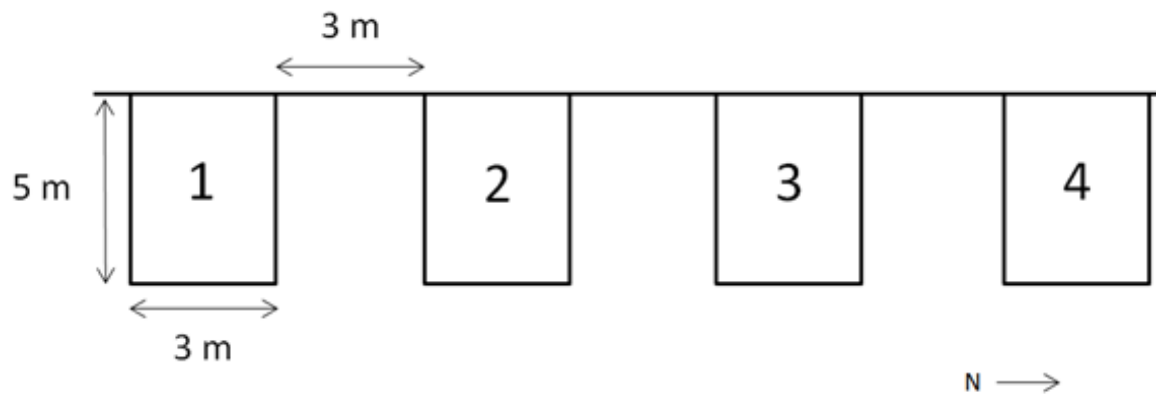
Figure 7.2: Schematic diagram the pasture-based pens used in all trials.

Figure 7.3: Pasture-based pens used in Trials 1 to 4.

For Days 1-7 of Trial 1, each of the pasture-based pens was irrigated with a sprinkler located on the northerly side of each pen. This method of irrigation was discontinued at Day 8 for reasons that are outlined in the Results for Trial 1. Thereafter, moisture was maintained by hosing each pen two-to-three times per day for 5 minutes. The output of the hose was 18 L per minute. This practice was continued for Trials 2 to 4. The level of irrigation required was determined by visually assessing how moist the pasture and soil appeared.

7.2.4 Challenge methods

Four challenge methods were evaluated across the four trials, with the aim of developing a challenge method that successfully induced foot lesions but required less intervention than the method used by Egerton et al. (1969), and that better reflected the way in which *D. nodosus* is naturally transmitted (Table 7.1). All sheep were challenged with virulent *D. nodosus* prototype strain A1001. A control group was also included in each trial to confirm that the sheep did not have a pre-existing *D. nodosus* infection.

7.2.4.1 Method 1 (Bandages)

Sheep were challenged with *D. nodosus* in accordance with the method reported by Egerton et al. (1969). Briefly, *D. nodosus* was cultured on 4% HA (Thomas, 1958) for four days, as described previously (Buller and Eamens, 2014). The culture was raked using a sterile scalpel blade to dislodge the colonies, and the inoculum collected into a small mound in the centre of the plate. A 2.0 cm^2 piece of agar immediately beneath the mound was excised with the scalpel blade and placed onto a piece of wet cotton wool, which was then placed between the digits against the interdigital skin and secured with cotton gauze bandaging. The growth on one whole plate was used to inoculate each foot. The gauze bandages were removed after five days

7.2.4.2 Method 2 (Swab)

D. nodosus was cultured on 4% HA plates (Thomas, 1958). The culture was raked using a sterile scalpel blade to dislodge the colonies, and the inoculum collected into a small mound in the centre of the plate. A sterile cotton-tipped swab (CLASSIQswabs; Copan Italia, Brescia, Italy) was coated with the inoculum by rolling it in the mound of bacteria. The inoculum was then applied to the entire surface of the interdigital skin and skin-horn junction with the swab. The growth on one whole HA plate was applied to each foot.

7.2.4.3 Method 3 (Transfer pipette)

D. nodusus was cultured on 4% HA plates. The growth on sixteen HA plates (equivalent to one per foot) were harvested using the same technique described in Method 2. The volume was made up to 16 mL with additional TAS broth. One millilitre of the suspension was deposited directly onto the interdigital skin of each foot with a transfer pipette (Livingstone, Roseberry, Australia).

7.2.4.4 Method 4 (Pasture inoculation)

D. nodosus was cultured on 4% HA plates (Thomas, 1958). The culture was harvested by flooding the surface of the plate with 1.0 mL of trypticase arginine serine (TAS) broth, dislodging the culture by raking the surface of the plate with a sterile scalpel blade, collecting the suspended culture with a 1.0 mL pipette, and depositing it into a sterile 500 mL wash bottle. Additional TAS broth was added to a total volume of 300 mL. The final concentration was approximately 2.8×10^4 cfu/mL, as determined with a Helber counting chamber. The wash bottle was stored in an anaerobic jar along with an anaerobic gas pack (GasPak, BD, Cockeysville, U.S.A.) and an anaerobic indicator (Oxoid, Hampshire, U.K.) at room temperature until required. The suspension was sprayed directly onto the pasture in the appropriate pasture-based pen, taking care to distribute the suspension evenly throughout the pasture-based pen. Sheep were transfered into the pasture-based pen immediately afterwards.

Table 7.1: Details of experimental design for Trials 1 to 4. All groups were maintained in pasture-based pens, with the exception of Group 4 in Trial 1, which were maintained in an indoor pen.

Trial No.	Group	No. sheep	Challenge method	Pen size (m^2)	Irrigation method
1	Control	4	…	15.0	Sprinker/Hose
Jan 31 – Feb 12, 2015	1	4	Bandages	15.0	Sprinkler/Hose
	2	4	Swab	15.0	Sprinkler/Hose
	3	4	Pasture inoculation	15.0	Sprinkler/Hose
	4	10	Bandages	12.0 (indoor)	Hose
2	Control	4	…	15.0	Hose
Apr 27 – May 27, 2015	1	4	Swab	15.0	Hose
	2	4	Transfer pipette	15.0	Hose
	3	4	Pasture inoculation	15.0	Hose
3	Control	4	…	15.0	Hose
Nov 19 – Dec 14, 2015	1	4	Swab	15.0	Hose
	2	4	Transfer pipette	15.0	Hose
	3	4	Pasture inoculation	15.0	Hose
4	Control	4	…	15.0	Hose
Jan 18 – Feb 15, 2016	1	4	Swab	15.0	Hose
	2	4	Transfer pipette	15.0	Hose
	3	4	Pasture inoculation	15.0	Hose

7.2.5 Flock examination

The feet of all sheep used in this study were examined prior to being transported to The University of Sydney's facilities at Camden, and at regular intervals during the course of each trial. At each examination, each sheep was placed in dorsal recumbency, each foot was inspected, and a score was allocated to each foot using a previously described scoring system (Egerton and Roberts, 1971). Briefly, mild inflammation of the interdigital skin was assigned a score of 1; if severe, a score of 2 was assigned; where underrunning of the sole and horn at the heel was observed, a score of 3 was assigned; if the underrunning extended to the abaxial wall of

the hoof, a score of 4 was assigned. A total weighted footscore (TWFS) was calculated for each sheep, and an average TWFS calculated for each group, using the system devised by Whittington and Nicholls (1995b).

7.2.6 Isolation of *D. nodosus*

To confirm that *D. nodosus* was successfully transferred to the sheep by means of each of the four challenge methods, swabs were collected from all active lesions (≥ score 1) observed at the first insepction to confirm the presence of *D. nodosus*. If no lesions were observed in a particular group, two swabs were collected from two randomly selected sheep. Specimens were collected from the interdigital skin or underrun hoof with a sterile, cotton-tipped swab (CLASSIQswabs; Copan Italia, Brescia, Italy), which was immediately used to inoculated 4% HA plates whilst in the field. The plates were transported to the laboratory in an anaerobic jar. I attempted to isolate *D. nodosus* from each swab, as described in Chapter 2.2

7.2.7 DNA extraction

DNA was prepared from each swab by magnetic bead separation using the BioSprint 96 One-For-All Vet Kit (Qiagen, Hilden, Germany) in accordance with the BS96 Vet 100 protocol, as described in Chapter 2.6.3.

7.2.8 cPCR amplification of the *D. nodosus fimA* gene

Each *D. nodosus* isolate was assigned to a serogroup by means of a multiplex cPCR amplification of variable regions of the *fimA* gene (Dhungyel et al., 2002), as described in Chapter 2.7.

7.2.9 qPCR amplification of the *D. nodosus* 16S rRNA gene

D. nodosus was detected by qPCR amplification of a variable region of the 16S rRNA gene (Frosth et al., 2012), as described in Chapter 2.8.

7.2.10 Animal welfare

Animal welfare standards were maintained in accordance with the principles outlined in the NHMRC *Australian code for the care and use of animals for scientific purposes* (NHMRC, 2013). All sheep were provided with a mixture of lucerne and oaten chaff, and water, *ad libitum*.

All sheep were treated with an broad-spectrum anthelmintic drench (Nilverm; Coopers, Macquarie Park, Australia) to treat gastroinestinal parasite infections. Trials were concluded when there was sufficient evidence that the infection had been transferred, indicated by the presence of progressive, active lesions from which *D. nodosus* was isolated in at least one sheep in each group deliberately infected with *D. nodosus*. After the trial was concluded, all sheep were walked several times through a foot-bathed containing a 10% zinc sulphate solution several times and put onto dry pasture. This process was repeated four times across four consecutive days. Sheep with severe underrun lesions were treated with a single intramuscular injection of long-acting oxytetracycline. All methods were approved by The University of Sydney Animal Ethics Committee (AEC Approval Number 2014/621).

7.2.11 Weather data

Weather data, including rainfall and air temperature, were drawn from the BOM weather station located at Camden Airport (Site No. 068192), which is located approximately 2.5 km from the trial site at The University of Sydney farms.

7.2.12 Soil temperature

Soil temperature was recorded at 15 minute intervals using data loggers (1921G, Thermocron®, Thermodata Pty Ltd, Eight Mile Plains, Australia), each of which was enclosed in a thin-walled waterproof polypropylene tube (Techo Plas, St Marys, South Australia). One tube containing a data logger was buried in each pen approximately 50 mm below the soil surface in an area that did not receive shade at any point in the day. Temperature data were downloaded to a computer using commerical software (Thermodata v3.2.23, Thermodata Pty Ltd, Eight Mile Plains, Australia). Data from all temperature loggers were collated into a Microsoft Access database and then imported into SAS (SAS Institute Inc, Lane Cove, Australia). Minimum, maximum and mean daily soil temperatures were calculated.

7.2.13 Degree days

Degree days are a measure of amount of thermal energy that an organism receive from its environment. They are typically used to predict and describe the development rates and life cycles of plants and insects (Wilson and Barnett, 1983), and to predict the response of ectothermic vertebrates to pathogens and toxins (Ellis, 2001). Degree days could also be used to

describe or predict the point at which a particular bacterial species is metabolically active, and could help to explain variation in the expression of footrot under different environmental conditions. Degree days were calculated using both the air and soil temperature data.

Degree days were calculated on a daily basis using the following formula:

$$\text{Degree days} = \frac{T_{\max} + T_{\min}}{2} - T_{\text{base}}$$

Where:

$T_{\max}$ = maximum daily temperature (°C).

$T_{\min}$ = minimum daily temperature (°C).

T_{base} = the minimum daily temperature (°C) at which the organism of interest is metabolically active. In the present case, T_{base} = 10°C, which is the minimum temperature at which *D. nodosus* is thought to be metabolically active (Beveridge, 1941).

7.2.14 Statistical analysis

A challenge method was deemed successful where a progressive lesion, indicated by an increase in TWFS over time, was observed on one or more feet of at least three sheep in the relevant group. Viable *D. nodosus* must also have been isolated from at least one foot or lesion in the group. A valid trial was one in which at least one challenge method was deemed successful according to these criteria.

Restricted maximum likelihood (REML) analysis was used to compare soil temperatures between pens across all four trials to determine if there was a pen effect on soil temperature. Pen was included in the fixed effects model, and Trial was included in the random model. REML analysis was also used to compare the TWFS for each challenge method. TWFS from each trial were analysed separately. Statistical analyses were performed with GenStat 16th Edition (VSN International Ltd, Oxford, U.K.).

7.3 Results

7.3.1 Trial 1

7.3.1.1 Environmental data

The first trial was undertaken between January 31 to February 12, 2015. Average daily air and soil temperatures and daily rainfall for the trial period are presented in Figure 7.4. Average daily air and soil temperatures did not fall below 10°C during the trial period. Rainfall was sporadic, with the exception of Day 5 where the Camden area received >40 mm of rainfall. Average daily soil temperature was mostly one or two degrees higher than the average daily air temperature. Soil temperature did not differ significantly between pens ($P > 0.05$).
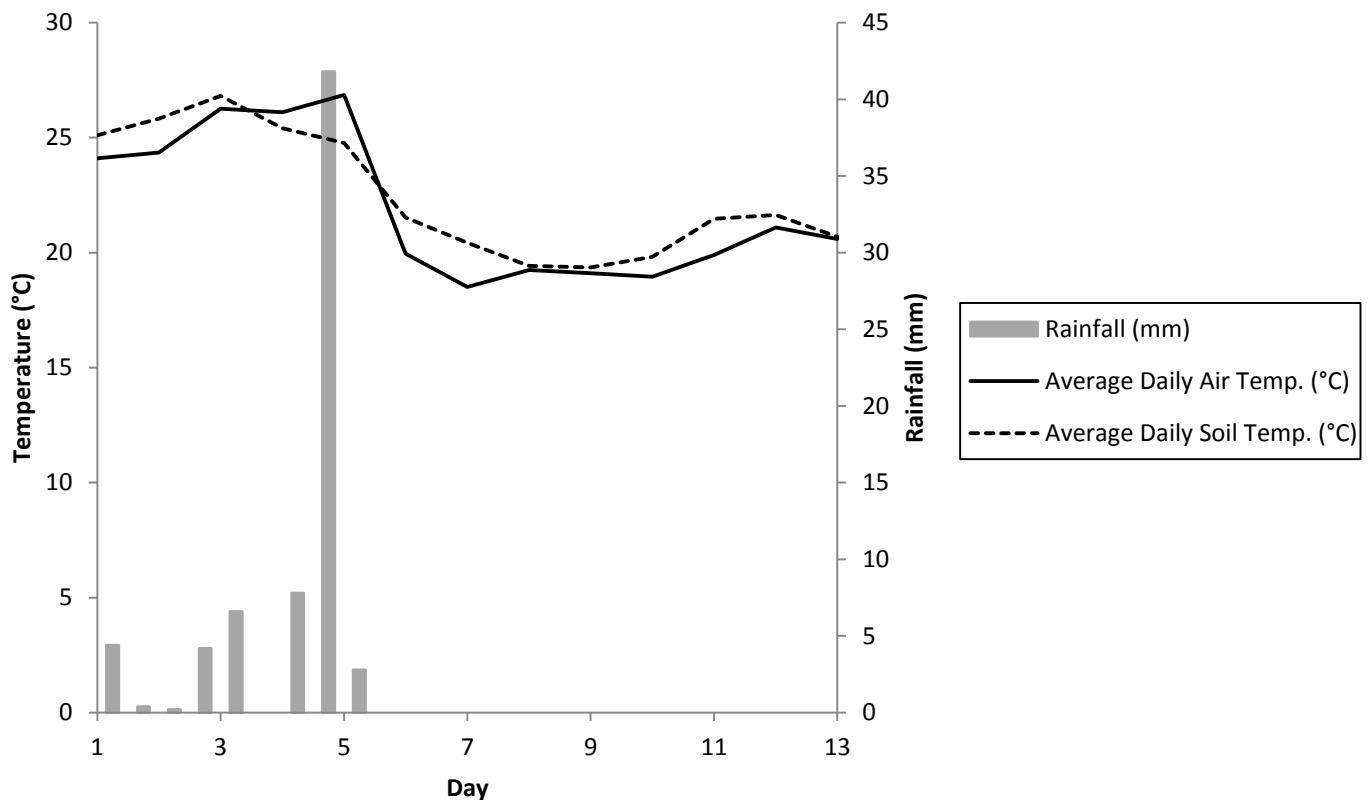
Figure 7.4: Average daily air and soil temperatures for the post-challenge phase of Trial 1 (January 31 to February 12, 2015). Soil temperature was obtained in each pasture-based pen with a single data-logger buried 50 mm below the soil surface. The average daily soil temperature across all four pens is reported. Rainfall and air temperature data were drawn from the BOM weather station located at Camden Airport (Station ID: 068192).

7.3.1.2 Foot scores

Clinical examinations of each group were conducted on Days 8 and 13 post-challenge. Foot scores from each examination are presented in Table 7.2. Between Day 8 and 13 the lesions progressed rapidly; at Day 13 the average TWFS for Group 1 (bandages, pasture-based pen), 2 (swab, pasture-based pen), and 3 (direct inoculation of pasture) were all >30.0. Groups 2 and 3 had the highest average TWFS (36.0). The lowest average TWFS was for Group 4 (12.5). The average TWFS recorded for Groups 1 to 3 were all significantly higher than the TWFS recorded for Group 4 ($P < 0.001$). There was no signficant difference between the average TWFS recorded for Groups 1 to 3 ($P > 0.05$) at any point during the trial. The day on which the sheep were examined had a significant effect on TWFS ($P < 0.001$). A score of 0 was recorded for all feet of all four sheep in the Control group at each time point. The trial was concluded at Day 13 for animal welfare reasons.

Table 7.2: Foot scores for Trial 1 (January 31 to Feb 12, 2015). Foot scores were assigned using a previously described scoring system (Egerton and Roberts, 1971). TWFS are based on a previously devised system (Whittington and Nicholls, 1995b). LF = left fore, RF = right fore, LH = left hind, RH = right hind.

							Foot scores					
				Day 8					Day 13			
Group	**Challenge Method**	**Tag No.**	**LF**	**RF**	**LH**	**RH**	**TWFS**	**LF**	**RF**	**LH**	**RH**	**TWFS**
Control	Control	5084	1	0	0	1	2	0	1	0	1	2
		5086	0	0	0	0	0	1	0	0	0	1
		5091	0	0	0	0	0	0	0	0	0	0
		5093	0	0	0	0	0	0	1	0	0	1
			Average TWFS				**0.5**	**Average TWFS**				**1.0**
1	Bandages	5075	1*	1*	0	1*	3	3	3	3	3	36
		5080	2*	2*	2*	2	8	1	2	3	3	21
		5083	2*	0	1	0	3	3	3	3	3	36
		5087	2*	2*	1	1*	6	3	3	3	3	36
			Average TWFS				**5.0**	**Average TWFS**				**32.3**
2	Swab	5077	1*	1	1	2*	5	3	3	3	3	36
		5082	1	1	2*	2*	6	3	3	3	3	36
		5088	2*	1	1*	2*	6	3	3	3	3	36
		5090	2*	1*	1*	2*	6	3	3	3	3	36
			Average TWFS				**5.8**	**Average TWFS**				**36.0**
3	Pasture inoculation	5076	1*	1*	1*	1*	4	3	3	3	3	36.0
		5081	1	1*	1	2*	5	3	3	3	3	36.0
		5092	1*	2*	3*	2*	14	3	3	3	3	36.0
		5094	0*	1	0	1*	2	3	3	3	3	36.0
			Average TWFS				**6.3**	**Average TWFS**				**36.0**
4	Bandages	316	0*	0	0	0	0	1	0	0	1	2
		322	1*	0	2*	1	4	2	1	3	1	13
		403	1	1*	0	1	3	2	2	0	2	6
		480	1	1	0	2*	4	2	2	0	3	13
		499	0	2*	1	2*	5	1	3	1	2	13
		506	1	2*	0	0	3	1	2	0	0	3
		538	2*	1	0	1	4	3	2	0	1	12
		605	0	1	2*	2*	5	1	2	3	2	14
		706	0	0	2*	2*	4	1	3	1	2	13
		5074	2*	1	0	0	3	3	2	1	0	12
			Average TWFS				**3.5**	**Average TWFS**				**12.5**

*Indicates *D. nodosus* was detected on this foot by culture.

7.3.1.3 Detection of *D. nodosus*

Swabs were collected from all lesions observed at Day 8 post-challenge for microbiological culture and PCR detection of *D. nodosus*. *D. nodosus* was detected most frequently in Group 4, which was challenged by bandaging. All *D. nodosus* isolates belonged to serogroup A. Culture and PCR results are presented in Table 7.3. *D. nodosus* was not detected in the control group by culture or 16S rRNA PCR.

Table 7.3: Culture and 16S rRNA PCR results for swabs collected from active foot lesions at Day 8 of Trial 1.

Group	No. sheep sampled	No. feet sampled	% Culture positive	% PCR positive
Control	2	2	0	0
1	4	13	79.9	100
2	4	16	68.8	100
3	4	14	85.7	100
4	10	15	93.3	100

7.3.2 Trial 2

7.3.2.1 Environmental data

The second trial was undertaken between April 27 and May 27, 2015. Average daily air and soil temperatures and daily rainfall for the trial period are presented in Figure 7.5. Average daily air and soil temperatures did not fall below 10°C during the trial period. Rainfall was more consistent during Trial 2 than during Trial 1, with periods of consistent rainfall towards the beginning and end of the trial. Average daily soil temperature was mostly one to two degrees higher than the average daily air temperature. Soil temperature did not differ significantly between pens ($P > 0.05$).
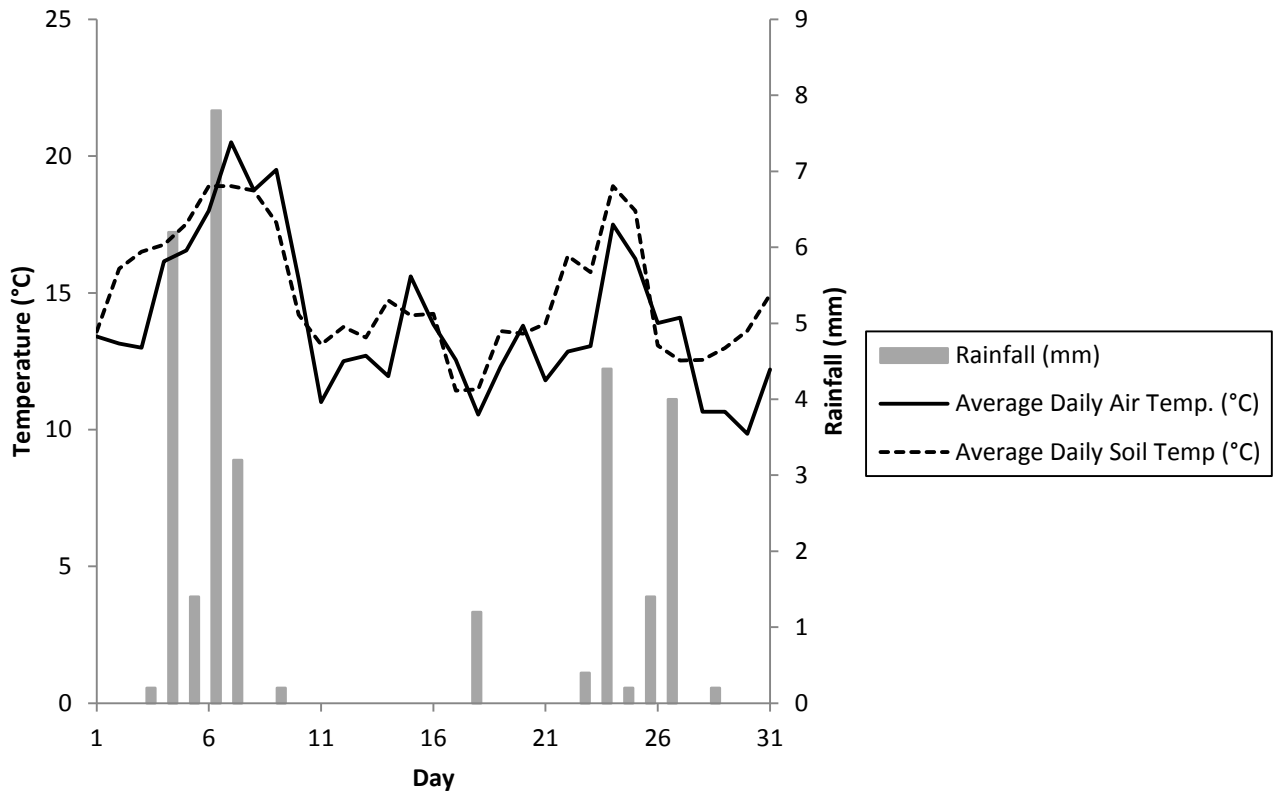
Figure 7.5: Average daily air and soil temperatures for the post-challenge phase of Trial 2 (April 27 to May 27, 2015). Soil temperature was obtained in each pasture-based pen with a single data-logger buried 50 mm below the soil surface. The average daily soil temperature across all four pens is reported. Rainfall and air temperature data were drawn from the BOM weather station located at Camden Airport (Station ID: 068192).

7.3.2.2 Foot scores

Feet were inspected on Days 11, 16, 21, 26, and 31 post-challenge. Foot score data for each inspection are provided in Table 7.4. Progressive lesions were observed on two feet of one sheep (Tag no. 7085) in Group 1 (Swab), and one sheep (Tag no. 5098) in Group 2 (Transfer pipette). TWFS were not significantly different across groups ($P = 0.467$). The day on which sheep were examined did not have a significant effect on TWFS ($P = 0.506$). A score of 0 was recorded for all feet of all four sheep in the Control group at each time point.

7.3.2.3 Detection of *D. nodosus*

Swabs were collected from all active lesions identified at Day 11 post-challenge. Two swabs were collected from two sheep in the Control group. Culture and 16S rRNA PCR results are presented in Table 7.5. *D. nodosus* was detected by PCR in all samples collected from sheep that were challenged; however, the bacterium was cultured from two feet of one sheep in Group 1 only. All *D. nodosus* isolates belonged to serogroup A. *D. nodosus* was not detected in the Control group.

Table 7.4: Foot scores for Trial 2 (April 27 to May 27, 2015). Foot scores were assigned using a previously described scoring system (Egerton and Roberts, 1971). TWFS are based on a previously devised system (Whittington and Nicholls, 1995b).

				Day 11					Day 16					Day 21					Day 26					Day 31		
																			Foot scores								
Group	Tag No.	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS
Control	6548	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	7082	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	1	**1**	0	0	0	0	**0**	0	0	0	0	**0**
	7083	0	0	0	0	**0**	0	0	0	0	**0**	0	0	1	0	**1**	0	0	1	0	**1**	0	0	1	0	**1**
	7091	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
				Average TWFS		**0**			Average TWFS		**0**			Average TWFS		**0.50**			Average TWFS		**0.25**			Average TWFS		**0.25**
1	5100	1*	0	1	1	**3**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	7084	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	7085	0	0	1*	1	**2**	0	0	1	0	**1**	0	0	1	1	**2**	0	0	2	2	**4**	0	0	2	2	**4**
	7092	0	0	0	0	**0**	0	0	0	1	**1**	0	1	0	0	**1**	0	0	0	0	**0**	0	0	0	0	**0**
				Average TWFS		**1.25**			Average TWFS		**0.50**			Average TWFS		**0.75**			Average TWFS		**1.0**			Average TWFS		**1**
2	5098	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	3	2	**11**	0	0	3	2	**11**
	6547	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	7081	0	0	1	1	**2**	0	1	0	0	**1**	0	1	0	0	**1**	0	0	0	1	**1**	0	0	0	1	**1**
	7094	0	0	0	0	**0**	0	1	1	1	**3**	0	0	1	0	**1**	0	0	0	1	**1**	0	0	0	0	**1**
				Average TWFS		**0.5**			Average TWFS		**1.0**			Average TWFS		**0.50**			Average TWFS		**3.25**			Average TWFS		**3.25**
3	3404	0	0	0	1	**1**	1	0	0	1	**2**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	6545	0	0	0	1	**1**	1	0	0	0	**1**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	6546	0	0	0	0	**0**	1	0	0	0	**1**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	5014	0	0	0	0	**0**	0	1	0	1	**2**	0	0	1	0	**1**	0	0	1	0	**1**	0	0	1	0	**1**
				Average TWFS		**0.5**			Average TWFS		**1.50**			Average TWFS		**0.25**			Average TWFS		**0.25**			Average TWFS		**0.25**

*Indicates *D. nodosus* was detected on this foot by culture.

Table 7.5: Culture and 16S rRNA PCR results for foot swabs collected at Day 11 of Trial 2.

Group	No. sheep sampled	No. feet sampled	% culture positive	% PCR positive
Control	2	2	0	0
1	2	5	40	100
2	1	2	0	100
3	2	2	0	100

7.3.3 Trial 3

7.3.3.1 Environmental data

The third trial was undertaken between November 19 and December 14, 2015. Average daily air and soil temperatures and daily rainfall for the trial period are presented in Figure 7.6. Average daily air and soil temperatures did not fall below 10°C during the trial period. Rainfall was sporadic throughout the trial period. Average daily soil temperature was mostly one to two degrees higher than the average daily air temperature. Soil temperature did not differ significantly between pens ($P > 0.05$).
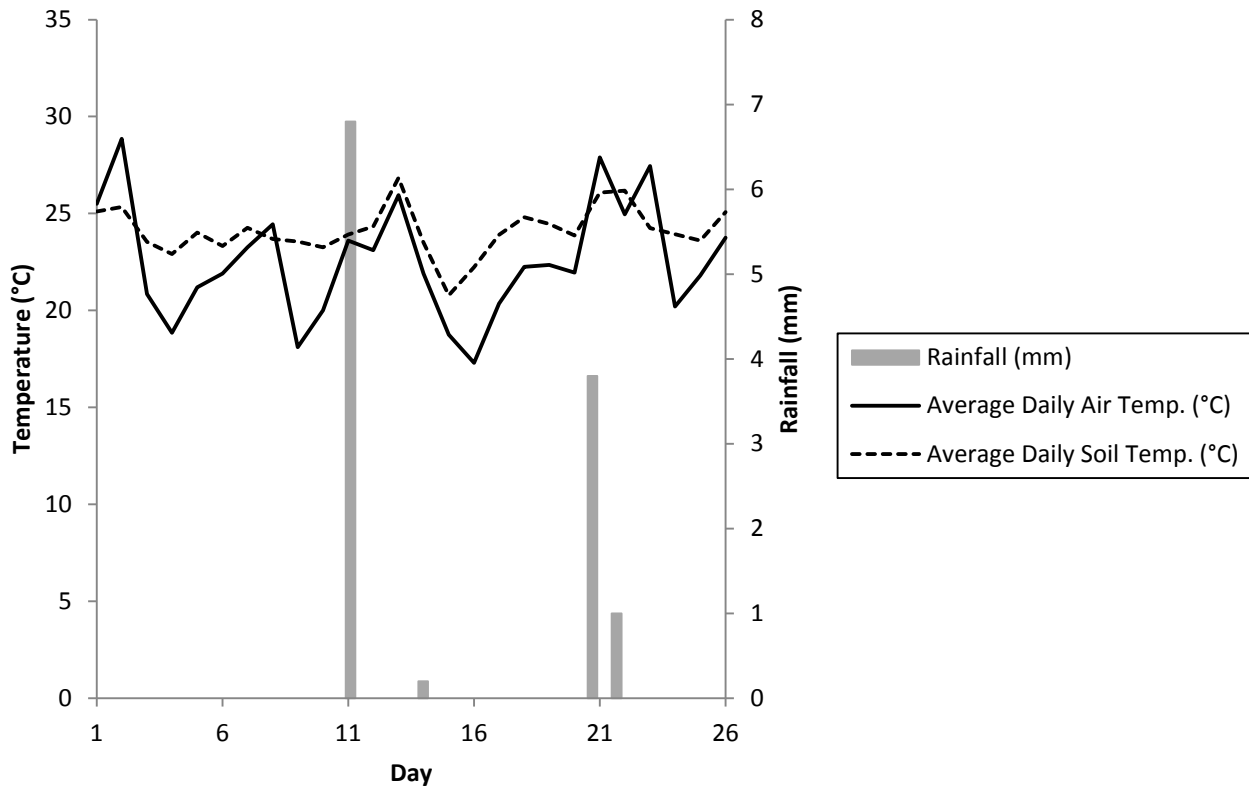
Figure 7.6: Average daily air and soil temperatures and daily rainfall for the post-challenge phase of Trial 3 (November 19 to December 14, 2015). Soil temperature was obtained in each pasture-based pen with a single data-logger buried 50 mm below the soil surface. The average daily soil temperature across all four pens is reported. Rainfall and air temperature data were drawn from the BOM weather station located at Camden Airport (Station ID: 068192).

7.3.3.2 Foot scores

Examinations were conducted on Day 11, 16, 21, and 26 post-challenge. Progressive lesions were not observed in any group during the course of the trial (Table 7.6). Several score 1 lesions were observed in each of the treatment groups, however none of these lesions progressed. TWFS did not differ significantly between groups at any point in the trial ($P = 0.831$). The day on which sheep were examined did not have a significant effect on TWFS ($P = 0.506$). A score of 0 was recorded for all feet of all four sheep in the Control group at each time point.

Table 7.6: Foot scores for Trial 3 (November 19 to December 14, 2015). Foot scores were assigned using a previously described scoring system (Egerton and Roberts, 1971). TWFS are based on a previously devised system (Whittington and Nicholls, 1995b).

		Day 11					Day 16					Day 21					Day 26				
												Foot scores									
Group	Tag No.	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS
Control	5111	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	5118	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	1	**1**	0	1	0	0	**1**
	5120	0	0	0	0	**0**	0	0	1	0	**1**	0	0	1	0	**1**	0	0	0	0	**0**
	5125	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
		Average TWFS				**0**	**Average TWFS**				**0.25**	**Average TWFS**				**0.5**	**Average TWFS**				**0.25**
1	5112	1	0	0	0	**1**	0	0	0	0	**0**	0	1	0	0	**1**	0	1	0	0	**1**
	5116	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	5122	0	0	1	0	**1**	0	0	1	0	**1**	0	0	1	1	**2**	0	0	1	0	**1**
	5123	0	0	0	0	**0**	0	0	0	1	**1**	0	1	0	0	**1**	0	0	0	0	**0**
		Average TWFS				**0.5**	**Average TWFS**				**0.5**	**Average TWFS**				**1.0**	**Average TWFS**				**0.5**
2	5114	0	0	0	0	**0**	0	0	0	0	**0**	0	1	1	0	**2**	0	0	1	1	**2**
	5115	0	0	0	0	**0**	1	1	0	0	**2**	0	0	0	0	**0**	0	0	0	0	**0**
	5119	0	0	1	1	**2**	0	0	0	0	**0**	0	1	0	0	**1**	0	0	0	1	**1**
	5124	0	0	0	0	**0**	0	1	0	1	**2**	0	0	0	0	**0**	0	0	0	1	**1**
		Average TWFS				**0.5**	**Average TWFS**				**1.0**	**Average TWFS**				**0.75**	**Average TWFS**				**1.0**
3	5110	0	0	0	1	**1**	1	0	0	1	**2**	0	1	0	0	**1**	0	0	0	0	**0**
	5113	0	0	0	1	**1**	0	0	1	0	**1**	0	0	1	0	**1**	0	1	0	0	**1**
	5117	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	5121	0	0	0	0	**0**	0	1	0	1	**2**	0	0	1	0	**1**	0	0	1	0	**1**
		Average TWFS				**0.5**	**Average TWFS**				**1.25**	**Average TWFS**				**0.75**	**Average TWFS**				**0.5**

7.3.3.3 Detection of *D. nodosus*

D. nodosus was not isolated from any of the foot swabs sampled during the course of the trial, and was only detected by 16S rRNA PCR in small proportion of the foot swabs (Table 7.7).

Table 7.7: Culture and 16S rRNA PCR results for foot swabs collected on Day 7 of Trial 3.

Group	No. sheep sampled	No. feet sampled	% culture positive	% PCR positive
Control	2	2	0	0
1	2	2	0	50
2	1	2	0	0
3	2	2	0	50

7.3.4 Trial 4

7.3.4.1 Environmental data

The fourth trial was undertaken between January 18 and February 15, 2016. Average daily air and soil temperatures and daily rainfall for the trial period are presented in Figure 7.7. Average daily air and soil temperatures did not fall below 10°C during the trial period. Rainfall was consisted through the middle of the trial period. Average daily soil temperature was mostly one to two degrees higher than the average daily air temperature. Soil temperature did not differ significantly between pens ($P > 0.05$).
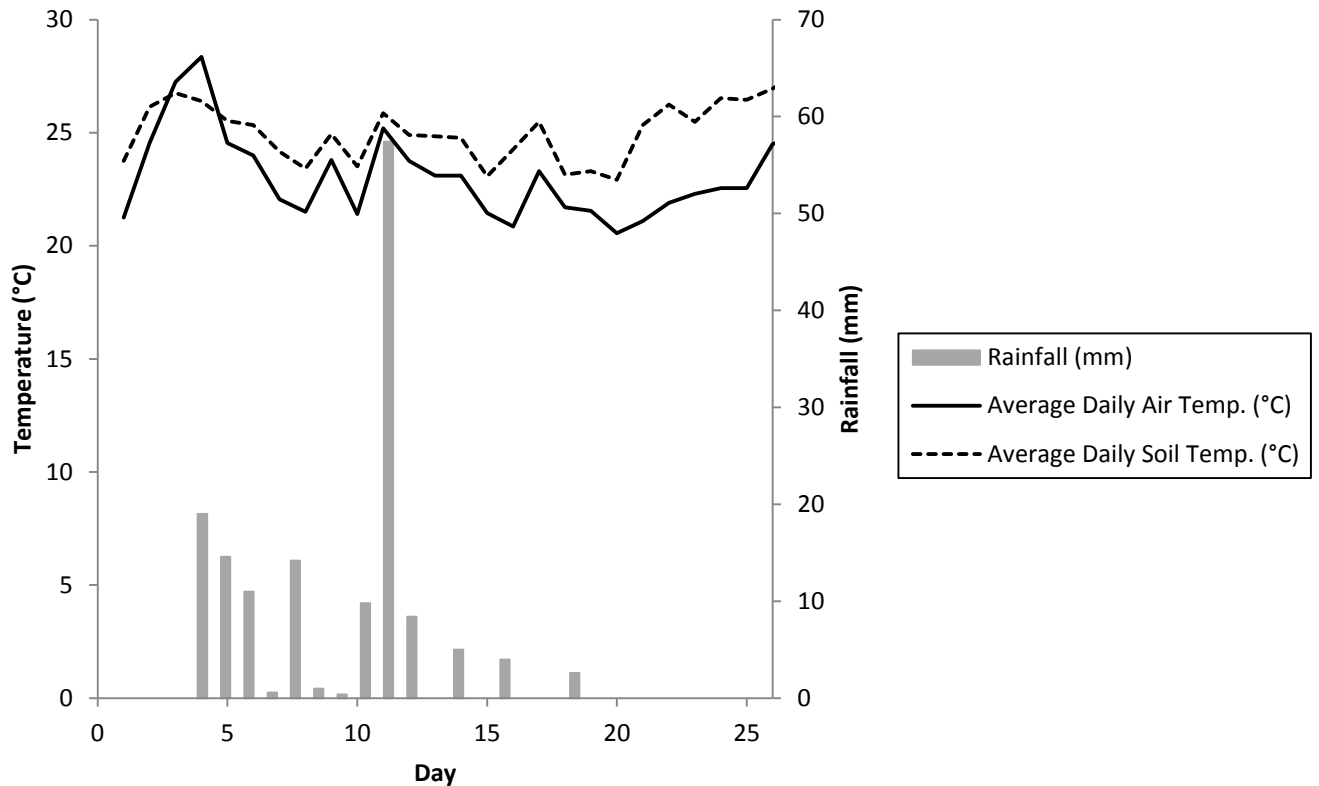
Figure 7.7: Average daily air and soil temperatures, and daily rainfall, for the post-challenge phase of Trial 4 (January 18 to February 15, 2016). Soil temperature was obtained in each pasture-based pen with a single data-logger buried 50 mm below the soil surface. The average daily soil temperature across all four pens is reported. Rainfall and air temperature data were drawn from the BOM weather station located at Camden Airport (Station ID: 068192).

7.3.4.2 Foot scores

Examinations were conducted Days 7, 12, 17, 22, and 29 on post-challenge. Progressive lesions were observed at Day 22 post-challenge (Table 7.8). TWFS for Group 1 (swab) were significantly higher than Groups 2 (transfer pipette) and 3 (pasture inoculation) ($P = 0.013$). Day was not a significant factor except for Day 29 ($P < 0.001$). Progressive foot lesions were identified in the Control group at Day 22. At Day 29 three of the four sheep in the Control group had at least one underrun (score 3) lesion.

Table 7.8: Foot scores for Trial 4 (January 18 to February 15, 2016). Foot scores were assigned using a previously described scoring system (Egerton and Roberts, 1971). TWFS are based on a previously devised system (Whittington and Nicholls, 1995b).

				Day 7					Day 12					Day 17					Day 22					Day 29	
Group	Tag No.	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS	LF	RF	LH	RH	TWFS
Control		0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	1	0	0	0	**1**
		0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	3*	2	2	0	**13**
		0	0	0	1	**1**	0	0	0	1	**1**	0	0	0	1	**1**	0	0	0	2	**2**	0	3	2	2	**13**
		0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	1	0	0	**1**	3	3*	0	0	**18**
			Average TWFS			**0.25**		Average TWFS			**0.25**		Average TWFS			**0.25**		Average TWFS			**0.75**		Average TWFS			**11.25**
1		0	0	1*	1	**2**	0	0	0	1	**1**	0	0	0	0	**0**	0	0	1	0	**1**	3	0	2	3	**20**
		0	0	1*	0	**1**	1	0	1	1	**3**	1	1	1	0	**3**	2	2	1	1	**6**	2	2	3	2	**15**
		0	0	0	0	**0**	0	0	0	0	**0**	0	0	1	0	**1**	0	1	1	1	**3**	3	2	3	2	**22**
		0	0	0	0	**0**	0	0	0	1	**1**	0	0	0	0	**0**	1	0	0	1	**2**	3	0	3	3	**27**
			Average TWFS			**0.75**		Average TWFS			**1.25**		Average TWFS			**1.0**		Average TWFS			**3.0**		Average TWFS			**21.0**
2		0	0	0	0	**0**	0	0	1	0	**1**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	3	**9**
		0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	1	**1**	0	0	0	3	**9**
		0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	3	2	**11**
		0*	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	2	1	1	0	**4**	3	3	3	0	**27**
			Average TWFS			**0**		Average TWFS			**0.25**		Average TWFS			**0**		Average TWFS			**1.25**		Average TWFS			**14.0**
3		0	0	0*	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	3	0	0	**9**
		0	0	0	0	**0**	2	0	0	0	**2**	1	1	0	0	**2**	2	2	0	0	**4**	2	2	2	0	**6**
		0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
		0	0*	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	1	**1**	0	0	1	3	**9**
			Average TWFS			**0**		Average TWFS			**0.5**		Average TWFS			**0.25**		Average TWFS			**1.25**		Average TWFS			**6.0**

*Indicates *D. nodosus* was detected on this foot by culture.

7.3.4.3 Detection of *D. nodososus*

Swabs were collected from a selection of feet with active lesions on Day 7 of Trial 4. Culture and 16S rRNA PCR results are presented in Table 7.9. Viable *D. nodosus* was isolated from the feet of sheep in Groups 1 to 3. All foot swabs collected from Groups 1 to 3 were PCR positive. Swabs were collected from two randomly selected feet of two sheep in in the Control group at Day 7, both of which were negative for *D. nodosus* by culture and PCR. Swabs were collected from the feet of two sheep in the Control group at Day 29 after progressive lesions were observed in this group. *D. nodosus* was detected by both culture and PCR. The *D. nodosus* isolates obtained from the Control group belonged to serogroup A.

Table 7.9: Culture and 16S rRNA PCR results for foot swabs collected at Day 7 of Trial 4.

Group	No. sheep sampled	No. feet sampled	% culture positive	% PCR positive
Control	2	2	0	0
1	2	3	66.6	100
2	2	2	50	100
3	2	2	100	100

7.3.5 Degree days

Cumulative degree days were calculated for each trial using average daily air and soil temperatures (Figure 7.8). Soil and air cumulative degree days were similar during the early stages of the trial, but diverged towards the latter stages of the trial as the average daily soil temperature was typically one to two degrees higher than the average daily air temperature.

(a) Trial 1
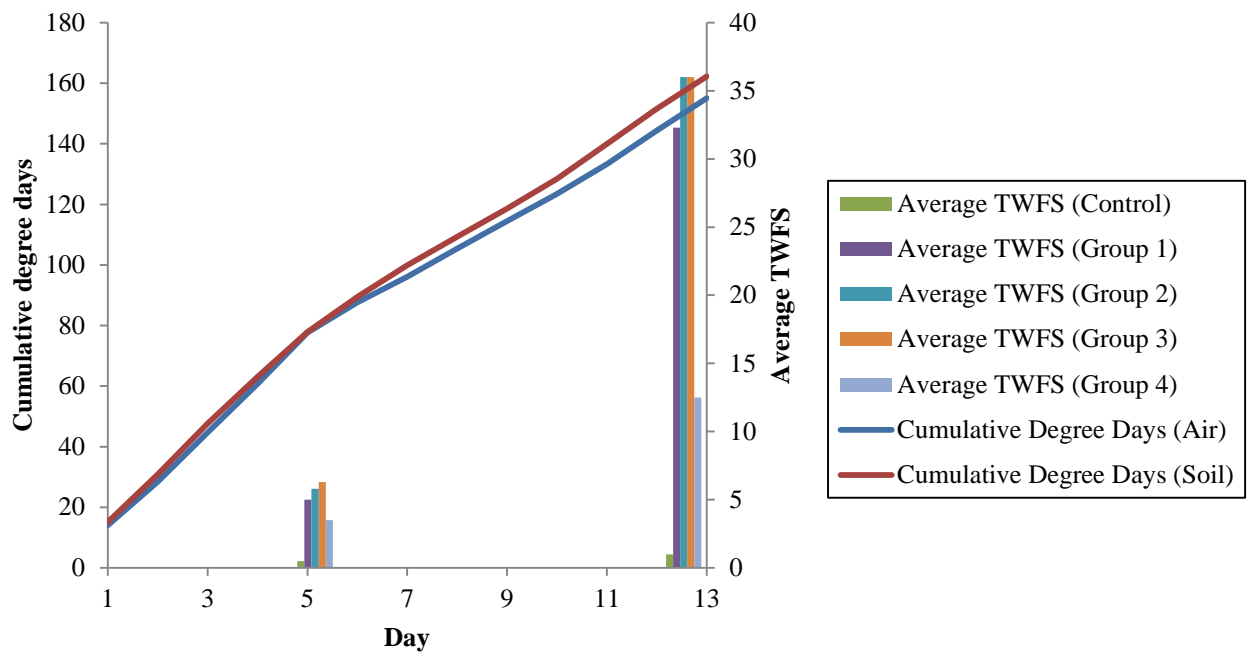
(b) Trial 2
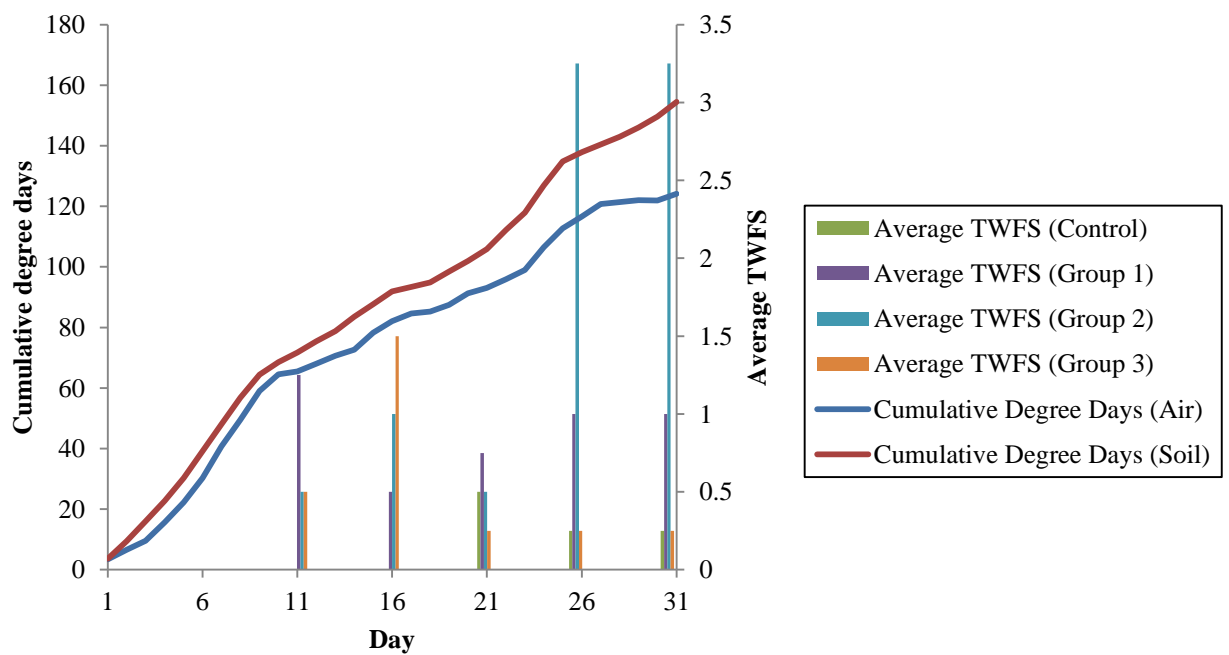
(c) Trial 3
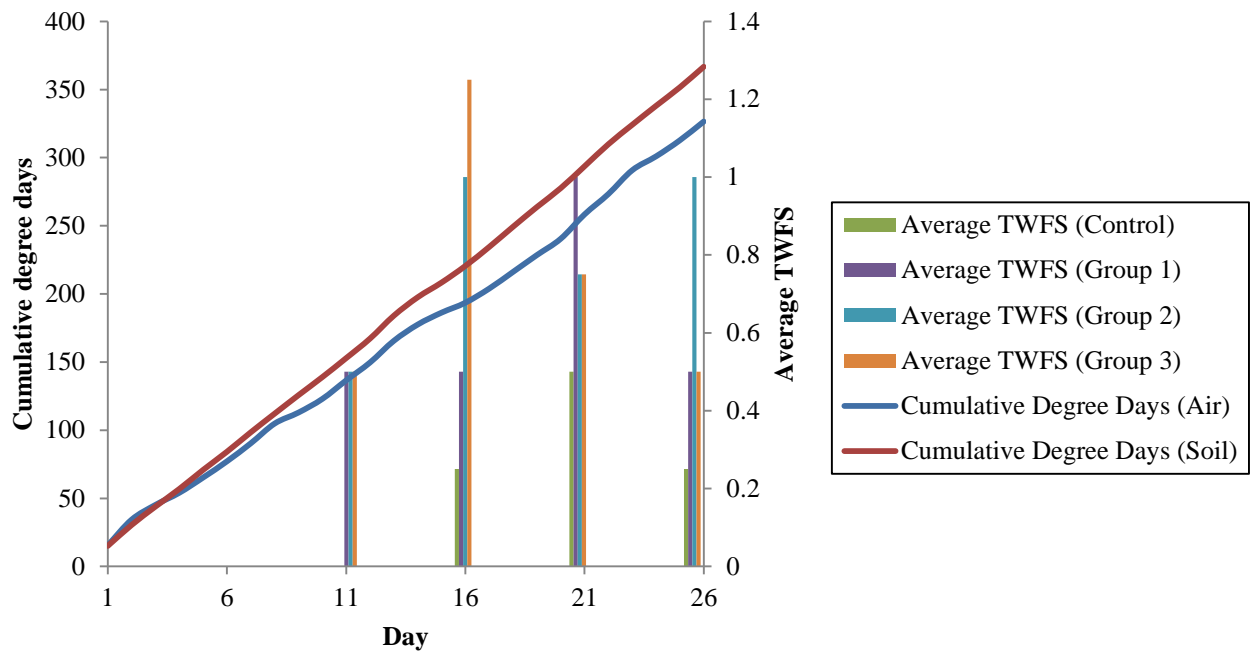
(d) Trial 4
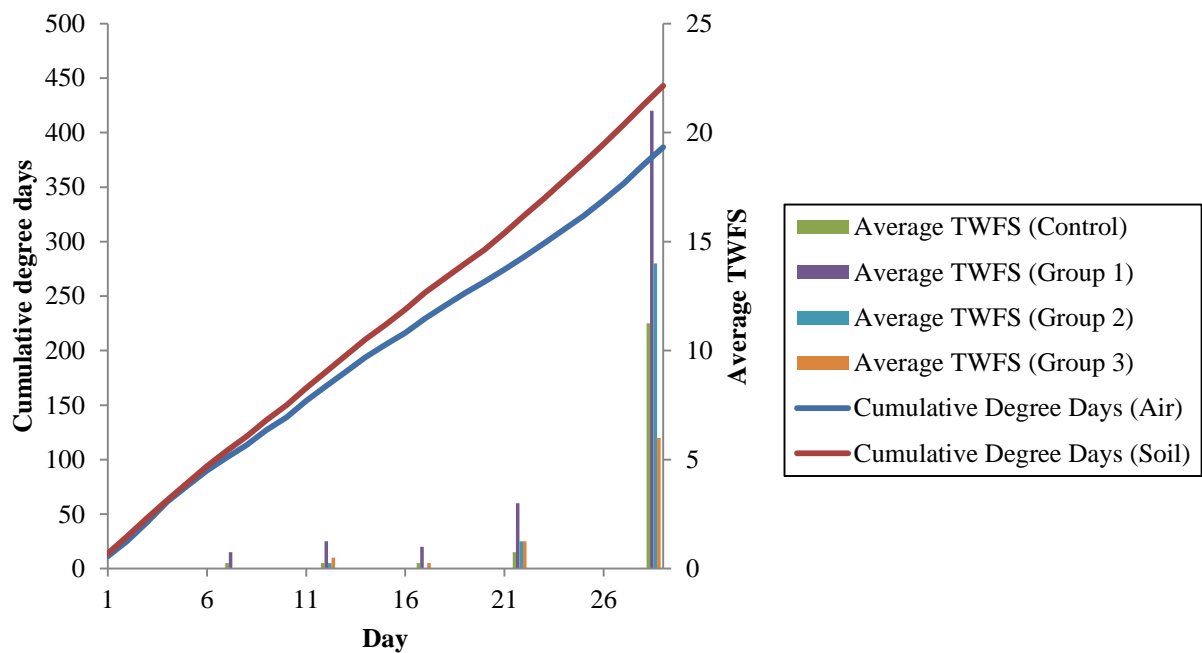
Figure 7.8: Cumulative air and soil degree days and average TWFS for Trials 1 to 4. Soil temperature was obtained in each pasture-based pen with a single data-logger buried 50 mm below the soil surface. Rainfall and air temperature data were drawn from the BOM weather station located at Camden Airport (Station ID: 068192).

7.4 Discussion

The aim of this study was to develop a low-intervention, pasture-based experimental model for the transmission of footrot to sheep. The bandaging challenge method devised by Egerton et al. (1969) was evaluated in both an indoor pen and a pasture-based pen in Trial 1. *D. nodosus* was transferred to fewer sheep in the indoor pens than in pasture-based pens using this method. This suggested that one or more environmental factors that were unique to the pasture-based system enhanced the rate at which the disease progressed. The alternative challenge methods evaluated in Trial 1 (swab inoculation and pasture inoculation) were both effective. As such, bandaging the foot was deemed unnecessary to transfer *D. nodosus* to the feet of sheep, and only the alternative challenge methods were evaluated in trials two to four, with the addition of the transfer pipette method. The alternative challenge methods evaluated in Trials 1 to 4 all require less intervention than the bandaging method, do not cause any additional mechanical damage to the foot that might alter the way in which the disease progresses, and more closely resemble the way in which the *D. nodosus* is naturally transferred. The fourth alternative method that was evaluated (direct inoculation of the pasture) most closely resembles this process; conceptually, as the sheep walk through the paddock, the pasture passes between the digits and transfers the bacterium onto the interdigital skin.

The seasonal nature of footrot outbreaks has been apparent since the disease was first described (Beveridge, 1941; Youatt, 1837); outbreaks occur primarily in spring when mild air temperatures and consistent rainfall support disease expression (Graham and Egerton, 1968). The second trial was conducted in late autumn with the aim of evaluating the repeatability of the alternative challenge methods under less favourable environmental conditions. The trial ran for 31 days but was ultimately deemed invalid as only two sheep developed progressive lesions during this time. The failure to transfer *D. nodosus* to a sufficient number of sheep during this trial is perplexing; although the trial was conducted in late autumn, which is typically less favourable for the expression of footrot than spring (Graham and Egerton, 1968), average daily temperatures were suitable for disease expression (Figure 5). The Camden area also received moderate but consistent rainfall across the first week of the trial period. It is possible that the some of the *D. nodosus* cultures that were used to challenge the sheep were non-viable, which might explain why lesions were observed on only a few feet. It is also possible that *D. nodosus* was transferred to sheep other than those that developed lesions, but that an insufficient number of organisms were present to trigger the disease

process. Detection of the organism on more feet by PCR than by culture would support this conclusion.

The third trial was also deemed invalid, despite sufficient irrigation and suitable average daily air temperatures (>10°C). However, maximum daily air temperatures recorded on Days 1 and 2 of the trial, respectively, were 37.5°C and 40.6°C, which may have reduced the survival of the bacterium on the pasture. The failure of the third trial highlights the vulnerability of pasture-based experimental models to fluctuating environmental conditions. Although a minimum average daily temperature of 10°C can be achieved by conducting trials at the appropriate time of year, and minimum levels of moisture can be achieved with irrigation, pasture-based systems are susceptible to high temperatures and rainfall, particularly during the first few days post-challenge when an infection has not yet been established. This can be mitigated to some extent by commencing the trial during a period where extreme temperatures and rainfall are not predicted.

The fourth trial was deemed valid, but progressive lesions were not observed until Day 22. The average TWFS (21.0) recorded for Group 1, which was challenged with the swab method, was significantly higher at Day 22 than the average TWFS recorded for Groups 2 and 3 ($P = 0.013$), which were challenged with the transfer pipette and pasture inoculation methods, respectively. Progressive lesions were observed in the Control group at Day 22, which suggests that a biosecurity failure occurred at some prior point in the trial. *D. nodosus* was not detected by culture or PCR in this group at Day 7 of the trial, so the failure probably occurred during one of the examinations, most likely due to the presence of soil on one of the investigators boots. This was confirmed by *fimA* PCR testing of the isolates obtained from this group, which belonged to serogroup A. This does not affect the validity of the trial, however, as culture and PCR results indicated that the sheep did not have a pre-existing *D. nodosus* infection at the commencement of the trial. In fact, this reinforces the ease with which *D. nodosus* can be transmitted by means of contaminated pasture.

The range of environmental conditions under which footrot is capable of expressing are yet to be comprehensively described, but previous reports indicate that outbreaks largely occur following periods in which rainfall was high and evenly distributed (Glynn, 1993; Graham and Egerton, 1968), and moisture has long been recognised as an essential requirement for the disease to be transmitted and expressed (Beveridge, 1941; Marsh and Tunnicliff, 1934; Youatt, 1837). As such, it was important to maintain a minimum level of moisture,

particularly when air temperatures were >25°C and evaporation was likely to be high (no data are available for daily evaporation from the BOM weather station at Camden Airport). Across the four trials, I evaluated two different irrigation methods. These were chosen to utilise available irrigation infrastructure and thereby minimise labour requirements. Simple sprinkler systems were set up in each pen for Trial 1, with the exception of Group 4, which were housed in an indoor pen, but this method was discontinued at Day 8 as it was difficult to avoid wetting the wool of the sheep despite there being sufficient space around the edges of the pen for them to avoid being wetted directly. Thus for the remainder of Trial 1, and each subsequent trial, each pen was irrigated using a hose fitted with a simple spray nozzle. This method is labour intensive, but is able to keep the pasture and soil moist without wetting the sheep and without the pasture becoming sodden. The use of in-ground sprinklers or irrigation tubing may be worthwhile investigating in future studies.

Average daily soil and air temperatures were recorded throughout each trial. These were then used to calculate daily air and soil degree days, a metric that is used describe the amount of thermal energy an organism receives from its environment (Wilson and Barnett, 1983). This metric is typically used to predict the response of ectothermic vertebrates to drugs or pathogens (Wilson and Barnett, 1983). To my knowledge, this is the first time the concept has been applied to the study of ovine footrot. The use of degree days as a potential predictor of disease expression was considered. Unfortunately, the study was not designed for the explicit purpose of examining the relationship between degree days and disease expression, therefore statistical analysis of this relationship was not possible with the available data. However, some trends are apparent in Figure 7.8. Cumulative degree days increased steadily over the course of each trial, with the exception of Trial 2, during which the air and soil temperature varied more between days. Cumulative air and soil degree days diverged as the trial progressed, which indicates that the feet of the sheep received greater thermal energy from the soil than the air during the course of each trial. Given that the feet of sheep are in continuous contact with the soil, this additional thermal energy might provide a more favourable environment for *D. nodosus*, and therefore support disease progression. Future investigation is necessary to establish whether or not there is a threshold, in terms of cumulative degree days, beyond which the disease begins to express.

The NHMRC *Australian code for the care and use of animals for scientific purposes* (NHMRC, 2013) highlights the need to refine experimental procedures to minimise pain, distress, and other adverse events. The pasture-based experimental model and challenge

systems developed in this chapter enhance animal welfare standards relative to the indoor system evaluated in Trial 1, enabling them to undertake normal behaviours such as grazing to some extent. The pasture-based system also minimises exposure to ammonia, which can be problematic in indoor systems. Direct inoculation of the pasture was an effective method for transmitting *D. nodosus* to experimental animals. In contrast to the other methods evaluated in this chapter, this method requires very little handling of the experimental animals.

7.5 Conclusions

This chapter describes the development of a low-intervention, pasture-based model of ovine footrot. A pasture-based model was shown to be suitable for transmission and expression of the disease but vulnerable to extreme weather conditions, particularly high air temperatures, as two out of four trials were invalid. Consequently, the repeatability of the model may be limited. Three challenge methods were shown to be effective: applying an inoculum of *D. nodosus* directly to the interdigital skin with a swab or transfer pipette, or indirectly by applying a liquid *D. nodosus* culture to the pasture. These methods require less intervention than earlier methods requiring the application of bandages to the feet, and thus improve animal welfare.

Chapter 8

General discussion

8.1 Introduction

Ovine footrot is a complex, multifactorial disease; the characteristic clinical manifestations of the disease are the result of synergistic interactions between the essential causative agent, *D. nodosus*, and the bacterial community of the foot (Egerton et al., 1969; Roberts and Egerton, 1969). The severity of these manifestations is largely contingent upon the virulence of the infecting *D. nodosus* strain(s) (Stewart et al., 1984; Stewart et al., 1986);however, the extent to which the disease progresses is governed by host and environmental factors (Beveridge, 1941), with moisture and mild air temperatures required for the disease to fully express (Graham and Egerton, 1968). As such, infection with a virulent strain of *D. nodosus* does not necessarily result in virulent footrot, and clinical severity is not always a reliable indicator of virulence (McPherson et al., 2017; Stewart et al., 1986)

This has necessitated the development of phenotypic and genotypic virulence tests to differentiate virulent and benign strains of *D. nodosus* (Cheetham et al., 2006; Every, 1982; Frosth et al., 2015; Palmer, 1993; Stäuble et al., 2014a; Stewart, 1979). New virulence tests are published periodically; however, a definitive virulence test has not yet been developed. This is due, at least in part, to our fragmented understanding of the genotypic and phenotypic factors that govern the virulence of *D. nodosus*.

8.2 Evaluation of virulence genotypic and phenotypic tests

The most recent published genotypic virulence tests, which were developed in Europe, differentiate putative virulent and benign strains according to the presence or absence of the *aprV2* gene, which encodes acidic protease isoenzyme 2 (AprV2) (Frosth et al., 2015; Stäuble et al., 2014a). Research conducted in Australia indicated that the expression of AprV2 is the defining phenotypic characteristic of virulent strains of *D. nodosus* (Kennan et al., 2010). Benign strains of *D. nodosus* express an analogous protease, AprB2, encoded by the gene *aprB2* (Kennan et al., 2010; Riffkin et al., 1995). Both tests were reported to have high diagnostic sensitivity and specificity (Kennan et al., 2010; Riffkin et al., 1995); however, validation data were limited and based on samples from European sheep only.

The aim of the study presented in Chapter 3 was to compare the analytical performance of the two qPCR tests, before subjecting one to the initial steps of the validation pathway proposed

by the OIE (OIE, 2016), with the aim of establishing the diagnostic performance of the tests in an Australian context. Specimens were collected from 40 sheep flocks in south-eastern Australia, 24 of which presented with clinically virulent footrot, and 16 of which presented with clinically benign footrot. The qPCR test published by Frosth et al. (2015) was compared with clinical diagnosis and the elastase test (Palmer, 1993) at both the flock and isolate levels

The key finding of this study was that the relationship between the *aprV2* gene and virulence is not as unequivocal as previous studies would suggest; a total of 363 *D. nodosus* isolates were obtained from 11/16 sheep flocks with clinically benign footrot, all of which had delayed or reduced elastase activity (weak elastase activity observed at 16 to 28 days, or not at all), relative to the virulent control strain (marked elastase activity observed at 8-12 days). The diagnostic specificity of the qPCR test was therefore deemed to be poor in an Australian context.

Interestingly, there was strong agreement between the elastase test and clinical diagnosis at both the flock and isolate level, which suggests that the expression of the AprV2 protease, with forms the basis of the elastase test (Kennan et al., 2010) is in indeed a good indicator of virulence. This discrepancy between genotype and phenotype suggests that our understanding of the genotypic factors that govern virulence is still incomplete. Indeed, the expression of an active protease is a complex phenotypic trait involving a network of genes and their products (Han et al., 2008; Han et al., 2012; Kennan et al., 2001; Kennan et al., 2010), and a single mutation in any one of these genes that results in a loss or change of function of the translated protein could potentially result in an attenuation of virulence. Whole-genome analysis was beyond the scope of the study presented in Chapter 3, but the presence of a non-synonymous SNP in one or more genes related to protease expression may explain the observed discrepancy between genotype and phenotype. This should be investigated in future studies, and might help to inform the development of new diagnostic tests.

The qPCR test does have some practical applications in an Australian context, despite having poor specificity. It is apparent from the study presented in Chapter 3 that there is good agreement between the presence of the benign protease allele (*aprB2*) and clinical diagnosis. As such, the qPCR test might be useful as a screening tool to distinguish putative virulent (*aprV2*-posititive) and benign (*aprB2*-positive) strains of *D. nodosus*. However, the qPCR test should not be used as the sole basis for diagnosis in Australia given the risk of a false-positive diagnosis.

8.3 Direct serogrouping of *D. nodosus*

Strains of *D. nodosus* are divisible into ten immunologically distinct serogroups (A to I, and M), by means of the slide agglutination test (Claxton et al., 1983) or cPCR amplification of variable regions of the *fimA* gene (Dhungyel et al., 2002). Each test is typically applied to pure cultures of the infecting *D. nodosus* strain(s), which requires the bacterium to be cultured from specimens of lesion materal. This is a slow, difficult task that many laboratories are not equipped to undertake. Further, neither test is capable of detecting all serogroups in a flock unless there is an extensive sampling strategy (Hill et al., 2010), which is both impractical and cost prohibitive

The aim of the study presented in Chapter 4 was to develop a direct (culture-independent) serogrouping procedure based on the multiplex *fimA* cPCRs published by Dhungyel et al. (2002). A direct testing procedure was optimised for a 16S rRNA cPCR (La Fontaine et al., 1993). Swabs collected into LB or mSTM, the latter of which was also used for culture of *D. nodosus*, were evaluated. Direct testing of both LB swabs and mSTM swabs was more sensitive for the detection of *D. nodosus* than culture. The optimised procedure was then applied to the *fimA* cPCR (Dhungyel et al., 2002). Direct *fimA* PCR of LB and mSTM swabs was compared to testing of pure cultures with the slide agglutination test and the *fimA* cPCR.

The greatest number of serogroups were detected by direct *fimA* cPCR testing of swabs collected into LB, with up to six serogroups detected in a single flock and up to four serogroups detected on a single foot. In most cases there was good agreement between the culture-based test and direct cPCR testing of mSTM swabs, which suggests that direct testing of mSTM swabs following culture might be satisfactory in circumstances where the collection of two swabs is impractical. The ability to collect one swab for both culture and direct PCR testing would obviously be advantageous for veterinarians and animal health officers given that microbiological culture of *D. nodosus* remains a prerequisite to virulence testing.

The direct testing procedure developed in this study provides diagnosticians with a rapid, sensitive means of detecting and serogrouping *D. nodosus*. Indeed, the procedure has since been applied in our laboratory to diagnostic specimens received from veterinarians and animal health officers in south-eastern Australia. In some cases only one or two serogroups have been detected in a flock by direct PCR testing, which has enabled the respective

producers to forego the cost of microbiological testing and proceed directly to the administration of serogroup-specific mono- or bivalent vaccines.

Direct serogrouping might also provide an early indication of the number of serogroups that are present in a flock and might help to inform preliminary management decisions, such as the decision to embark on a vaccination programme, while culture results are pending. The development of a real-time PCR serogrouping test is a logical next step, and may provide even greater sensitivity when coupled with the direct testing procedure developed in this study; however, the development of a real-time PCR assay was beyond the scope of this study.

6.4 Development of a serogroup M cPCR

Despite reports that serogroup M is prevalent in Australia, N.Z., Nepal, Norway, and the U.K. (Chetwin et al., 1991; Day et al., 1986; Dhungyel et al., 2015; Ghimire et al., 1998; Gilhuus et al., 2013), a serogroup M-specific PCR has not been published. The aim of the study presented in Chapter 5 was to develop a conventional serogroup M-specific PCR assay to accompany the serogroup A to I multiplex *fimA* PCRs published by Dhungyel et al. (2002). A serogroup M PCR was developed, producing a 94 bp amplicon.

A direct comparison of the serogroup M PCR with the slide agglutination test, which was previously the only means of detecting serogroup M, demonstrated that the PCR test had a greater analytical sensitivity; three *D. nodosus* isolates, which were previously assigned to serogroup M by means of the slide agglutination test, were shown to belong to serogroup B. To my knowledge, this is the first time that serological cross-reactivity between serogroup B and serogroup M antisera has been reported. This finding, coupled with those of previous studies which have reported serological cross-reactivity between serogroup M and serogroup F and I antisera, demonstrates the extent to which the serogroup M cPCR developed in this study could improve the specificity of the serogrouping procedure. This test might also help to determine the true prevalence of serogroup M in Australia.

The serogroup M PCR presented in Chapter 5 could be coupled with the direct testing procedure reported in Chapter 4. The serogroup M PCR had an analytical sensitivity of 250 *D. nodosus* cells, which approached that of the multiplex *fimA* PCRs published by Dhungyel et al. (2002), which were reported to be capable of detecting 50-100 *D. nodosus* cells.

However, foot swabs collected from the feet of sheep infected with serogroup M were not available for direct testing during this study.

6.5 Microbiome of the Merino foot

The Merino is particularly susceptible to footrot, but the basis of this susceptibility is contentious (Beveridge, 1941; Egerton and Morgan, 1972; Emery et al., 1984; Skerman et al., 1982). The interdigital skin of the foot of the Merino sheep appears to be less resistant to bacterial invasion than that of British sheep breeds (Emery et al., 1984). Therefore, it is possible that the foot of the Merino is colonised by a greater number or diversity of opportunistic pathogens following environmental predisposition and infection with *D. nodosus* than that of British breeds. The aim of the study presented in Chapter 6 was to characterise the bacterial communities on the feet of a group of healthy Merino sheep, and two groups of Merino sheep with footrot using next-generation sequencing and analysis of the bacterial 16S rRNA gene. To my knowledge, this is the first time that the bacterial community of the Merino foot has been characterised using these technologies.

Briefly, the results indicated that a qualitative shift in the bacterial community of the Merino foot is triggered by infection with *D. nodosus*. The communities of healthy Merino feet were dominated by Gram-positive, aerobic genera such as *Corynebacterium* and *Staphylococcus*. In contrast, the communities of footrot-affected feet were dominated by Gram-negative, anaerobic genera such as *Porphyromonas* and *Fusobacterium*. Fifteen bacterial genera were preferentially abundant on the feet of Merino sheep with footrot (see Table 6.2), only four of which were reported to be abundant on the feet of British breed sheep with footrot in the U.K. (Maboni et al., 2017). This could indicate breed-specific differences in the aetiology of ovine footrot, although geographic variation no doubt explains some of this variation. Surprisingly, the bacterial community of the footrot lesion did not differ between time points, irrespective of lesion severity, which indicated that the same bacterial genera were abundant in both mild and severe lesions. Further investigation is required to establish the significance of these genera to the disease process.

6.6 Pasture-based experimental model

Experimental models are used extensively in the study of ovine footrot footrot (Beveridge, 1941; Depiazzi and Richards, 1985; Egerton et al., 1969; Graham and Egerton, 1968; Han et al., 2008; Jelinek et al., 2000; Kennan et al., 2001; Kennan et al., 2010; Roberts and Egerton, 1969). They are preferable to natural systems as they enable investigators to manipulate

environmental parameters. The outcomes of the study presented in Chapter 3 indicate that challenge of penned sheep under experimental conditions is probably the only reliable means of determining the virulence of a *D. nodosus* isolate.

The experimental model developed by Egerton et al. (1969), in which sheep are maintained in indoor pens on wet foam rubber matting, appears to be the most widely-used model (Bhardwaj et al., 2014; Depiazzi and Richards, 1985; Depiazzi et al., 1991; Depiazzi et al., 1998; Egerton, 1974; Egerton and Merritt, 1973; Egerton and Roberts, 1971; Emery et al., 1984; Ghimire et al., 1999; Han et al., 2008; Hunt et al., 1994; Jelinek and Depiazzi, 2003; Jelinek et al., 2000; Kennan et al., 2001; Kennan et al., 2010; Marshall et al., 1991a; Skerman et al., 1982; Stewart et al., 1991). Sheep are challenged with *D. nodosus* by placing a HA culture between the digits and securing it with gauze bandages. This method is highly effective for inducing footrot lesions, as indicated by the aforementioned studies, however it is apparent that this method is not an accurate representation of the means by which the disease is naturally transmitted, nor the environment in which the disease occurs.

The aim of Chapter 7 was to develop a pasture-based experimental model. Sheep were maintained in pasture-based pens constructed from portable fencing panels, and challenged with one of five different methods. The bandaging method described by (Egerton et al., 1969) was evaluated in both an indoor system and a pasture-based system. The bandaging method was more successful in the pasture-based system than in the indoor system, based on the number of feet that developed lesions and the rate at which the lesions progressed. This outcome indicates that one or more environmental factors unique to the pasture-based system accelerated the disease process. Several factors could have contributed to this outcome, including exposure to soil microorganisms, and differences in temperature.

Three alternative challenge methods were evaluated in the pasture-based system, all of which were equally as effective as bandaging the foot, including the application of a liquid suspension of *D. nodosus* directly to the pasture with a plastic spray bottle. This method directly mimics the way in which *D. nodosus* naturally transmitted between sheep; conceptually, as the sheep walk through the pen, the pasture passes between the digits and applies the inoculum directly to the interdigital skin. This method requires minimal intervention, which also enhances animal welfare outcomes. This model and challenge method has since been used successfully to challenge donor sheep for use in a vaccine trial (MLA Project No. 2017/1210), and is currently being used for pathogenicity testing of *D.*

nodosus isolates obtained from a flock on Kangaroo Island, Australia, during the course of the study presented in Chapter 3.

8.7 Future directions and conclusions

The development of accurate laboratory virulence tests is hampered by our fragmented understanding of the genotypic and phenotypic factors that govern the virulence of *D. nodosus*. In light of the data presented in Chapter 3 of this thesis, it is apparent that virulence is probably multigenic and that the presence of a SNP in a single gene is insufficient to categorise an isolate as virulent or benign. The complex, multifactorial nature of virulence needs to be acknowledged moving forward if we are to develop accurate molecular diagnostic tests. Furthermore, investigators also need to acknowledge that there is spectrum of virulence and that *D. nodosus* strains with intermediate phenotypic characteristics are prevalent in Australia and probably elsewhere; and that virulent and intermediate strains probably share common virulence genes, including *aprV2*. Unfortunately, there are only small number of studies that directly address intermediate strains of *D. nodosus* and the majority of these were published prior to advent of sequencing technologies. Whole-genome sequencing of isolates with discrepant genotypes and phenotypes might help to explain the existence of strains with intermediate phenotypic traits and inform the development of more accurate virulence tests. The need for this work is apparent given the increased prevalence of intermediate footrot in Australia in recent years.

In Chapter 4, I developed a culture-independent procedure for the identification of *D. nodosus* serogroups based on direct cPCR testing of foot swabs. However, a transition to entirely culture-independent serogrouping is hampered by the potential for multiple serogroups and multiple virulence genotypes in a single sheep flock, as demonstrated in Chapters 3 and 4. At present, there is no way of linking serogroup and virulence in such circumstances without isolating and testing pure cultures of the infecting *D. nodosus* strains (Buller and Eamens, 2014). The development of novel diagnostic tools that are capable of simultaneous serogrouping and virulence testing should be pursued in future studies. It would also be advantegeous to evaluate the direct serogrouping procedure in the context of a vaccination programme.

The microbiome of the Merino foot was characterised in Chapter 6, resulting in the identification of 15 bacterial genera that were preferentially abundant in footrot lesions. Many of these are recognised as pathogens of significance to human and veterinary medicine.

Additional, targeted investigaton is necessary to define the role of bacteria in these genera in the disease, using both *in vitro* and *in vivo* methods. In particular, it would be worth investigating the role of *Porphyromonas*, given the role of this genus in the aetiopathogenesis of BDD and periodontal disease, both of which are characterised by a distinct bacterial dybiosis, and which share common aetiological agents with ovine footrot. The results of this study indicate potential breed-specific agents of disease, given that the composition of the bacterial communities in active lesions differed to those reported in previous studies in the U.K. that collected specimens from British sheep breeds. However, geographic variation would have contributed to this result. Further investigation of breed-specific variation with sheep maintained on the same property would address this concern.

The concept of a degree days was introduced in Chapter 7. This metric has not previously been used in the study of ovine footrot; however, given that expression ovine footrot is heavily dependent upon air temperatures, the use of this metric should be investigated as a predictive tool in future studies. In doing so, it might be possible to identify a threshold (in cumulative degree days) beyond which the disease begins to express. This could be used as a simple predictive tool to inform management decisions, for example in the form of a smart-phone application, drawing on temperature data from a local BOM weather station.

In summary, this thesis addresses aspects of the aetipathogenesis and diagnosis of ovine footrot. The results presented in Chapter 3 demonstrate the strengths and limitations of current laboratory virulence tests and demonstrated the importance of clinical diagnosis as a primary means of differentiating virulent and benign footrot. This chapter also highlights the need for a greater understanding of the factors that govern virulence. I have developed a direct serogrouping procedure that will enhance the specificity of the serogrouping procedure, and enhance the implementation of serogroup-specific vaccination. I have also developed a serogroup M-specific cPCR that provides diagnosticians with a rapid test for the detection of serogroup M. The PCR test has a greater specificity than the slide agglutination test, which was previously the only means of detecting serogroup M. The serogroup M cPCR has not yet been evaluated with the direct testing procedure developed in Chapter 4; this should be the focus of a future investigation. In Chapter 6, the bacterial community of the Merino foot was characterised using NGS and amplicon-based metagenomic techniques. To my knowledge, this is the first time the bacterial community of the Merino foot has been characterised using these technologies. In doing so, 15 bacterial genera were shown to be abundant on the feet of Merino sheep with footrot. This finding opens up several new avenues of investigation and is

another step towards developing a more thorough understanding of the complex aetiology of ovine footrot. Finally, I developed a pasture-based experimental model that can be used to evaluate and validate diagnostic tests, study the aetiopathogenesis of the disease, and evaluate the virulence of *D. nodosus* strains.

Table A.1: Results of the GLMM for the detection of *D. nodosus* by microbiological culture. Lesion score, lesion type, and lesion contamination were accounted for in the fixed model, and farm of origin was accounted for in the random model.

Variable	P-value	Std Error	Category	Parameter Estimate	Odds Ratio*	95% Confidence Intervals[#]	
						Lower	Upper
Lesion score	0.021	0.58	Score 1	0	…	…	…
			Score 2	0.55	1.72	0.55	5.40
			Score 3	1.99	7.32	2.34	22.93
			Score 4	0.64	1.90	0.61	5.96
Lesion type	0.027	0.61	Active	0	…	…	…
			Inactive	-1.35	0.26	0.08	0.85
Lesion contamination	<0.001	0.49	High	0	...	...	…
			Moderate	1.61	4.99	1.90	13.07
			Low	2.42	11.21	4.28	29.39
Random (Farm)	…	0.71	…	1.04	…	…	…

*Odds of a category testing positive for *D. nodosus* by culture compared with the reference category

[#]Where the 95% confidence intervals contain the value 1.00, there is no significant difference between the respective category and the reference category

Table A.2: Results of the GLMM for detection of the *D. nodosus* 16S rRNA gene using DNA prepared from swabs collected into mSTM. Lesion score, lesion type, and lesion contamination were accounted for in the fixed model and farm of origin was accounted for in the random model.

Variable	P-value	Std Error	Category	Parameter Estimate	Odds Ratio*	95% Confidence Intervals[#]	
						Lower	Upper
Lesion score	<0.001	0.86	Score 1	0	…	…	…
			Score 2	3.69	40.13	7.47	215.57
			Score 3	3.23	25.25	4.70	135.68
			Score 4	2.55	12.74	2.37	68.46
Lesion type	<0.001	0.56	Active	0	…	…	…
			Inactive	-2.41	0.09	0.03	0.27
Lesion contamination	<0.001	0.57	High	0	…	…	…
			Moderate	2.66	14.32	4.72	43.47
			Low	3.23	25.15	8.29	76.34
Random (Farm)	…	3.90	…	5.96	…	…	…

*Odds of a category testing positive for *D. nodosus* by PCR testing of DNA prepared from swabs collected into mSTM compared with the reference category

[#]Where the 95% confidence intervals contain the value 1.00, there is no significant difference between the respective category and the reference category

Table A.3: Results of the GLMM for detection of *D. nodosus* 16S rRNA gene using DNA prepared from swabs collected into LB. Lesion score, lesion type, and lesion contamination were accounted for in the fixed model, and farm of origin was accounted for in the random model. Categories in which there were fewer than five observations were collapsed.

Variable	P-value	Std Error	Category	Parameter Estimate	Odds Ratio*	95% Confidence Intervals[#]	
						Lower	Upper
Lesion score	0.422	0.57	Score 1/2	0	…	…	…
			Score 3/4	0.57	…	…	…
Lesion type	0.008	0.87	Active	0	…	…	…
			Inactive	-2.28	0.10	0.02	0.56
Lesion contamination	0.007	0.79	High	0	…	…	…
			Low/Moderate	2.14	8.50	1.81	39.92
Random (Farm)	…	1.57	…	2.00	…	…	…

*Odds of a category testing positive for *D. nodosus* by PCR testing of DNA prepared from swabs collected into LB compared with the reference category

[#]Where the 95% confidence intervals contain the value 1.00, there is no significant difference between the respective category and the reference category

Table B.1: Culture results, including serogrouping and virulence testing.

Month	Group	Tag No.	Foot	Score	Sample No.	Isolate No.	Serogroup Slide agglutination	*fimA* PCR	Elastase	Gelatin gel
Mar	Control	130	RF	0	16/063/1	NA	Not tested	Not tested	Not tested	Not tested
	1	317	LF	1	16/063/2	16/063/2.1	D	A, E	28	Non-benign
	1	318	LF	1	16/063/3	16/063/3.1	A	A	12	Non-benign
						16/063/3.2	A	A	20	Non-benign
	1	320	LF	2	16/063/4	16/063/4.1	E	E	Contaminated	Non-benign
						16/063/4.2	E	A, E	Negative	Non-benign
	1	325	RF	2	16/063/5	16/063/5.1	B, E	E	Negative	Non-benign
						16/063/5.2	E	E	16	Non-benign
	1	326	RH	1	16/063/6	16/063/6.1	E	E	Contaminated	Non-benign
						16/063/6.2	E	E	Contaminated	Not tested
	2	382	RF	2	16/063/7	16/063/7.1	E	E	24	Non-benign
						16/063/7.2	E	E	Contaminated	Not tested
	2	387	LH	2	16/063/8	16/063/8.1	D	E	24	Not tested
						16/063/8.2	A	A	Contaminated	Non-benign
	2	475	RH	2	16/063/9	16/063/9.1	E	E	Contaminated	Non-benign
						16/063/9.2	E	A, E	Contaminated	Non-benign
	2	482	RH	1	16/063/10	16/063/10.1	B	A, E	Contaminated	Non-benign
						16/063/10.2	E	A, E	Contaminated	Not tested
July	Control	287	RF	0	16/156/2	NA	Not tested	Not tested	Not tested	Not tested
	Control	130			16/156/12	NA	Not tested	Not tested	Not tested	Not tested
	1	326	RH	2	16/156/4	16/156/4.1	A	Not tested	Not tested	Non-benign
						16/156/4.2	A	Not tested	20	Non-benign
	1	323	RF	0	16/156/5	16/156/5.1	Not tested	Not tested	Not tested	Not tested
	1	323	LH	2	16/156/6	16/156/6.1	A	Not tested	24	Non-benign

Month	Group	Tag No.	Foot	Score	Sample No.	Isolate No.	Serogroup		Elastase	Gelatin gel
							Slide agglutination	*fimA* PCR		
						16/156/6.2	A	Not tested	28	Non-benign
	1	325	RF	0	16/156/7	16/156/7.1	Not tested	Not tested	Not tested	Not tested
	1	318	RH	2	16/156/8	16/156/8.1	A	Not tested	28	Non-benign
						16/156/8.2	A	Not tested	28	Non-benign
	2	475	RH	1	16/156/1	16/156/1.1	E	Not tested	24	Non-benign
						16/156/1.2	D	Not tested	12	Non-benign
	2	388	LF	1	16/156/9	16/156/9.1	Not tested	Not tested	Not tested	Not tested
						16/156/9.2	Not tested	Not tested	Not tested	Not tested
	2	290	LH	1	16/156/10	16/156/10.1	Not tested	Not tested	Not tested	Not tested
	2	386	RH	1	16/156/11	16/156/11.1	A	Not tested	Not tested	Non-benign
						16/156/11.2	A	Not tested	Not tested	Non-benign
Aug	Control	320	RH	0	16/164/1	NA	Not tested	Not tested	Not tested	Not tested
	1	325	RF	0	16/164/2	16/164/2.1	A	Not tested	12	Non-benign
						16/164/2.2	A	Not tested	16	Non-benign
	1	326	LH	0	16/164/3	16/164/3.1	A	Not tested	20	Non-benign
						16/164/3.2	A	Not tested	20	Non-benign
	1	326	RH	2	16/164/4	16/164/4.1	A	Not tested	20	Non-benign
						16/164/4.2	D	Not tested	Not tested	Non-benign
	2	387	LF	2	16/164/5	16/164/5.1	E	Not tested	Not tested	Non-benign
						16/164/5.2	E	Not tested	Not tested	Non-benign
	2	475	RF	2	16/164/6	16/164/6.1	E	Not tested	Not tested	Non-benign
						16/164/6.2	E	Not tested	Not tested	Non-benign
Sept	Control	86	LH	0	16/175/1	NA	Not tested	Not tested	Not tested	Not tested
	1	319	RH	3	16/175/2	16/175/2.1	A	Not tested	Contaminated	Non-benign
						16/175/2.2	A	Not tested	24	Non-benign
	1	318	RF	3	16/175/3	16/175/3.1	A	Not tested	24	Non-benign

Month	Group	Tag No.	Foot	Score	Sample No.	Isolate No.	Serogroup		Elastase	Gelatin gel
							Slide agglutination	*fimA* PCR		
						16/175/3.2	A	Not tested	16	Non-benign
	1	318	RH	2	16/175/4	16/175/4.1	A	Not tested	12	Non-benign
						16/175/4.2	A	Not tested	20	Non-benign
	1	323	RF	0	16/175/5	16/175/5.1	E	Not tested	Not tested	Non-benign
						16/175/5.2	Not tested	Not tested	Contaminated	Non-benign
	1	323	LH	3	16/175/6	16/175/6.1	Not tested	Not tested	Not tested	Non-benign
						16/175/6.2	D	Not tested	28	Non-benign
	1	326	LH	0	16/175/7	16/175/7.1	D	Not tested	20	Benign
						16/175/7.2	Not tested	Not tested	Not tested	Not tested
	2	90	RH	4	16/175/8	16/175/8.1	E	Not tested	Not tested	Not tested
						16/175/8.2	E	Not tested	Not tested	Not tested
	2	326	RF	0	16/175/9	16/175/9.1	Not tested	Not tested	Not tested	Not tested
						16/175/9.2	Not tested	Not tested	Not tested	Not tested
	2	482	RH	0	16/175/10	16/175/10.1	Not tested	Not tested	Not tested	Not tested
						16/175/10.2	Not tested	Not tested	Not tested	Not tested
Oct	Control	130	RF	0	16/182/1	NA	Not tested	Not tested	Not tested	Not tested
	1	322	RH	1	16/182/2	16/182/2.1	A	Not tested	16	Non-benign
						16/182/2.2	A	Not tested	8	Non-benign
	1	325	RF	0	16/182/3	16/182/3.1	A	Not tested	8	Non-benign
						16/182/3.2	A	Not tested	12	Non-benign
	1	319	LH	2	16/182/4	16/182/4.1	D	Not tested	Not tested	Non-benign
	1	319	RH	2	16/182/5	16/182/5.1	D	Not tested	Contaminated	Non-benign
						16/182/5.2	Not tested	Not tested	Contaminated	Non-benign
	1	326	LH	2	16/182/6	16/182/6.1	Not tested	Not tested	Not tested	Not tested
						16/182/6.2	Not tested	Not tested	Not tested	Not tested
Nov	Control	86	RH	0	16/196/1	NA	Not tested	Not tested	Not tested	Not tested

Month	Group	Tag No.	Foot	Score	Sample No.	Isolate No.	Serogroup		Elastase	Gelatin gel
							Slide agglutination	*fimA* PCR		
	1	322	RH	1	16/196/3	16/196/3.1	A	Not tested	10	Not tested
						16/196/3.2	A	Not tested	10	Non-benign
	1	323	RF	0	16/196/4	16/196/4.1	D	Not tested	16	Non-benign
						16/196/4.2	D	Not tested	16	Non-benign
	1	323	LH	2	16/196/5	16/196/5.1	E	Not tested	16	Non-benign
						16/196/5.2	Not tested	Not tested	Not tested	Not tested
	1	319	LH	2	16/196/6	16/196/6.1	D	Not tested	Contaminated	Not tested
						16/196/6.2	D	Not tested	Contaminated	Not tested

Table B.2: Sample metadata.

Swab No.	Tag No.	Foot	Month	Group	Forward primer (319F) (5' to 3')	Reverse primer (806R) (5' to 3')	No. reads*
1	130	LH	Mar	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	106,668
2	287	RF	Mar	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	106,585
3	130	LH	Apr	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	40,307
4	287	RF	Apr	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	100,512
5	130	LH	May	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	84,339
6	287	RF	May	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	67,574
7	130	LH	June	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	76,306
8	287	RF	June	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	86,033
9	130	LH	July	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	52,536
10	287	RF	July	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	70,674
11	130	LH	Aug	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	77,482
12	287	RF	Aug	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	81,313
13	130	LH	Sept	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	64,084
14	287	RF	Sept	Control	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	132,244
15	317	RH	March	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	116,352
16	318	RF	March	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	74,110
17	318	RH	March	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	89,956
18	319	RH	March	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	108,272
19	317	RH	April	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	71,072

Swab No.	Tag No.	Foot	Month	Group	Forward primer (319F) (5' to 3')	Reverse primer (806R) (5' to 3')	No. reads*
20	318	RF	April	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	76,230
21	318	RH	April	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	86,400
22	319	RH	April	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	124,904
23	317	RH	May	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	87,756
24	318	RF	May	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	43,322
25	318	RH	May	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	72,179
26	319	RH	May	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	70,908
27	317	RH	June	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	93,322
28	318	RF	June	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	70,320
29	318	RH	June	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	90,874
30	319	RH	June	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	82,239
31	317	RH	July	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	91,983
32	318	RF	July	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	99,207
33	318	RH	July	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	95,929
34	319	RH	July	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	90,985
35	317	RH	August	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	74,891
36	318	RF	August	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	64,174
37	318	RH	August	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	81,750
38	319	RH	August	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	66,787
39	317	RH	September	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	75,819

Swab No.	Tag No.	Foot	Month	Group	Forward primer (319F) (5' to 3')	Reverse primer (806R) (5' to 3')	No. reads*
40	318	RF	September	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	71,873
41	318	RH	September	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	65,220
42	319	RH	September	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	96,570
43	317	RH	October	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	79,057
44	318	RF	October	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	110,725
45	318	RH	October	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	73,339
46	319	RH	October	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	110,703
47	317	RH	November	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	111,276
48	318	RF	November	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	96,080
49	318	RH	November	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	89,290
50	319	RH	November	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	70,650
51	317	RH	December	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	124,294
52	318	RF	December	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	83,024
53	318	RH	December	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	81,968
54	319	RH	December	1	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	102,710
55	389	RF	Mar	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	90,833
56	389	RH	Mar	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	120,450
57	390	RH	Mar	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	127,157
58	475	RH	Mar	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	118,944
59	389	RF	Apr	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	95,617

Swab No.	Tag No.	Foot	Month	Group	Forward primer (319F) (5' to 3')	Reverse primer (806R) (5' to 3')	No. reads*
60	389	RH	Apr	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	82,086
61	390	RH	Apr	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	69,980
62	475	RH	Apr	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	86,146
63	389	RF	May	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	83,513
64	389	RH	May	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	89,163
65	390	RH	May	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	82,366
66	475	RH	May	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	106,286
67	389	RF	June	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	87,926
68	389	RH	June	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	108,906
69	390	RH	June	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	74,488
70	475	RH	June	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	72,878
71	389	RF	July	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	111,048
72	389	RH	July	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	105,665
73	390	RH	July	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	87,445
74	475	RH	July	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	65,325
75	389	RF	Aug	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	74,809
76	389	RH	Aug	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	86,901
77	390	RH	Aug	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	98,266
78	475	RH	Aug	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	92,382
79	389	RF	Sept	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	71,641

Swab No.	Tag No.	Foot	Month	Group	Forward primer (319F) (5' to 3')	Reverse primer (806R) (5' to 3')	No. reads*
80	389	RH	Sept	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	66,053
81	390	RH	Sept	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	88,354
82	475	RH	Sept	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	95,844
83	389	RF	October	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	124,014
84	389	RH	October	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	103,424
85	390	RH	October	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	94,970
86	475	RH	October	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	76,276
87	389	RF	November	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	67,414
88	389	RH	November	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	65,138
89	390	RH	November	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	64,683
90	475	RH	November	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	71,939
91	389	RF	December	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	88,027
92	389	RH	December	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	77,883
93	390	RH	December	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	87,171
94	475	RH	December	2	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT	105,385
						Total	8,179,973

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