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Dichelobacter nodosus and Fusobacterium necrophorum in ruminants with lameness and footrot

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at
Lincoln University
by
G. N. Bennett

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Declaration of parts of thesis that have been accepted for publication

Chapter 2


Chapter 5


Chapter 6


Chapters 5 and 6

Footrot is a destructive hoof disease found predominantly in sheep and goats that is caused by *Dichelobacter nodosus* (*D. nodosus*), a gram-negative, anaerobic bacterium. It has been shown that *D. nodosus* requires a second, gram-negative, anaerobic bacterium, *Fusobacterium necrophorum* (*F. necrophorum*), to initiate footrot. However, once footrot is established, the role, if any, that *F. necrophorum* plays is unknown. In this thesis, the roles *D. nodosus* and *F. necrophorum* play in footrot were investigated.

To facilitate the study of *F. necrophorum* a new diagnostic Polymerase Chain Reaction (PCR) specific for *F. necrophorum* was developed and tested. This method was used in combination with an existing *D. nodosus*-specific PCR to study the prevalence of *D. nodosus* and *F. necrophorum* in sheep and goats with footrot in the field. This found *D. nodosus* and *F. necrophorum* were associated with under-running footrot and tended to be detected together. This supported the contention that *F. necrophorum* acts cooperatively with *D. nodosus* to cause under-running footrot in sheep and goats as well as that ovine and caprine footrot have similar bacteriologies.

To ascertain the possible roles *D. nodosus* and *F. necrophorum* play during footrot development, two footrot trials were conducted. These trials ran disease-free sheep and
sheep with footrot together in an experimental group as well as a control group of disease free sheep. Data were collected describing hoof pathologies and bacterial prevalence which was assessed using a combination of case study and statistical analyses.

The case studies suggested that *F. necrophorum* may be involved in the most rapidly developing and destructive under-running pathologies, both *D. nodosus* and *F. necrophorum* persist together in old cryptic lesions and that both *D. nodosus* and *F. necrophorum* can be found in sheep with no clinical signs of footrot. Furthermore, it was observed that before under-running occurred, only *F. necrophorum* could be detected and *D. nodosus* only became detectable once under-running began. These case studies not only suggested that *F. necrophorum* is involved with *D. nodosus* in initiating footrot, but also supports the contention that it is involved in under-running lesions and persists in cryptic lesions with *D. nodosus*. The observation that *D. nodosus* was also detected in sheep with no signs of disease suggested *D. nodosus* was either transmitted to, or able to persist in these animals, without causing disease.

Statistical analyses of the two footrot trials revealed that detection of *F. necrophorum* on the skin-horn junction and variance in the detection of *D. nodosus* were strongly correlated with disease. This suggested that *F. necrophorum* is important in disease and implied *D. nodosus* numbers “wax and wane” during disease. In turn, this suggests *D. nodosus* undergoes a "boom and bust" lifecycle during disease and in combination with the strong correlations between variables; it highlights the complex multi-factorial nature of footrot.
An investigation was undertaken to identify if *D. nodosus* could be persisting in the gastro-intestinal tract of sheep. This site was studied since it had the potential to act as a subclinical reservoir of *D. nodosus* as an anaerobic habitat in close proximity to the hoof. PCR was used to test if *D. nodosus* was detectable in the mouth, rumen, duodenum, caecum or colon of 25 culled sheep; or shed via faeces or the mouth of 36 monitored sheep. *D. nodosus* was not detected in the mouth, faeces, or gastro-intestinal tract of these sheep.

*F. necrophorum* carries a leukotoxin (*lktA*) gene that has previously only been described in *F. equinum* and *F. necrophorum*. Here, four variants (A, B, C and D) of the *lktA* gene of *F. necrophorum* were identified on lame cattle and cattle, sheep and goats with footrot. After sequence comparison, it was found that variant A of the *lktA* matched the *lktA* of the type strain of *F. necrophorum* sub sp. *necrophorum* (NCTC no.10575) while variants B, C and D have not been described previously. Of the four *lktA* variants observed, variants A and C were found most often. Furthermore, variant A was predominantly found in lame cattle (87.7% of cattle) while variant C was predominantly found in footrot-infected sheep and goats (83.3% of sheep and goats).

During the development of the *F. necrophorum* specific *lktA* PCR, a novel variant of the *lktA* gene was identified in *F. equinum* and, subsequently, detected in lesion material collected from cattle and sheep. This novel *lktA* sequence was different from other *F. necrophorum* type strains (67.8-68.1 % homologous). Moreover, blocks of sequence conservation were observed suggesting the *lktA* gene may be structurally conserved across species.
The work described here suggests that *F. necrophorum* may play an important role in ovine footrot, being found in under-running, cryptic and developing lesions. It was also shown that a distinctive variant of *F. necrophorum* may be present in footrot lesions and if the genetic variation in this portion of *lktA* gene is representatives of variation in the genome, it implies these variants represent an un-described species or sub-species of *Fusobacteria*.

While ovine footrot has been well investigated with regard to *D. nodosus* and its virulence, it is clear that the study of *F. necrophorum* has been neglected. The work presented here suggests that *F. necrophorum* plays an important role in ovine footrot and it should be studied further.

**Key words:** Footrot, lameness, *Dichelobacter nodosus, Fusobacterium necrophorum, Fusobacterium equinum*, leukotoxin, sheep, goats, cattle.
Publications arising from this thesis


**Sequences submitted to NCBI GenBank**

*LktA* sequences from *F. necrophorum*: FJ230830, FJ230831, and FJ230832

*LktA* sequence from *F. equinum*: EU836325
Popular press:


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Special thanks to my wife Eleanor, for her love and support which made this endeavour possible. Thanks to my brother, Michael and my parents, Shirley and Bob for their love and encouragement.
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<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>first</td>
</tr>
<tr>
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<td>2-β-mercaptoethanol</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
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<td>alternative sigma factor</td>
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<td>-</td>
<td>negative</td>
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<td>×</td>
<td>multiply</td>
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<td><em>Arcanobacterium pyogenes</em></td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>B. fragilis</td>
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CliFlo  National Climate Database
cm  centimetre
CO  Colorado
csr  carbon storage regulator
*D. nodosus*  *Dichelobacter nodosus*
dH2O  distilled water
DNA  deoxyribonucleic acid
dNTPs  deoxynucleoside triphosphates
*E. coli*  *Escherichia coli*
EDTA  ethylene di-amine-tetra-acetic-acid
*F. equinum*  *Fusobacterium equinum*
*F. necrophorum*  *Fusobacterium necrophorum*
*F. nucleatum*  *Fusobacterium nucleatum*
*F. pseudonecrophorum*  *Fusobacterium pseudonecrophorum*
*F. varium*  *Fusobacterium varium*
fim  fimbriae
*Fnn*  *F. necrophorum* sub sp. *necrophorum*
*Fnf*  *F. necrophorum* sub sp. *funduliforme*
h  hours
IA  Iowa
Int  integrase
kb  kilobase
kDa  kiloDalton
km  kilometres
KS  Kansas
Ltd  limited
lkt  leukotoxin
M  molar
MA  Massachusetts
MI  Michigan
max.  maximum
mb  mega base
MgCl2  magnesium chloride
<table>
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<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>min.</td>
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<tr>
<td>ng</td>
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<td>omp</td>
<td>outer membrane protein</td>
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<td><em>P. anaerobius</em></td>
<td><em>Peptostreptococcus anaerobius</em></td>
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<td><em>P. buccae</em></td>
<td><em>Prevotella buccae</em></td>
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<td><em>Porphyromonas levii</em></td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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SSCP  single-strand conformational polymorphism

spp       species

ssrA      E. coli 10Sa RNA

Sub sp.   sub-species

Taq       Thermus aquaticus

TBE       tris, boric acid, EDTA buffer

TE        tris, EDTA buffer

™         trade mark

tRNA      transfer RNA

tris      tris (hydroxyl-methyl) amino-methane

U         units

UV        ultra violet

V         volts

vap       virulence associated protein

V. fischeri  Vibrio fischeri

vrl       virulence related locus

UK        United Kingdom

v/v       volume per unit volume

WI        Wisconsin

w/v       weight per unit volume
1.1 Introduction

Footrot is a destructive hoof disease of sheep and other ungulates that impacts their production and welfare. It is caused by the bacterium *Dichelobacter nodosus* (*D. nodosus*); but it can only begin if a second bacterium, *Fusobacterium necrophorum* (*F. necrophorum*), is also present (Roberts and Egerton 1969).

Footrot has a broad range of pathology and virulence resulting from a variable and complex aetiology that is a consequence of interactions between the many factors known to affect the disease. These include the occurrence of warm wet weather, the genetics of the host, the effect of management practices and the virulence genetics of *D. nodosus*. However, little is known about the role *F. necrophorum* plays in the development of the disease.

The causative agent of footrot, *D. nodosus*, is a gram-negative, anaerobic bacterium that has a small genome, considerable genetic diversity and a broad continuum of virulence. Where a strain of *D. nodosus* lies on this continuum of virulence is a key determinant for the severity of an infection (Egerton and Parsonson 1969). In turn, the virulence of individual strains of *D. nodosus* is determined by virulence factors such as the production of an array of proteases, the structure and function of their type IV fimbriae, their twitching motility, the presence of genes *omp1A, B, C, D* that produce outer-membrane proteins and the presence or absence of genetic elements such as *vrl* and *int* (previously known as *vap*). These genetic elements do not encode for virulence factors per se, rather they are hypothesised to affect virulence by their insertion in, or near, global genetic regulators.

The second bacterium involved in footrot, *F. necrophorum*, is a gram-negative, anaerobic bacterium that has been isolated from a range of hosts and diseases. As part of its pathogenicity, *F. necrophorum* produces a well characterised and distinctive leukotoxin. This leukotoxin is a large, secreted protein (~300 kD) encoded by the 10 kb *lktA* gene that has only been identified in *F. necrophorum* and *F. equinum* (a phenotypically similar, but
genetically distinct relative of *F. necrophorum* found in horses). *F. necrophorum* may also be important in determining the course of footrot development, as it is found in new footrot lesions as they penetrate and destroy hoof tissue (Egerton *et al.* 1969). While *F. necrophorum* had been only described in sheep on footrot lesions, it is assumed to be part of the normal gut flora shed by sheep (Roberts and Egerton 1969). This is in contrast with *D. nodosus* which appears to be a specialised inhabitant of the hoof (Myers *et al.* 2007) that will only persist in the environment outside the hoof for a maximum of 2-3 weeks (Egerton *et al.* 1989).

Given that *F. necrophorum* is a pathogen in its own right (Lemierre 1936), carries potent virulence factors (Nagaraja *et al.* 2005), such as a leukotoxin, and that ovine footrot has a complex aetiology with a broad range of virulence, it is possible that *F. necrophorum* may be involved in footrot as it progresses and develops, rather than merely being required for an infection by *D. nodosus* to begin.

In this literature review, research in these areas is summarised to justify an investigation of *D. nodosus*, *F. necrophorum* and footrot.

1.2 Footrot

Footrot is a contagious hoof disease of sheep that results in production losses and welfare issues throughout the world (Egerton *et al.* 1989). This disease is difficult to manage, being expensive to treat and hard to eradicate. Footrot is also aetiologically complex with many factors thought to be involved in the disease process. These include interactions between two anaerobic, gram-negative bacteria, *D. nodosus* and *F. necrophorum* (Roberts and Egerton 1969), the occurrence of warm and wet weather (Graham and Egerton 1968), the genetics of the host (Raadsma *et al.* 1999), management practices such as stocking rates (Egerton *et al.* 1989) and the use of foot-paring (Wassink *et al.* 2003a).

1.2.1 A brief history of footrot

Footrot was prevalent within English sheep in the 18th century and by the 19th century; it was recognised as a contagious disease in France (Egerton *et al.* 1989). Footrot was also identified on sheep farms in the United States of America, Australia, Italy and Germany by the early 19th century. The impact of this disease was severe on some
Australian sheep farms in the 19th century, with many deaths being recorded (Egerton et al. 1989). Footrot did, and still can, result in reduced feed intake, losses in production, and a reduction in wool strength and, in the worst cases, death from a combination of starvation, thirst and systemic bacterial infections caused by necrosis of the sternum resulting from contact with the soil (Egerton et al. 1989).

1.2.2 Why footrot is a problem

Apart from the short-term impacts that footrot in sheep has on production and welfare, it is particularly problematic in a commercial farming environment since it is expensive to treat and hard to eradicate. Footrot treatment focuses on curing footrot cases and preventing new infections, and may variously involve a combination of vaccination (Egerton and Roberts 1971, Liardet et al. 1989), use of zinc sulphate footbaths (Skerman et al. 1983), use of antibiotics (Egerton 1966) and culling or removal of diseased sheep (Egerton et al. 1989). Treatment can be combined with the quarantine of uninfected sheep to create an eradication programme. Eradication aims to eliminate footrot on a farm (Egerton et al. 1989) or regional level (Egerton et al. 2002, Egerton et al. 2004) and to prevent footrot being reintroduced into the quarantined area. However, eradication is expensive and does not guarantee that a footrot outbreak will not occur at a later date (Egerton et al. 1989). If eradication of footrot is unsuccessful, or is impractical to attempt, other long-term strategies are required to reduce the impact of footrot outbreaks; for example, using selective breeding programmes to decrease the susceptibility of a sheep population to footrot (Emery et al. 1984, Skerman and Moorhouse 1987).

1.3 The aetiology of footrot

Beveridge first outlined the role *D. nodosus* played in footrot, describing it as a causative agent able to be isolated from footrot lesions (Beveridge 1941). Subsequent pathological studies suggested that *F. necrophorum* could also be involved in the footrot process (Egerton et al. 1969). This hypothesis was confirmed by small pen trials, which showed that footrot tended to be induced much more frequently if cultures of *F. necrophorum* and *D. nodosus* were applied together, rather than individually (Roberts and Egerton 1969). This suggested that a mechanism of aetiology involving both *D. nodosus* and *F. necrophorum* acting together was the cause of footrot. It should be noted that during these pen trials, conditions were far removed from what would be experienced by sheep grazing on pasture, with pens lined in wet hessian and bacterial
cultures being administered by injection into the skin between the claws of the hoof (Roberts and Egerton 1969).

Footrot aetiology, however, is modulated by many factors other than the presence or absence of virulent *D. nodosus* and *F. necrophorum* on the foot (Egerton et al. 1969, Egerton and Parsonson 1969, Roberts and Egerton 1969). The aetiology is complex and may be modulated by other bacterial ecologies, host genetics (Skerman and Moorhouse 1987, Escayg et al. 1997), the acquired immunity of the sheep (Egerton and Roberts 1971), which may not be mutually exclusive from the host’s genetics, farm management practices (Wassink et al. 2003a, Abbott and Lewis 2005, Green et al. 2007) and environmental factors (Graham and Egerton 1968). It is not known how the bacterial ecology of the hoof may affect footrot, but in other anaerobic diseases it has been recognised that multiple bacterial species interact with a host to cause the disease (Otto et al. 2002, Aas et al. 2003, Nishihara and Koseki 2004).

### 1.3.1 The bacterial ecology of *D. nodosus*, *F. necrophorum* and footrot

*D. nodosus* has been found in footrot lesions in sheep (Beveridge 1941), goats (Beveridge 1983, Claxton and O’Grady 1986, Wani et al. 2007), cattle (Egerton and Parsonson 1966), pigs (Piriz et al. 1996), deer (Skerman 1983) and some wild ungulates such as ibexes and mouflons (Belloy et al. 2007). Ovine footrot lesions act as a habitat and are affected by a microbial community from which a large variety of bacteria are able to be identified (Duran et al. 1990a, Calvo-Bado et al. 2011). These include a widespread but as yet unidentified spirochete observed in histological sections (Egerton et al. 1969), that is however unable to induce virulent footrot on its own, or with *D. nodosus* (Thomas 1962a).

How such a microbial community may affect footrot, *F. necrophorum* and *D. nodosus* populations, or the mobile genetic elements of *D. nodosus* is unknown. However, genomic studies have implicated *Staphylococcus* in the disease (Calvo-Bado et al. 2011) and in other anaerobic infections; the ecology of the microbial community is recognised as important. Examples where diseases are driven by changing anaerobic communities include periodontitis (Nishihara and Koseki 2004), *Escherichia coli* (*E. coli*) and *Bacteroides fragilis* (*B. fragilis*) in abdominal abscesses (Otto et al. 2002) and *Clostridium difficile* (*C. difficile*) - associated diarrhoea syndromes (Aas et al. 2003).
While it is acknowledged that the entire microbial community may play an important role in ovine footrot, this thesis will focus specifically on *F. necrophorum* and *D. nodosus* since Roberts and Egerton’s (1969) work suggests they are species that must be present for footrot to occur. Prior to looking at these two bacterial species it is, however, necessary to have some understanding of the other factors that affect footrot and, therefore, may affect both of these bacteria.

1.3.2 The genetics of footrot resistance in sheep

It has been observed that some breeds and lines of sheep are vulnerable or resistant to footrot (Emery *et al.* 1984, Skerman and Moorhouse 1987), suggesting that footrot resistance is a heritable trait. Footrot resistance has also been linked to variation in the Major Histocompatibility Complex (MHC) genes (Outeridge *et al.* 1989, Litchfield *et al.* 1993, Escayg *et al.* 1997). Currently, in New Zealand, testing of the ovine MHC-DQA2 gene is used to identify particularly vulnerable sheep within a population and provide information for making selective breeding decisions (Bishop and Morris 2007, Ennen *et al.* 2009).

1.3.3 Acquired immunity to footrot

It has been demonstrated that it is possible for a sheep to acquire immunity to *D. nodosus* and that this can affect footrot prevalence (Egerton and Burrel 1970, Egerton and Roberts 1971). If a sheep is exposed to footrot or vaccinated against *D. nodosus* (Egerton and Roberts 1971) with a vaccine such as Footvax™ (Liardet *et al.* 1989), some measure of footrot resistance can be acquired. Despite this immune response, footrot protection is incomplete and varies from host to host (Liardet *et al.* 1989). Vaccine responses have also been shown to include a genetic component and can vary widely from sheep to sheep (Emery and Stewart 1984, Liardet *et al.* 1989, Outeridge *et al.* 1989, Litchfield *et al.* 1993). Antigenic competition (Schwartzkoff *et al.* 1993), combined with the effect of multi-strain infections (Claxton *et al.* 1983, Zhou and Hickford 2000a) may also reduce vaccine effectiveness.

1.3.4 Farm management practices and how they affect footrot

Farm management can affect how widespread and damaging a footrot outbreak is. When footrot is spreading from animal to animal, stocking rates are thought to play an important role in transmission (Egerton *et al.* 1989) and high stocking rates (>8.8...
ewes/hectare) are associated with other diseases associated with footrot such as inter-digital dermatitis (Wassink et al. 2004). In Britain, the indiscriminate use of foot-paring has been linked to an increase in the risk of having footrot (Wassink et al. 2003a, Green et al. 2007, Kaler et al. 2010).

1.3.5 Environmental factors that increase the prevalence of footrot

Warm, wet weather has been reported to be required for footrot outbreaks to occur (Graham and Egerton 1968). Several hypotheses have been proposed for the possible mechanisms by which footrot can be modulated by this warm, wet weather. These include physical changes to the hoof that make it more vulnerable to attack (Graham and Egerton 1968), or other unknown factors that increase the risk that a sheep will develop footrot and coincide with warm, wet weather.

1.4 Footrot pathology and virulence

Footrot is described using three virulence classifications: benign, intermediate (Stewart et al. 1986, Egerton et al. 1989,) and virulent (also known as severe footrot [Thomas 1962b]). These virulence classifications can somewhat confusingly be used to describe a diagnosis at the level of the flock, of an individual case, or to describe a particular strain of *D. nodosus* (Stewart et al. 1986).

1.4.1 Diagnosis of footrot at the flock level

Flock level diagnoses are made by monitoring lameness rates and the prevalence of pathologies within a flock. A diagnosis of virulent footrot is made once a “certain percentage” of animals have clinical signs of footrot including the presence of under running, in which the hard horn becomes detached from the rest of the hoof (Egerton et al. 1989). In contrast, benign footrot is characterised by a lack of under-running, despite the presence of widespread inter-digital dermatitis in the flock. Intermediate footrot describes a disease that causes under-running, but when observed at the flock level, causes disease in a smaller percentage of animals than when conditions are favourable for footrot development (Egerton et al. 1989). Flock level diagnosis is modulated by the wide variety of factors that may affect a footrot outbreak. This makes footrot outbreaks highly variable and, as a result, comparisons between different outbreaks are difficult to make.
1.4.2 Diagnosis of footrot in individual cases

Individual footrot cases are diagnosed by observing hoof pathology (Thomas 1962b) which can be described using foot scoring systems. One of the better recognised systems, created by Egerton and Roberts (1971), catalogues footrot pathologies, including "limited mild inter-digital dermatitis" (foot score 1), "extensive inter-digital dermatitis" (foot score 2), "severe inter-digital dermatitis and under-running of the horn of the heel and sole" (foot score 3) to "with under-running extended to the walls of the hoof" (foot score 4).

While individual cases of footrot are classified using pathology (Thomas 1962b, Egerton et al. 1989), this classification is undermined by the complex aetiology of footrot and the effect of the host’s immune response. These immune responses include phagocytosis of \textit{D. nodosus} and passive killing of \textit{D. nodosus} (Roberts and Egerton 1969). Such responses appear variable and important in disease since, if animals are vaccinated against \textit{D. nodosus}, both the response to vaccination (Outteridge 1993) and the protective immunity conferred varies between individuals (Liardet et al. 1989). Individual variation in footrot resistance or vulnerability also appears to be heritable (Skerman et al. 1988) as the result of genetic variation in the host (Raadsma et al. 1999). Given the effect that such host variation can have on footrot, when classifying individual footrot cases into virulent, intermediate or benign categories, it is important to consider the limitations of such an approach.

1.4.2.1 Virulent footrot

Virulent footrot is described as a destructive disease that attacks the hoof or horn (Thomas 1962c). The disease begins as an infection of the inter-digital skin characterised by inflammation and ulceration of the skin and accompanied by the presence of a "thin film of moist greyish necrotic material" (Thomas 1962c). This develops further with the horn and skin separating as an under-running lesion forms within the hoof. A distinctive smell is usually present and this can aid in field diagnosis, since the breakdown of the sulphur-rich keratin within the hoof is unique to footrot and the resulting release of sulphur compounds has a distinctive smell.

1.4.2.2 Intermediate footrot

In individual cases, intermediate footrot is indistinguishable from virulent footrot. The only way of identifying intermediate footrot is at the flock level (Egerton et al. 1989),
or by isolating and identifying *D. nodosus* with intermediate virulence characteristics (Stewart *et al.* 1986).

### 1.4.2.3 Benign footrot

Benign footrot is a disease with similar pathology to the early stages of virulent footrot, with signs of inflammation, ulceration and the presence of moist necrotic material on the inter-digital skin (Thomas 1962c, Parsonson *et al.* 1967). While inflammation and inter-digital dermatitis can be seen, no under-running, delamination or destruction of the hoof, is observed (Thomas 1962c, Egerton and Parsonson 1969). This disease is also known as "scald" and can be a precursor to both virulent footrot and under-running (Thomas 1962c).

Somewhat confusingly, two pathologically similar forms of disease can be described: "benign footrot", caused by benign strains of *D. nodosus* (Egerton and Parsonson 1969) and "ovine inter-digital dermatitis" attributed to infection by *F. necrophorum* only (Parsonson *et al.* 1967). While "benign footrot" is perhaps incorrectly described as "pathologically indistinguishable from ovine inter-digital dermatitis", "benign footrot" is a more chronic disease uniquely distinguishable by the presence of *D. nodosus* in smears (Egerton and Parsonson 1969). The difficulty in distinguishing "benign footrot" from "ovine inter-digital dermatitis" is problematic given that *D. nodosus* can be transmitted to, and found on, the feet of healthy sheep (Beveridge 1941). This creates an incongruity within the postulate that "benign footrot" is able to be distinguished from "ovine inter-digital dermatitis" by the presence or absence of *D. nodosus*.

### 1.5 *D. nodosus* virulence

Individual *D. nodosus* strains vary considerably in virulence (Egerton and Parsonson 1969). This is linked to the stability and potency of key virulence factors such as the production of proteases (Egerton and Parsonson 1969) and variation in fimbriae-mediated motility (Depiazzi and Richards 1985). These characteristics form the basis of current virulence testing regimes for *D. nodosus* strains. Typical tests include the elastase test (Stewart 1979), protease thermo-stability tests (Depiazzi and Rood 1984) and motility assays (Depiazzi and Richards 1985).
Tests have been also developed to detect genetic elements associated with virulence, such as the intA (previously known as vap [Whittle et al. 1999]) or vrl loci (Rood et al. 1996, Cheetham et al. 2006). Using the above tests, most D. nodosus strains fall into the virulent or benign categories, but some strains have been described with intermediate virulence and have characteristics found in both the virulent and benign strains (Stewart et al. 1986).

It should be noted that there are some problems with virulence testing, since some isolates of D. nodosus from virulent footrot outbreaks can test as benign, while others from benign footrot outbreaks can test as virulent (Every 1982, Depiazzi and Richards 1985, Stewart et al. 1986). This suggests either that the virulence tests do not work for a proportion of strains, or that the strains that are being isolated, grown and tested, are not the ones causing the observed disease. This is possible, given that D. nodosus is known to form multi-strain infections (Claxton et al. 1983, Zhou and Hickford 2000a) and it is not known what proportion, if any, of D. nodosus strains may be un-cultivable using various isolation protocols.

1.5.1 Virulent D. nodosus

Virulent strains of D. nodosus have been shown to induce under-running footrot (Egerton and Parsonson 1969) and have characteristic virulence factors such as the production of thermo-stable proteases and high levels of fimbriae-mediated motility (Egerton and Parsonson 1969, Depiazzi and Richards 1985). Virulent strains also typically carry the intA and vrl genetic elements more frequently than benign strains (Katz et al. 1991). Details of these genetic elements, fimbriae, proteases and other virulence factors are described below.

1.5.2 Intermediate D. nodosus

D. nodosus strains with an intermediate virulence have been described by virulence testing as having characteristics typical of both benign and virulent strains (Stewart et al. 1986). These intermediate strains of D. nodosus are not a-virulent and have some ability to cause disease, but have been described as "not as transmittable as virulent strains" (Stewart et al. 1986, Egerton et al. 1989).
1.5.3 Benign *D. nodosus*

Benign strains of *D. nodosus* have been shown to cause benign footrot and, typically, carry proteases that are less thermo-stable than those of virulent strains (Egerton and Parsonson 1969). Benign strains also tend to carry the *intA* and *vrl* genetic elements less often than virulent strains (Katz *et al.* 1991, Rood *et al.* 1996).

1.5.4 The virulence factors of *D. nodosus*

*D. nodosus* strains have a wide range of virulence with several virulence factors having been described. These include the large number of fimbriae *D. nodosus* carries (Walker *et al.* 1973, Stewart 1973), the ability to secrete thermo-stable proteases (Thomas 1962b, Riffkin *et al.* 1995) and the occurrence of a variety of genetic elements with a postulated gene regulatory function (Katz *et al.* 1991, Myers *et al.* 2007).

1.5.4.1 Fimbriae

The *D. nodosus* genome carries 21 fimbrial biogenesis genes and isolates typically express type IV polar fimbriae (Myers *et al.* 2007). Variation within the fimbrial subunit gene, *fimA* is responsible for determining serotype and fimbriae have been used as the primary antigen in vaccines developed against *D. nodosus* (Egerton 1973, Stewart 1978, Liardet *et al.* 1989).

Fimbriae are known to be glycosylated in some strains of *D. nodosus* (Cagatay and Hickford 2008) and several functions of the type IV fimbriae have been described. These include: allowing bacterial binding to epithelial cells (Kennan *et al.* 2001, Parker *et al.* 2006), having a role in twitching motility (Kennan *et al.* 2001, Parker *et al.* 2006), facilitating the ability to take up extra-cellular DNA (Kennan *et al.* 2001, Han *et al.* 2007) and a role in a secretion system in *D. nodosus* that is able to export proteases from the bacterial cytoplasm (Kennan *et al.* 2001, Parker *et al.* 2006, Han *et al.* 2007, Myers *et al.* 2007). In other species such as *Pseudomonas aeruginosa* (*P. aeruginosa*), type IV fimbriae have also been shown to be necessary for biofilm growth (O’Toole and Kolter 1998) and the attachment of bacteriophages (Bradley 1973).

1.5.4.2 Proteases

*D. nodosus* carries three protease genes and the activities of the secreted proteases of *D. nodosus* are important during the development of footrot (Thomas 1962b, Egerton and
Chapter 1: Literature review

Parsonson 1969, Riffkin et al. 1995, Myers et al. 2007). The proteases of *D. nodosus* are able to hydrolyse keratin (Thomas 1964) and can be used to predict the virulence of an isolate of *D. nodosus* (Egerton and Parsonson 1969). This has lead to the measurement of protease stability as a predictor of the virulence of *D. nodosus* strains *in-vitro*, since it was highly associated (P<0.001) with the "virulent" classification of an individual *D. nodosus* strain (Egerton and Parsonson 1969). The genes encoding these proteases have been found on a putative "genomic island" in *D. nodosus*, suggesting an extra-chromosomal source, such as from a horizontal gene-transfer event (Myers et al. 2007).

### 1.5.4.3 *D. nodosus* has a potentially phase-variant antigenic outer-membrane protein

The outer-membrane protein of *D. nodosus* is encoded by four structurally variant, but linked genes (*omp1A, B, C, D*) (Moses et al. 1995). The *omp1* protein is a major antigen of *D. nodosus* and elicits a strong antibody response in sheep during an infection (O’Donnell et al. 1983, Moses et al. 1995). The *omp1* genes are also able to undergo site-specific inversions leading to the potential for phase-variance to occur in the *omp1* protein (Moses et al. 1995) thus creating antigenic shifts in *D. nodosus* populations during infection.

### 1.5.4.4 *D. nodosus* has a putative RTX toxin gene

Through whole genome sequencing, a putative RTX (repeat in toxin) toxin gene was described in *D. nodosus* (Myers et al. 2007). RTX genes encode a pore-forming extracellular cytotoxin found in a wide range of gram-negative bacteria (Strathdee and Lo 1989). Such toxins are toxic, or lytic, to host immune cells and can induce inflammatory responses that can ultimately lead to necrosis, apoptosis and further inflammation (Frey and Kuhnert 2002). In *D. nodosus*, RTX expression and secretion appears to be co-regulated with fimbrial expression by PilR/S (a two component signal transduction system) and an alternative sigma factor (σ\(^{54}\)) (Parker et al. 2006).

### 1.5.4.5 DNO_0690

The gene *DNO_0690* has also been linked to virulence in *D. nodosus* (Myers et al. 2007). *DNO_0690* encodes a large secreted protein made up of 32, nine-amino-acid repeats and it has a postulated function in adhesion to host cells (Myers et al. 2007). This
gene has been found to harbour considerable inter-strain diversity with differences in arrangement and composition noted (Calvo-Bado et al. 2010).

1.5.5 Mobile genetic elements and virulence

The broad range of virulence of *D. nodosus* (Egerton and Parsonson 1969) has been linked to the presence or absence of several potentially mobile genetic elements (Katz et al. 1991, Katz et al. 1994, Haring et al. 1995, Whittle et al. 1999). The bacterium has been sequenced and this suggests that up to 20% of the 1.4 MB chromosome may be derived from mobile elements such as viruses, transposons and plasmids (Myers et al. 2007). What is more, the particularly small genome reveals evidence of notable specialisation and genomic reduction (e.g. *D. nodosus* can only synthesise two amino acids [Myers et al. 2007]). As well as being small, the genome has a small set of regulatory genes with only 3% of its genome apparently devoted to regulation (Myers et al. 2007). By comparison, species such as *E. coli* and *P. aeruginosa* use 8% and 8.4% of their genomes, respectively, for regulation (Myers et al. 2007). This makes the presence or absence of mobile elements that affect the regulation of virulence particularly important, since their impact could be much larger in the smaller regulatory system used by *D. nodosus*.

1.5.5.1 The Vrl, IntA, intB, intC and intD elements

The best studied of the mobile genetic elements of *D. nodosus* are *vrl* and *intA* (formerly known as *vap* [Whittle et al. 1999]). *Vrl* is associated with virulence, being found in 87% of virulent strains and 6% of benign strains (Katz et al. 1991, Rood et al. 1996). *IntA* is more widespread and less specifically associated with virulence, being found in 98% of virulent strains and 28% of benign strains (Katz et al. 1991, Rood et al. 1996). Complicating these associations, in the 872 isolates studied, while *intA* could be detected alone, *vrl* was never detected unless *intA* was also present (Katz et al. 1991, Rood et al. 1996).

*IntB* is found with either *intA*, or replacing *intA*, depending on the insertion site (Whittle et al. 1999). *IntC* can replace *intA* at certain insertion sites and the loss of *intC* has been found to coincide with a decrease in protease thermo-stability (Whittle et al. 1999), while *intD* (GenBank, AY847513) is associated with benign strains (Tanjug et al. 2009) and is not found in virulent strains (Cheetham et al. 2006).
Vrl is a 27 kb element and has many characteristics found in integrated phage genomes, such as an unusually high GC ratio compared to the rest of the *D. nodosus* chromosome (75-80% compared to an average of 45%), and having helicases, methylases, bacteriophage resistance mechanisms, and also a total of 22 genes in closely spaced open-reading frames that are orientated in the same direction (Billington *et al.* 1996). However, no integrase gene has been found as part of vrl, suggesting that this gene has been lost or has been supplied from another source, such as intA. The intA element is a candidate for this as it encodes an integrase, all isolates of *D. nodosus* that carry vrl also carry intA, and both elements have similar insertion sites in the chromosome (Billington *et al.* 1996, Rood *et al.* 1996). A possible vector for vrl and intA has been described in the form of the bacteriophage, DinoHI, (Cheetham *et al.* 2008). Cheetham *et al.* (2008) showed that a 4.6 kb portion of the DinoHI genome can be found adjacent to vrl and it shares many regulatory sequences with the int family of elements, as well as having an integrase homologous to the integrase found in the int elements.

### 1.5.5.2 Plasmid pJIR896 and insertion sequence IS1253

A 10 kb plasmid designated pJIR896 has been identified in *D. nodosus*. This plasmid appears to be made up of a portion of the intA region and 1689bp insertion sequence designated IS1253 (Billington *et al.* 1996). IS1253 is 98.2% homologous to a region that has been found adjacent to the outer-membrane protein gene (*omp1*) found in the *D. nodosus* chromosome (Billington *et al.* 1996). This provides a mechanism that may explain how intA may have evolved over time via a plasmid intermediary and a section of the genome that may be involved in production of the outer-membrane protein, an essential virulence factor.

### 1.5.5.3 Functions of intA and vrl

Despite the intA and vrl regions being tightly associated with virulence (Rood *et al.* 1996), no genes have been found in these regions that encode for a virulence factor that would increase the pathogenicity of *D. nodosus* (Katz *et al.* 1991, Myers *et al.* 2007). This suggests that these elements may be affecting the regulation of virulence as both intA and vrl are inserted near potential global genetic regulators of *D. nodosus*. IntA is found inserted in a tRNA gene (Whittle *et al.* 1999) adjacent to genes homologous to csrA (Lawhon *et al.* 2003) and pnpA (Clements *et al.* 2002), both global regulators of virulence in *Salmonella spp*. Vrl is inserted in a gene homologous with ssrA, a global regulator of
gene expression in *E. coli* (Retallack and Friedman 1995). The impact of these elements on *D. nodosus* regulation may be considerable given its small and, apparently, specialised regulatory system (Myers *et al.* 2007).

### 1.5.5.4 Genetic elements and recombination changing the virulence and antigenic profile of *D. nodosus*

Changes in genetic elements and recombination events in the genome of *D. nodosus* have been linked to changes in virulence, antigenic profile and phase-shifting. For example, the loss of *intC* has been observed to lead to the loss of protease thermo-stability, which is an indicator of isolate virulence (Whittle *et al.* 1999). Additionally, the *omp1* genes can undergo inversions, resulting in changes in the antigenic profile of *D. nodosus* and this may be linked to the plasmid-borne insertion sequence IS1253 found adjacent to the *omp1* genes (Moses *et al.* 1995).

Recombination may also be occurring within the *fimA* genes of *D. nodosus*. Over time, *in-vivo* changes in fimbrial subunit molecular weight and strain serotype have been observed in footrot, suggesting recombination may be occurring (Ghimire *et al.* 1998, Moore *et al.* 1990). This has been supported by sequence analysis of novel *fimA* sequences, which suggests that they could have arisen by a recombination event involving two different *fimA* sequences (Zhou and Hickford 2000b). Furthermore, changes in *fimA* sequence and serotype have been shown to occur in laboratory cultures following transformation (Kennan *et al.* 2003). This confirms that *fimA* sequences can change over time and may result in antigenic changes, (Zhou and Hickford 2000b, Ghimire *et al.* 1998, Moore *et al.* 1990) suggesting that benign strains of *D. nodosus* may act as a reservoir of genetic variation, able to donate sequences to other strains leading to antigenic variation (Kennan *et al.* 2003) and changes in virulence.

The shifting virulence genetics of *D. nodosus* may have consequences for management of footrot especially in the context of "symptomless carriers", sheep that are able to be infected with *D. nodosus* without showing signs of disease (Thomas 1962b, Egerton *et al.* 1989). While it is unknown if such infected, un-diseased sheep are vectors for *D. nodosus* transmission (Green and George 2008), it is conceivable that a sheep infected by a benign, non-contagious strain of *D. nodosus* could act as a source of disease if the *D. nodosus* strain became virulent, or donated virulence genetics to other strains.
Investigating such hypotheses is hampered by the complexities of virulence testing and because *D. nodosus* is considered by some to be difficult to reliably isolate or detect in footrot cases (Wassink *et al.* 2003b, Hussain *et al.* 2009). This highlights an ongoing problem in the field of footrot research and diagnosis, where it has proved difficult to reliably link the presence or absence of *D. nodosus*, or the virulence of individual strains to presentation of a disease that is inherently variable due its complex, multi-factorial aetiology.

1.6 *F. necrophorum*

1.6.1 *F. necrophorum* nomenclature

The naming of *F. necrophorum* has undergone several changes, revisions and divisions, as conventional and molecular techniques have developed and allowed more strains, biovars or sub-species to be resolved (Shinjo *et al.* 1981, Shinjo *et al.* 1991, Garcia *et al.* 1992). As a result, *F. necrophorum* has been re-classified into several species and sub-species.

The bacterium was formerly known as *Fusiformis necrophorus*, before becoming *F. necrophorum* biovars A, B, C, and AB (Shinjo *et al.* 1991, Garcia *et al.* 1992). Biovar A is now considered to be *F. necrophorum* sub-species *necrophorum* and biovar B is now considered to be *F. necrophorum* sub-species *funduliforme* (Shinjo *et al.* 1991). Biovar C was previously described as *F. pseudonecrophorum* (Shinjo *et al.* 1990) and is now considered to be part of *F. varium* (Jin *et al.* 2002). *F. necrophorum* biovar AB remains unclassified (Citron 2002). A new species, *F. equinum* (also known as *F. equorum*), has also been isolated from horses, and it is biochemically and phenotypically similar to *F. necrophorum*, but genetically distinct (Dorsch *et al.* 2001).

1.6.2 The virulence of *F. necrophorum*

*F. necrophorum* is thought to play an important role in footrot aetiology by initiating the disease process (Roberts and Egerton 1969) and is found in the most recently developed region of an under-running footrot lesion (Egerton and Roberts 1969). However, the role *F. necrophorum* plays in footrot pathologies and how this may affect the progression of disease in sheep is unknown. In other hosts, virulence factors that allow *F. necrophorum* to act as a pathogen have been described and *F. necrophorum* has been demonstrated to
produce a wide variety of endotoxins and exotoxins which contribute significantly to its virulence (Nagaraja et al. 2005).

The best described virulence factor of *F. necrophorum* is a leukotoxin that has only been found in *F. necrophorum* sub-species *necrophorum* (Narayanan et al. 2001, Oelke et al. 2005), *F. necrophorum* sub-species *funduliforme* (Tadepalli et al. 2008a, Oelke et al. 2005) and *F. equinum* (Tadepalli et al. 2008c). The leukotoxin induces the apoptosis of leukocytes (Roberts 1967, Narayanan et al. 2001), is encoded by a 10 kb *lktA* gene and is expressed as a large secreted protein of over 300 kDa in molecular weight (Narayanan et al. 2001). The *lktA* gene is part of the *lktBAC* operon that includes a promoter region, an *lktB* gene that appears to be a transmembrane transporter, the *lktA* structural gene and an *lktC* gene of unknown function (Narayanan et al. 2001, Oelke et al. 2005).

Considerable variation in sequence has been observed between the *lktA* genes of *F. necrophorum* sub-species *necrophorum* and *F. necrophorum* sub-species *funduliforme* (Narayanan et al. 2001, Tadepalli et al. 2008a). Variation has also been observed in the leukotoxin gene promoter length, the distance from the structural genes, the sequence and activity of the promoter (Zhang et al. 2006), levels of expression of the gene (Okwumabua et al. 1996, Narayanan et al. 1997), the potency of the leukotoxin (Narayanan et al. 1997) and the *lktA* gene sequence and length (Narayanan et al. 2001, Tadepalli et al. 2008a, Tadepalli et al. 2008b). These variants and sub-species of *F. necrophorum* can cause a wide range of diseases and have been isolated from either humans or cattle.

1.6.3 Role of *F. necrophorum* in human diseases

*F. necrophorum* is described as a human pathogen causing Lemierre’s syndrome (Lemierre 1936). Lemierre’s syndrome is characterised as a *F. necrophorum* infection where the patient initially presents with a sore throat, which then progresses to the formation of intra-venal abscesses followed by organ failure and death (Lemierre 1936). Once antibiotic use became widespread, this syndrome was reported with far less frequency, but in the last two decades a resurgence of *F. necrophorum* infections in human clinical cases has been reported (Hagelskjaer et al. 1998, Brazier 2006). These include *F. necrophorum* being isolated from a variety of deep bone infections, tissue infections, ear and sinus infections, necrobacillosis, lung and respiratory tract infections and new cases of Lemierre’s syndrome (Hagelskjaer et al. 1998, Brazier 2006, Hagelskjaer Kristensen
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In Britain, a survey found that 1 in 10 people presenting with a sore throat to a medical practitioner had *F. necrophorum* sub-species *funduliforme* detected on throat swabs (Aliyu et al. 2004). *F. necrophorum* sub-species *funduliforme* tends to be isolated from human cases and has a different profile of haemagglutinin (Shinjo et al. 1981, Tan et al. 1994a, Narongwanichgarn et al. 2003, Tadepalli et al. 2008a) and leukotoxic activity compared with *F. necrophorum* sub-species *necrophorum* (Tadepalli et al. 2008b).

1.6.4 Role of *F. necrophorum* in animal diseases

Within an agricultural setting, *F. necrophorum* is well known as a pathogen associated with many animal diseases such as necrobacillosis (Langworth 1977, Nagaraja et al. 2005), calf diphtheria (Mackey 1968), acidosis, rumenitis, liver abscess complex (Jensen et al. 1954), lameness in cattle (Clark et al. 1985, Emery et al. 1985) and ovine footrot (Roberts and Egerton 1969).

1.6.4.1 Role of *F. necrophorum* in necrobacillosis

Necrobacillosis is a disease usually found in neonatal stock or aborted foetuses (Langworth 1977). Initially, an infection enters the abdominal cavity via the umbilical cord and it can develop into a fatal abdominal infection (Agerholm et al. 2007, Langworth 1977). Necrobacillosis is also used as a general term describing necrotic soft tissue infections attributed to *F. necrophorum* (Nagaraja et al. 2005).

1.6.4.2 Role of *F. necrophorum* in calf diphtheria

Calf diphtheria or necrotic laryngitis is an often fatal syndrome of young cattle that display inflammation and disease of the larynx and/or pharynx (Mackey 1968). *Fusobacteria* have been thought to be involved in calf diphtheria since the 19th century and were originally described by Loeffler ([Loeffler 1884] as reported by Langworth 1977). *F. necrophorum* has been recognised as playing a role during the disease along with other factors such as physical damage or infection by other bacteria such as *Arcanobacterium pyogenes* (*A. pyogenes*) (Mackey 1968, Panciera et al. 1989).

1.6.4.3 Role of *F. necrophorum* in the acidosis, rumenitis and liver abscess complex

The "Acidosis, rumenitis and liver abscess complex" (Jensen et al. 1954) is a chronic, bovine syndrome that can result in substantial losses in production and the
condemning of carcasses (Nagaraja and Chengappa 1998). Initially, the rumen becomes acidic (also known as acidosis), due to excess acid being produced by the micro-flora in the rumen, is caused by the high sugar content of the feed entering the rumen (Jensen et al. 1954). Following acidosis, damage to the rumen wall may occur (rumenitis) and an increase in the numbers of *F. necrophorum* can be observed in the rumen (Tan et al. 1994b). Animals in this state are more likely to get liver abscesses caused by *F. necrophorum* as the result of the bacterium invading the liver via the portal circulation (Tadepalli et al. 2009). Vaccination using the Fusogard™ vaccine (a deactivated whole cell vaccine produced by Novartis Animal Health) (Checkley et al. 2005) and the Centurion™ vaccine (a bi-valent toxoid of *F. necrophorum* leukotoxin and *A. pyogenes* haemolysin produced by Merck) have both been shown to reduce liver abscess rates in feedlot cattle (Jones et al. 2004). In other animals, truncated leukotoxin vaccines have been used to protect mice from *F. necrophorum* challenges (Narayanan et al. 2003).

### 1.6.4.4 Role of *F. necrophorum* in lameness in cattle

Some research suggests that *F. necrophorum* may be involved as an agent in lameness of cattle (Clark et al. 1985, Emery et al. 1985, Dopfer et al. 1997). In a Canadian feedlot system, vaccination using Fusogard™ resulted in 6% - 72% lower rates of lameness in vaccinated herds (Checkley et al. 2005). It should be noted that lameness in cattle is not a single disease; rather a syndrome with a wide range of causes (Choquette-Levy et al. 1985, Chesterton et al. 1989) and that lameness is not eliminated by this vaccine, merely reduced in prevalence.

### 1.6.4.5 Role of *F. necrophorum* in ovine footrot

In small pen trials, histological and challenge studies showed that *F. necrophorum* pre-disposes sheep to footrot (Egerton et al. 1969, Roberts and Egerton 1969). Egerton showed that *F. necrophorum*-like cells were seen to invade the epidermis several days before a *D. nodosus* infection occurred and *F. necrophorum* could be seen in the leading-edge of the newly developing footrot lesions (Egerton et al. 1969). Furthermore, Roberts demonstrated that footrot was able to be induced by injection of cultures of both *D. nodosus* and *F. necrophorum* together into the inter-digital skin, but neither bacterium was able to induce footrot consistently when injected alone (Roberts and Egerton 1969). Vaccination against *F. necrophorum* was attempted to control footrot, but *F. necrophorum*
antigens were found to generate poor immune responses in sheep and give little protective effect (Egerton and Roberts 1971).

*F. necrophorum* is also involved in the invasion of tissue during ovine inter-digital dermatitis (also known as benign footrot or scald) and histological and bacteriological studies suggest it could be acting as a causative agent (Parsonson *et al.* 1967). A foot abscess model has been demonstrated where *F. necrophorum* biovar AB cultures were able to induce foot abscesses if the feet were first devitalised with liquid nitrogen and the inter-digital skin injected with *F. necrophorum* biovar AB (Corner *et al.* 1996).

1.7 Justification for research into the role of *F. necrophorum* in footrot

The role of *D. nodosus* in footrot is understood to be as the primary infective agent whose changeable, broad range of virulence can determine the course and severity of disease (Egerton *et al.* 1989). *F. necrophorum* infection appears to predispose sheep to infection by *D. nodosus*, allowing subsequent development of footrot (Parsonson *et al.* 1967, Egerton *et al.* 1969, Roberts and Egerton 1969). *F. necrophorum* is present in under-running lesions (Egerton *et al.* 1969).

What role *F. necrophorum* may be playing in the footrot after the disease has begun and how it may affect the course of the disease is less well understood and it has not been as extensively studied as *D. nodosus*. Given that footrot has a broad range of pathology and virulence at the individual and flock level, and that *F. necrophorum* is a destructive pathogen (Nagaraja *et al.* 2005), it is conceivable *F. necrophorum* may play a role in determining the course of footrot or an outbreak once it has begun. Therefore, *F. necrophorum* would appear to require further study to identify what role it could be playing in footrot.

Consequently, in this thesis, *F. necrophorum* was investigated along with *D. nodosus* to identify how these bacteria could be behaving and interacting during footrot. This was facilitated by the use of molecular identification techniques that were not limited by the requirement to anaerobically sample, isolate and culture bacteria. This limitation is noteworthy in the case of *F. necrophorum* since footrot lesions are both a microbiologically varied and highly contaminated environment (Duran *et al.* 1990a) which would further aggravate the lack of reliable isolation techniques for this organism from
ovine footrot lesions. To circumvent these problems, the diagnostic molecular techniques used have been broadly described as both sensitive and specific approaches (Johansson et al. 2000, Fenollar et al. 2006) as these are able to detect difficult to culture or isolate organisms (Johansson et al. 2000) such as anaerobes (Fenollar et al. 2006).

The use of such techniques in footrot has been assessed by Moore et al. (2005) who observed that diagnostic molecular techniques were 17% more sensitive than culture for the detection of *D. nodosus* from footrot lesions. Given that *F. necrophorum* is a strict anaerobe (Tally et al. 1975) and more sensitive to oxygen than *D. nodosus* (Myers et al. 2007), it is likely that *F. necrophorum* is much more difficult to reliably isolate than *D. nodosus*. As a result, in this thesis, diagnostic molecular techniques were selected in preference to culture for the detection of both *F. necrophorum* and *D. nodosus* in a clinical setting. Such techniques also allow the study of un-described sources of pathogens that could exist outside a clinical setting such as subclinical habitats or hosts other than sheep.

1.8 Aims of this thesis

1) To test if *F. necrophorum* is associated with footrot in the field and confirm its involvement in "natural" ovine footrot.

2) To study what footrot pathologies of *D. nodosus* and *F. necrophorum* are present as the disease develops and changes. This involves challenging sheep with footrot and monitoring what pathologies and pathogens are evident as disease develops.

3) To survey the gastro-intestinal tract of sheep for *D. nodosus* to ascertain if it can be found outside the hooves of sheep.

4) To survey lame dairy cows for *D. nodosus* and *F. necrophorum* to ascertain if *D. nodosus* and *F. necrophorum* are prevalent in lame dairy cattle.

5) To study the variation in the leukotoxin gene of *F. necrophorum* and to identify if different variants of *F. necrophorum* tend to be found in different pathologies or hosts.
Chapter 2: Development, validation and application of methods to survey animals with footrot for the presence of *Dichelobacter nodosus* and *Fusobacterium necrophorum*

2.1 Introduction

Given the observation that *F. necrophorum* is required for *D. nodosus* to initiate footrot in small pen trials (Roberts and Egerton 1969), and that *F. necrophorum*-like cells are observed with *D. nodosus* at the leading-edge of developing lesions in histological analysis of hooves (Egerton et al. 1969), it is plausible that "in the field", *F. necrophorum* is also required for *D. nodosus* to initiate ovine footrot and may be involved in the formation and progression of under-running lesions. This could be tested by conducting a survey of *D. nodosus* and *F. necrophorum* prevalence that compares healthy (disease free) sheep and sheep with footrot in a pastoral production system.

Undertaking a survey of healthy sheep and sheep with footrot would have three additional benefits. First, it would confirm if *F. necrophorum* is part of the natural footrot biota in New Zealand. Secondly, it is a logical extension of Roberts and Egerton’s (1969) pen trials, since these were very artificial and involved faecal contamination or injection of soft tissues with cultures. Thirdly, it facilitates the development, validation and demonstration of molecular techniques that are able to detect *D. nodosus* and *F. necrophorum* *in-situ*. Such techniques have the advantage of being specific, sensitive and are not limited by the requirements of anaerobic isolation.

Once techniques to detect *D. nodosus* and *F. necrophorum* are developed, validated and used on ovine footrot, they can also be applied to study if *F. necrophorum* may be involved in footrot in other species. For example, caprine footrot, described as similar to ovine footrot, is caused by *D. nodosus* (Stewart et al. 1986) and *F. necrophorum* has been cultured from caprine footrot lesions (along with many other bacterial species) [Duran et al. 1990b]. However, it has not yet been confirmed if *D. nodosus* requires *F. necrophorum* to cause caprine footrot. To test if *F. necrophorum* could be involved in caprine footrot, a survey could be conducted to compare healthy goats and goats with footrot. This would identify if *F. necrophorum* is associated with *D. nodosus* in under-running caprine footrot in the field.
In this chapter, highly species-specific PCR-based methods to detect *F. necrophorum* and *D. nodosus* were developed, validated and applied to survey sheep and goats, with and without footrot, in the field.

### 2.2 Materials and methods

#### 2.2.1 Survey of the prevalence of *D. nodosus* and *F. necrophorum* in disease free sheep and sheep with footrot

A survey was undertaken to compare disease free sheep to sheep with under-running footrot in a pastoral sheep farming system. Eighty sheep farmers were contacted via mail and supplied with sterile cotton swabs in sterile 15 mL tubes for the purpose of collecting hoof scrapings. The farmers received instructions (Appendix A) to take swabs from the skin-horn junction on the axial wall of the hoof (Figure 2.1). A total of three swabs from disease free sheep and three swabs from sheep with under-running footrot were requested from each farmer. The swabs were returned by post in the tubes supplied and stored at -80°C until required.

![Figure 2.1. Extract of sampling instructions.](image)

**Figure 2.1. Extract of sampling instructions.** Diagram of sampling site that farmers received as part of sampling instructions, for further details of these instructions see Appendix A.

Of the 80 farmers contacted, 14 returned swabs taken from disease free sheep and sheep with footrot. Three further farmers reported their farms were footrot free and returned six swabs from disease free sheep. Twelve sheep with under-running footrot from

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1 Diagram was extracted from "Footrot in sheep: 2. Diagnosis" Agriculture note AG0446, published by the Department for Primary Industry, Victoria, Australia.
Chapter 2: Development, validation and application of methods to survey animals with footrot for the presence of *Dichelobacter nodosus* and *Fusobacterium necrophorum*

The Lincoln University research farm were swabbed in a similar manner. A total of 50 swabs from disease free sheep and 42 swabs from sheep with under-running footrot were received. DNA was extracted from swabs by the following method and the presence of *D. nodosus* and *F. necrophorum* sub sp. *necrophorum* ascertained using species-specific PCR methods.

### 2.2.2 Survey of the prevalence of *D. nodosus* and *F. necrophorum* in disease free goats and goats with footrot

As part of a science fair project, Ms Ayla van Loenen, a Year 11 high school student, used the methods described above to conduct a survey of *D. nodosus* and *F. necrophorum* prevalence in goats by comparing disease free goats to goats with footrot. Ms van Loenen contacted farmers, carried out most of the DNA extraction and performed a single *fimA* PCR and *lktA* PCR on each sample.

### 2.2.3 Development of diagnostic Polymerase Chain Reactions for detecting hoof microorganisms

The application of species-specific PCRs to detect the *fimA* gene of *D. nodosus* and the *lktA* gene of *F. necrophorum* from footrot lesions required the development and validation of DNA extraction techniques as well as PCRs that could amplify a portion of the *fimA* gene of *D. nodosus* or the *lktA* gene of *F. necrophorum*. These PCRs had their *in situ* detection limits measured using sensitivity testing.

#### 2.2.3.1 DNA extraction from footrot swabs

DNA was extracted from footrot swabs by placing each of them in a sterile 1.75 mL tube with 400 µL of sterile 1 × TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), then mixing for 20 seconds in a vortex mixer. The swabs were removed and 40 µL of 10% SDS (sodium dodecyl sulphate) was added to each tube together with 220 µL of Tris-buffered phenol (pH 7.8) and 220 µL of chloroform. The tubes were mixed using a vortex mixer, then frozen overnight at -20°C. After thawing, the suspensions were mixed by inverting and then centrifuged at 5000 × g for 5 minutes. The aqueous layer was aliquoted into a new tube and precipitated with 100 µL of 3 M sodium acetate (adjusted to pH 5.2) and 500 µL of ice cold isopropanol. The precipitated DNA was centrifuged at 14 500 × g for 15 minutes and the supernatant was removed. The DNA pellet was air-dried before being
suspended in 50 µL of sterile dH₂O. These DNA solutions were stored at 4°C until required.

2.2.3.2 *D. nodosus* fimbrial (fimA) PCR

A PCR able to detect the *fimA* gene of *D. nodosus* was performed using the previously described primers u1, u2, d1, d2 and d3 (Zhou 2000a). Each 50 µL PCR reaction contained 100 nM of each primer; 200 µM of each nucleotide (Quantum Scientific, Milton, Australia); 1 unit of *Taq* DNA polymerase (Qiagen, Hilden, Germany); 5 µL of the 10 × buffer supplied with the polymerase; a final concentration of 2.75 mM of MgCl₂ and 2.5 µL of the previously extracted DNA. The PCR was performed on an iCycler (Bio-Rad, Hercules, CA) using an initial denaturation of 94°C for 5 min, followed by 45 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 40 s, ending with a final extension of 72°C for 5 min. The PCR products were visualised using ethidium bromide staining and UV transillumination. All PCR reactions included a positive (*fimA* amplicon diluted 1:1000) and negative control (dH₂O).

2.2.3.3 *F. necrophorum* leukotoxin (lktA) PCR

A *F. necrophorum* species-specific PCR was developed to detect the leukotoxin A gene of *F. necrophorum* sub sp. *necrophorum*. Dr Huitong Zhou (Lincoln University) designed the primers lktA-up (5’-acaatcggagtagtaggttc-3’) and lktA-dn (5’-atttggtaactgccactgc-3’). These were designed based on the published *F. necrophorum* sub sp. *necrophorum* leukotoxin gene sequence (GenBank accession number DQ672338), to amplify a 403 bp amplicon of the *F. necrophorum* lktA coding sequence.

Each 50 µL PCR reaction contained 200 µM of each nucleotide (Quantum Scientific); 250 nM of each primer (Proligo LLC, Colorado, CO); 1 unit of *Taq* DNA polymerase (Qiagen); 5 µL of 10 × buffer supplied with the polymerase; 2.5 µL of DNA extracted previously and 2.5 mM of MgCl₂. The PCR reaction was performed in an iCycler (Bio-Rad) with an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s and a final extension of 5 min at 72°C. PCR products were visualised using ethidium bromide staining and UV transillumination. All PCR reactions included a positive (*lktA* amplicon diluted 1:1000) and negative control (dH₂O).
2.2.3.4 Validation of the *F. necrophorum* lktA PCR

The fidelity and specificity of the *F. necrophorum* sub sp. *necrophorum* lktA PCR was assessed by searching NCBI GenBank (http://www.ncbi.nlm.nih.gov) using a range of genomic DNA. Genomic DNA was extracted (by boiling colonies cultured on plates in dH2O) from the following species: *F. pseudonecrophorum* (ATCC 51644), *F. varium* (ATCC 8501), *F. necrophorum* sub sp. *funduliforme* (ATCC 51357), *F. necrophorum* sub sp. *necrophorum* (NCTC 10575), *F. nucleatum* sub sp. *nucleatum* (ATCC 25586), *F. equinum* (NCTC 13176, acquired from Dr Jacqueline Norris, University of Sydney, NSW, Australia, via Professor Julian Rood, Monash University, NSW, Australia, whose lab performed the DNA extraction) and *D. nodosus* (strain A198 acquired from Professor Julian Rood, Monash University, NSW, Australia). The various microorganisms were cultured anaerobically on Wilkins-Chalgren plates (Oxoid, Hampshire, United Kingdom) using Click-Clack™ jars, as described by Bennett *et al.* (2006).2

2.2.3.5 Sensitivity testing of the fimA and lktA PCRs

To determine the sensitivity of the *fimA* and *lktA* PCRs, genomic DNA from *D. nodosus* and *F. necrophorum* was quantified, serially diluted and used as a template in PCR reactions, as described above. *D. nodosus* genomic DNA was supplied by Dr Huitong Zhou, Lincoln University, Lincoln, New Zealand, and *F. necrophorum* cells were supplied by Craig Trotter, Lincoln University, Lincoln, New Zealand before a DNA extraction was carried out, as described above. DNA Quantification was performed on a Qubit™ fluorometer (Q32857, Invitrogen, Carlsbad, CA) using a Quant-iT™ dsDNA BR Assay Kit (Q32850, Invitrogen) according to the manufacturer's instructions. Calculations of genome number assumed that 978 mb of DNA weighed 1 pg (Dolezel 2003), *D. nodosus* has a 1.4 mb genome (Myers 2007) and *F. necrophorum* has a 2.17 mb genome. As the genome size of *F. necrophorum* was not described, it was assumed to be 2.17mb based on the genome sequence of *F. nucleatum*, a close relative (Kapatral *et al.* 2002).2

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2 This publication was an indirect outcome of this thesis and can be found in Appendix B.
2.2.3.6 Methods developed to compensate for the inhibition of PCR reactions using swab derived material

The DNA extracted from hoof swabs was tested for the presence of *fimA* and *lktA* using the PCR protocols described above. Each PCR was performed in triplicate. If any signs of PCR failure or inhibition could be seen, such as failure of positive controls or no extraneous primer-primer products from the *fimA* PCR (which used 45 replication cycles), it was assumed that the PCR had failed and it was repeated with the extracted DNA solution diluted 1/10 and/or 1/100.

2.2.3.7 Using BSA to reduce inhibition of PCRs performed on footrot swabs

During the course of this study, it was found that the use of 400 ng/µL bovine serum albumin (BSA) (New England Biolabs, Ipswich, MA) and an extra 2.5 mM MgCl$_2$ (at a final MgCl$_2$ concentration of 5.25 mM and 5.0 mM in the *fimA* and *lktA* PCRs, respectively) in PCR reactions greatly improved the consistency of PCR from hoof swabs (Figure 2.4). Approximately 1 in 3 sheep samples were able to be retested using this modified PCR and all the goat PCR reactions contained 400 ng/µL BSA (New England Biolabs) and an extra 2.5 mM MgCl$_2$.

2.2.4 Statistical methods used to test if *D. nodosus* and *F. necrophorum* are associated with footrot in sheep and goats

Statistical analyses to ascertain if *D. nodosus* and *F. necrophorum* were associated with footrot in sheep and goats were carried out by Dr Richard Sedcole of Lincoln University using a log-linear model and Poisson errors (GenStat version 10, 2007, Lawes Agricultural Trust, Rothamsted). The fit of the model was assessed by the residual deviance (-2×log likelihood), which is approximately Chi square distributed with residual degrees of freedom.

2.3 Results

2.3.1 Validation of the *F. necrophorum lktA* PCR

The fidelity and specificity of the *lktA* PCR was tested and it was found to only produce amplicons of the correct size with *F. necrophorum* sub sp. *necrophorum* (NCTC 10575) or sub sp. *funduliforme* (ATCC 51357). No amplicons were generated from DNA extracted from *F. pseudonecrophorum* (ATCC 51644), *F. varium* (ATCC 8501), *F. nucleatum* sub sp. *nucleatum* (ATCC 25586), *F. equinum* (NCTC 13176) or *D. nodosus*. 
### 2.3.2 Sensitivity testing of *fimA* and *lktA* PCRs

The quantification of stock solutions was carried out and the solution of *D. nodosus* genomic DNA was calculated to contain $1.96 \times 10^8$ genomes/µL while the solution of *F. necrophorum* genomic DNA was calculated to contain $1.14 \times 10^6$ genomes/µL. Upon sensitivity testing the *fimA* PCR produced a visible amplicon with 9.6 genomes/µL, but failed to produce an amplicon with 0.96 genomes/µL (Figure 2.2). The *lktA* PCR produced a visible amplicon with 6.3 genomes/µL but failed to produce an amplicon with 0.63 genomes/µL (Figure 2.3).

**Figure 2.2. FimA PCR sensitivity test.** *D. nodosus* genomic DNA was serially diluted and used as a template for a *fimA* PCR, as described above. Dilution began at 960000 genomes/µL and progressed to 0.096 genomes/µL. While 9.6 genomes/µL produced a band, 0.96 genomes/µL did not.

**Figure 2.3. LktA PCR sensitivity test.** *F. necrophorum* genomic DNA was serially diluted and used as a template for a *lktA* PCR, as described above. Dilution began at 63000 genomes/µL and progressed to 0.63 genomes/µL. While 6.3 genomes/µL produced a band, 0.63 genomes/µL did not.

### 2.3.3 Use of BSA in PCR amplification from footrot swabs

When testing swabs collected from the hooves of sheep with footrot, both the *fimA* and *lktA* PCR reactions were found to more reliably produce amplicons with the addition of 400 ng/µL BSA and 5.0 or 5.25 mM MgCl$_2$ in the *fimA* or *lktA* PCR reactions,
respectively (see Figure 2.4 for an example). The fimA PCR was also optimised further by reducing the primer concentration from 250 nM to 100 nM (Figure 2.4).

Figure 2.4. Optimisation of the fimA PCR to detect \(D. \textit{nodosus}\) on swabs collected from the hooves of sheep with footrot. Four different sets of PCR conditions using the same template DNA extracted from a footrot swab. Each PCR used 250 nM or 100 nM of each primer, 400 ng/µL BSA or no BSA. When BSA was added to a PCR, 5.0 mM of \(\text{MgCl}_2\) was used (rather than 2.5 mM) to prevent PCR inhibition by the absorption of \(\text{MgCl}_2\) to BSA.

### 2.3.4 Survey of the prevalence of \(D. \textit{nodosus}\) and \(F. \textit{necrophorum}\) in disease free sheep and sheep with footrot

Fifty swabs were taken from the feet of disease free sheep and 42 from sheep with under-running footrot. Of the swabs from disease free sheep, none tested positive for the fimA gene of \(D. \textit{nodosus}\) and only one out of 50 was positive for the lktA gene of \(F. \textit{necrophorum}\). In contrast, of the swabs tested from sheep with under-running footrot, 19 out of 42 tested positive for the fimA gene of \(D. \textit{nodosus}\) and 19 out of 42 tested positive for the lktA gene of \(F. \textit{necrophorum}\). Of the 19 swabs that tested positive for the fimA gene of \(D. \textit{nodosus}\), 17 out of 19 were also positive for the lktA gene of \(F. \textit{necrophorum}\). These results are summarised in Table 2.1.
Table 2.1. Frequency of \textit{fimA} and \textit{lktA} detection from disease free sheep and sheep with footrot

<table>
<thead>
<tr>
<th></th>
<th>Sheep with footrot (n=42)</th>
<th>Disease free sheep (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection of \textit{fimA}</td>
<td>19/42</td>
<td>0/50</td>
</tr>
<tr>
<td>Detection of \textit{lktA}</td>
<td>19/42</td>
<td>1/50</td>
</tr>
<tr>
<td>Detection of \textit{fimA} and \textit{lktA} together</td>
<td>17/42</td>
<td>0/50</td>
</tr>
</tbody>
</table>

A statistical analysis was carried out and revealed that detection of the \textit{fimA} gene of \textit{D. nodosus} and the \textit{lktA} gene of \textit{F. necrophorum} was highly associated with under-running footrot (P<0.01). It was also demonstrated that the \textit{fimA} gene of \textit{D. nodosus} and the \textit{lktA} gene of \textit{F. necrophorum} were detected together at a significantly higher rate than would be expected by random assortment (P<0.025). These results are summarised in Table 2.2.

Table 2.2. Statistical analysis of the detection of \textit{fimA} and \textit{lktA} from disease free sheep and sheep with footrot

<table>
<thead>
<tr>
<th>Association of:</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection of \textit{fimA} with footrot</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Detection of \textit{lktA} with footrot</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Detection of \textit{fimA} and \textit{lktA} together</td>
<td>P&lt;0.025</td>
</tr>
</tbody>
</table>

These results have been published (Bennett \textit{et al.} 2009a see Appendix B).

2.3.5 Survey of the prevalence of \textit{D. nodosus} and \textit{F. necrophorum} in disease free goats and goats with footrot

A total of 20 swabs were received from disease free goats and 24 swabs from goats with under-running footrot. Of the 20 swabs from disease free goats, none tested positive for the \textit{fimA} gene of \textit{D. nodosus} or the \textit{lktA} gene of \textit{F. necrophorum}. In contrast, of the swabs tested from the goats with footrot, 15 out of 24 were positive for the \textit{fimA} gene of \textit{D. nodosus} and eight out of 24 were positive for the \textit{lktA} gene of \textit{F. necrophorum}. Of the
15 swabs that tested positive for the *fimA* gene of *D. nodosus*, seven out of 15 were also positive for the *lktA* gene of *F. necrophorum*. These results are summarised in Table 2.3.

**Table 2.3. Frequency of *fimA* and *lktA* detection from disease free goats and goats with footrot**

<table>
<thead>
<tr>
<th></th>
<th>Goats with footrot (n=24)</th>
<th>Disease free goats (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection of <em>fimA</em></td>
<td>15/24</td>
<td>0/20</td>
</tr>
<tr>
<td>Detection of <em>lktA</em></td>
<td>8/24</td>
<td>0/20</td>
</tr>
<tr>
<td>Detection of <em>fimA</em> and</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lktA</em> together</td>
<td>7/24</td>
<td>0/20</td>
</tr>
</tbody>
</table>

A statistical analysis was carried out and revealed that detection of the *fimA* gene of *D. nodosus* and the *lktA* gene of *F. necrophorum* were both highly associated with under-running footrot in goats (P<0.01). It also demonstrated that the *fimA* gene of *D. nodosus* and the *lktA* gene of *F. necrophorum* were detected together at a significantly higher rate than would be expected from random assortment (P<0.039). These results are summarised in Table 2.4.

**Table 2.4. Statistical analysis of the detection of *fimA* and *lktA* from disease free goats and goats with footrot**

<table>
<thead>
<tr>
<th>Association of:</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection of <em>fimA</em> with</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>footrot</td>
<td></td>
</tr>
<tr>
<td>Detection of <em>lktA</em> with</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>footrot</td>
<td></td>
</tr>
<tr>
<td>Detection of <em>fimA</em> and</td>
<td>P&lt;0.039</td>
</tr>
<tr>
<td><em>lktA</em> together</td>
<td></td>
</tr>
</tbody>
</table>

These results have been published (Bennett *et al.* 2009b, see Appendix B).

### 2.4 Discussion

In this study, PCR-based bacterial detection methods were developed, validated and applied to footrot in sheep and goats, including a new, species-specific PCR able to detect the *lktA* gene of *F. necrophorum*. These new methods then allowed surveys to be
undertaken of sheep and goats on commercial farms with footrot, revealing that *D. nodosus* and *F. necrophorum* are associated with under-running footrot in both sheep and goats.

It was also demonstrated that *D. nodosus* and *F. necrophorum* are detected together at a higher rate in both sheep and goats than if they were randomly distributed. This suggests that both of these bacteria are associated with each other and footrot (as diagnosed by farmers) which, in turn supports the model that *F. necrophorum* causes of footrot with *D. nodosus* in the field.

This co-detection of *F. necrophorum* and *D. nodosus* in sheep and goats with under-running footrot lesions supports the hypothesis that footrot is caused by both these organisms (Roberts and Egerton 1969) and that *F. necrophorum* is involved in the development of under-running lesions, since it is found in the leading-edge of growing footrot lesions (Egerton *et al.* 1969).

This study found that in goats, both *D. nodosus* and *F. necrophorum* were highly associated with under-running footrot. In contrast with sheep, over half of the swabs from goats that tested positive for *D. nodosus* did not test positive for *F. necrophorum*. This suggests that *F. necrophorum* infections in goats with footrot are not necessarily identical to sheep, despite caprine footrot being described as having similar bacteriology to ovine footrot (Stewart *et al.* 1986). Other authors (Ghimire *et al.* 1999) have noted some differences between sheep and goats such as lower rates of under-running in goats and, when under-running was observed, it tended not to penetrate the hoof as deeply when compared to sheep.

Other explanations for the low prevalence of *F. necrophorum* on *D. nodosus* positive swabs in goats might be that the PCR detection of the lktA gene of *F. necrophorum* in goats is less effective than in sheep. For example, the *F. necrophorum* found on goat hooves may be different from that found in sheep for which the primers and PCR were optimised. It is also possible that these results are a reflection of the small number of caprine footrot swabs analysed (n=24).

A large proportion of the swabs from sheep (19/42) and goats (15/24) with footrot tested negative for both the fimA gene of *D. nodosus* and the lktA gene of *F. necrophorum*. 
Other authors have noted (Moore et al. 2005, Wani et al. 2007) the difficulty in using PCR to detect *D. nodosus* from virulent footrot cases with detection rates ranging from 50% in India (Wani et al. 2007) to 68.9% in the UK (Moore et al. 2005). In this study, this difficulty may have been compounded because the site of most active disease (the under-running lesion) was not sampled. Rather, farmers were instructed to sample the skin-horn junction, since it allowed consistent sampling between sheep and avoided the need for farmers to find and sample footrot lesions. Footrot lesions can be pathologically variable, difficult to access and sample consistently. This was an important consideration given that the nature of the study required it to be conducted in a commercial farming situation with farmers collecting the samples. A consequence of farmers conducting sampling, is that it was unknown if all samples were taken correctly from the skin-horn junction as requested. If the instructions were not followed, and samples were taken from the wrong part of the hoof, the impact of this on the study may be relatively minor since 14 different farmers contributed samples, diluting the effect of an individual's erroneous sampling. Ideally, samples would be collected by the same person on any test flocks and the sampling would be from the active footrot lesion and not just the skin-horn junction. Conversely given that the skin-horn junction was sampled rather than lesions, this still resulted in *D. nodosus* detection rates of 45% in sheep and 63% in goats, which is consistent with the detection rates of Wani et al. (2007) and Moore et al. (2005), who sampled lesions.

When conducting microbial sampling, the swab material, transport and storage all potentially impact the viability and numbers of bacteria retrieved. This had been highlighted by a comparison between cotton swabs and nylon flocked swabs (Moore and Griffith 2007) on surfaces. It was observed that despite nylon flocked swabs retrieving more bacteria from wet surfaces, cotton swabs were much more effective on dry surfaces since they allow much more mechanical force to be used to scrape samples from the surface. Moore and Griffith (2007) also demonstrated the role of bacterial replication during swab storage and transport in sampling regimes where it was observed that some bacteria will quadruple in numbers on swabs stored for 24 hours. While post-sampling bacterial replication is a general issue in sampling regimes, it is less relevant to the detection of anaerobes by PCR since anaerobes will not be viable or replicate during storage unless oxygen is removed and an aqueous environment maintained. Furthermore, since PCR diagnostics are capable of detecting non-viable cells, this decreases the need to maintain live, culturable cells in a sample. Rather, in a PCR diagnostic test, preventing
contamination and maintaining DNA stability is paramount. How inadvertent environmental contamination of swabs affected this study is difficult to assess since it is unknowable how often it occurs. To attempt to decrease the risk of contamination on farms, a simple to use, sterile 12 cm swab was given to farmers before being stored in sterile, dry transport tubes. While anaerobic cells may not be viable or stable under such a dry, oxygen-rich environment, DNA is stable in such conditions as evidenced by the routine use of dry storage of DNA in laboratories.

Beyond what site was examined and how sampling was conducted, there are other issues that need to be considered when using a diagnostic PCR on samples from the hoof environment. Numerous factors can conspire to limit the amount of bacterial material collected, the amount of the target genome extracted and inhibit of amplification of a genome (Wilson 1997). For example, phenolic compounds (also known as humic compounds), produced from decaying organic matter, inhibit PCR amplification (Tsai and Olson 1992, Kreader 1996) and by their nature are variable from sample to sample. As a result, if a diagnostic test works on one sample, there is no guarantee that it will work on the next, even if both samples have the same number of target bacteria present. Other sources of variation include the possibility that the DNA extraction may be inefficient or variable. Therefore, when using PCR diagnostics on clinical samples, analysis and conclusions should only be framed in terms of detection or non-detection and not the presence or absence of bacteria.

The importance of framing a conclusion in terms of non-detection or detection is particularly pertinent when using marker genes to detect a species. While such tests can be specific and sensitive, they risk not detecting novel strains that lack the marker gene or have genetic variation at primer binding sites. How important this is in the case of the lktA gene of *F. necrophorum* is unknown, because neither the genome nor the degree of inter-strain genetic variation of *F. necrophorum* has been described in detail. Despite lktA being found in the type strain of each sub-species, (Narayanan et al. 2001, Oelke et al. 2005, Tadepalli et al. 2008a) there are some reports describing the absence of lktA in *F. necrophorum* (Ludlam et al. 2009). However, Ludlam et al’s work (2009) has been criticised as unsound (Bennett et al. 2010) since it attempted to prove a negative finding using a detection-based PCR diagnostic; the diagnostic probe had mismatches with the
potential target sequences and the experiment attempted to exclude the possibility of false negatives due to genetic variation by using two geographically distinct \textit{lktA} sequences (with 99.75% homology) as controls. This is particularly dangerous in the case of \textit{lktA} since the strain variation of \textit{F. necrophorum} is un-described and it is known to have the potential to be variable, as illustrated by the recent description of \textit{F. equinum} (formally called \textit{F. necrophorum} [Dorsch et al. 2001]) whose \textit{lktA} gene is suspected of being substantively different to that found in \textit{F. necrophorum} (Tadepalli et al. 2008c). As a result, when using a diagnostic based on the \textit{lktA} gene, a lack of detection does not mean \textit{F. necrophorum} is not present, merely it was not detected and it is critically important that conclusions are drawn appropriately in this context.

In this chapter, a new diagnostic PCR was demonstrated to detect the \textit{lktA} gene of \textit{F. necrophorum} on footrot swabs. This PCR was also found to be sensitive, being able to detect 6 genomes/µL in a PCR reaction. Importantly, this sensitivity is similar to the \textit{fimA} PCR for \textit{D. nodosus} (9 genomes/µL). Since both PCRs have comparable sensitivity, it allows conclusions to be drawn on the detection or non-detection of both organisms. In turn, this demonstrates an advantage this approach has over isolation and culture-based systems, since different species will be selected for depending on culture conditions, which makes comparative studies difficult. These difficulties are compounded in an anaerobic culture system if you are attempting to compare anaerobes that have different oxygen tolerances, as \textit{D. nodosus} (Myers et al. 2007) and \textit{F. necrophorum} (Tally et al. 1975, Nagaraja et al. 2005) appear to have. The limitations of using culture as a diagnostic technique have been noted by other authors who observed that molecular techniques can be both more specific and more sensitive than culture alone (Johansson et al. 2000, Fenollar et al. 2006).

It was noted that the consistency of the \textit{lktA} PCR and the \textit{fimA} PCR was improved by the addition of BSA and MgCl$_2$ to the reaction. BSA's likely mode of action is its large binding capacity for phenolics (Weinbach and Garbus 1966, Loomis 1974, Kreader 1996). Therefore, the addition of BSA to a PCR reaction may push the DNA-phenolic and enzyme-phenolic equilibrium in favour of BSA-phenolic complexes. In turn, this would leave more unbound DNA that can be amplified as well as limiting the loss of \textit{Taq} polymerase activity (Kreader 1996). Other ways to deal with the issues of how a variable sample or DNA extraction results effects PCR reactions include the use of internal...
amplification and extraction standards to monitor PCR inhibition and DNA extraction efficiency. Such an approach would also allow a much better understanding of the occurrence of PCR inhibition and the effect BSA has on it. In turn this would have been useful in this study since not all samples were able to be retested using BSA and information on the incidence of PCR inhibition would have allowed stronger conclusions to be drawn.

The lack of detection of *F. necrophorum* and *D. nodosus* in healthy animals does not mean these organisms are not on these animals; merely that numbers are below the detection limits of the PCRs. This result has implications for the ecology of the organisms in healthy sheep since it suggests neither is frequently found in high numbers in healthy feet. For this implies that *F. necrophorum* may not be frequently shed in high numbers via faeces, as has been observed to occur in other mammalian species. For example, in one cattle study, *F. necrophorum* is infrequently shed in faeces (2/81 animals) despite being common in the rumen (13/15 animals) (Smith and Thornton 1993a). This incongruity was explained by these authors on the basis that *F. necrophorum* appears to be a poor competitor with normal intestinal flora and will only survive the passage through the intestine of mice if large doses of cells are consumed (~5 million cfu) or the normal flora is disrupted with antibiotics (Smith and Thornton 1993b). In humans, the *Fusobacterium* genus is detected in 10% (8/80) of faecal samples [taken at random from patients reporting gastrointestinal illness (Nagano et al. 2007)]. In conjugation with Smith and Thornton’s (1993a) findings of a low prevalence in cattle, this suggests it is plausible that *F. necrophorum* is not frequently shed in sheep faeces and, as a result, would not often be found on the feet of sheep. This would possibly have compromised Roberts and Egerton’s (1969) use of sheep faeces to provide an *F. necrophorum* challenge in small pen trials.

This study showed that either, *F. necrophorum* is causing under-running footrot with *D. nodosus* in the field or, if *F. necrophorum* does not cause footrot, it preferentially colonises footrot lesions that also have *D. nodosus* present. If *F. necrophorum* does not cause footrot, its presence in sheep and goats with footrot could still have consequences for footrot management. For example, if the secreted leukotoxin of *F. necrophorum* adversely affects the immune system of the sheep, it may be able to prolong the infection by *D. nodosus*. This effect is not implausible since *D. nodosus* is known to be phagocytised by ovine leukocytes (Emery and Stewart 1984) and *F. necrophorum* can prevent the phagocytosis of *D. nodosus* (Roberts and Egerton 1969). It should be noted, however, that
the survival time of *D. nodosus* did not increase when *F. necrophorum* was present, as it appears to be killed by other factors in the immune system (Roberts and Egerton 1969). Even if it is assumed that *F. necrophorum* does not cause footrot itself, Roberts and Egerton's results (1969), suggest that *D. nodosus* causes footrot more often when *F. necrophorum* is present, implying that *F. necrophorum* may provide a specific factor or environment that allows *D. nodosus* to be more virulent. Egerton and Roberts' (1969) histological studies suggest that at the very least, *F. necrophorum*-like cells can accompany *D. nodosus* during hoof invasion.

Future work could involve investigating the potential for synergistic mechanisms occurring between *D. nodosus* and *F. necrophorum* in-vitro. For example, *D. nodosus* may play a role in protecting *F. necrophorum* from hydrogen peroxide produced as a by-product of oxygen metabolism, since *D. nodosus* carries some genes that may allow aerotolerance, such as alkyl hydro-peroxide reductase (Myers *et al.* 2007). Such interactions may also be mediated by biofilm formation since biofilms can protect bacteria from environmental insults (Costerton *et al.* 1999). Such work may also need to consider the role of both the variable nature of fimbriae (Zhou and Hickford 2000a) and the potential for those fimbriae to be glycosylated (Cagatay and Hickford 2008), given the important role fimbriae play in biofilm formation (O'Toole and Kolter 1998).

The widespread prevalence of *F. necrophorum* on footrot-diseased hooves in this study may have implications for a wide-range of other diseases that *F. necrophorum* is associated with, such as necrobacillosis, calf diphtheria or Lemierre's syndrome (Lemierre 1936, Langworth 1977). In the management of these diseases it may be necessary to consider footrot as a potential source of *F. necrophorum* and/or the effect on the host's immune system if the leukotoxin of *F. necrophorum* is expressed in footrot lesions.

This study demonstrated a diagnostic species-specific PCR able to detect the *lktA* gene of *F. necrophorum*. This showed that both *D. nodosus* and *F. necrophorum* are associated with footrot in sheep and goats in the field. It was also found that *D. nodosus* and *F. necrophorum* tend to be detected together in sheep with footrot. This suggests that either, *F. necrophorum* is a cause of footrot in the field with *D. nodosus*, or it preferentially colonises footrot lesions that have *D. nodosus* present.
3.1 Introduction

Footrot has a variety of pathologies that can be observed as the disease begins, progresses and then either persists, heals, or forms a cryptic lesion (Thomas 1962b, Egerton et al. 1989). These pathologies or processes are variously described in the literature as "severe or virulent footrot" (Thomas 1962b, Egerton and Parsonson 1969), "benign footrot" (Thomas 1962b, Egerton and Parsonson 1969), "symptomless carriers" (Thomas 1962b, Egerton et al. 1989), "covert infections" (Egerton et al. 1989) and "self-curing" (Egerton et al. 1983, Egerton et al. 1989).

Footrot pathologies are identified by a set of clinical signs, combined with the use of bacterial isolation, bacterial detection in smears (Egerton et al. 1969, Egerton and Parsonson 1969) and the analysis of histological sections (Egerton et al. 1969). While both *D. nodosus* and *F. necrophorum* have been found in smears and histological sections (Parsonson et al. 1967, Egerton et al. 1969, Egerton and Roberts 1969), the development of species-specific PCRs has allowed a precise and rapid identification of *D. nodosus* and *F. necrophorum* to be made. The precise and rapid identification that PCR allows is useful in studying footrot pathology, since other techniques require isolation of a bacterium or analysis of bacterial morphology in a smear or section which can be imprecise, time consuming and difficult.

The precision that PCR allows for the identification of bacteria (compared with morphology) cannot be understated in the case of *F. necrophorum*, since it is described as having a pleomorphic morphology with either coccid, bacilli or filamentous forms (Krieg and Holt 1989). Bergy's Manual of Systemic Bacteriology goes on to state, "There is considerable doubt concerning the validity of "F. necrophorum" or "Sphaerophorus necrophorus" identified in many publications prior to 1970" (Krieg and Holt 1989). PCR is also more sensitive than techniques based on isolation or morphology since it is able to detect non-viable, dead and dying bacteria, rather than just those that are able to be isolated, cultured, or stained.
The combination of PCR with case studies describing the pathology observed over the course of footrot development, has allowed a more precise investigation of *F. necrophorum*, *D. nodosus* and pathology. In turn, this has allowed questions to be asked about the role of *D. nodosus* and *F. necrophorum* in various footrot pathologies and as the disease develops and changes.

### 3.2 Materials and methods

#### 3.2.1 The establishment of two experimental footrot "Challenges"

During the springs of 2006 and 2007, sheep were challenged with footrot in a field trial. Each trial consisted of two groups of sheep, a control group and an experimental group. Two sheep with footrot were added to the experimental group to provide a footrot challenge. During pre-trial sampling, sheep were sampled (see below) and assessed to identify which had no signs of footrot, or showed signs of disease and could act as carriers of footrot. Irrigation was applied on site as considered appropriate by farm management to replicate wet conditions (the demand for irrigation varied depending on the weather).

Each trial was conducted in a series of four adjacent, 0.4 hectare paddocks. This allowed the experimental and control groups to be separated at all times by an empty paddock. The groups were routinely moved to an adjacent paddock as demand for feed dictated. Both groups were moved at the same time to maintain an empty paddock between each group.

#### 3.2.2 Trial 1

The first trial was run for 48 days from 12/10/06 to 29/11/06. Irrigation was carried out by farm management from day 18, to replicate a wet spring. The sheep used in this trial were in-lamb Perendale ewes. Lambs were born throughout the early stages of the trial, before being weaned and removed on day 34. No samples were taken from lambs at any stage.

Pre-trial sampling was conducted on day -31. Candidate sheep were assessed for the presence of footrot by hoof observations and the collection of swabs for PCR analysis taken from the skin-horn junction of their hooves. To create an experimental group that was challenged with footrot, two sheep diagnosed with footrot were run with eight healthy
sheep. Eight control sheep were also selected that did not have signs of footrot or foot deformations. The experimental and control groups were managed in a similar manner.

### 3.2.2.1 Sampling procedure, Trial 1

Over the course of Trial 1, samples were taken from all the sheep three times a week, every Monday, Wednesday and Friday. Sheep were assessed for the presence of footrot and scored using the scoring system described below. All sheep had swabs taken from the foot with the highest footrot score or, if no clinical signs were apparent, from the front right foot. If a footrot lesion was present, it was sampled in preference to the skin-horn junction. A faecal sample was collected from the rectum of each sheep.

### 3.2.3 Trial 2

Trial 2 was run for 103 days from 29/8/07 to 23/12/08. Pre-trial sampling and footrot assessment was conducted day -13. Irrigation was carried out by farm management from day 55, to replicate a wet spring. Eight footrot-free, dry (non pregnant) sheep of various ages, breeds and sexes were used as controls. In the experimental group, a mixture of six sheep of various ages, breeds and sexes were challenged, by the addition of four sheep with footrot. Trial 2 was conducted in the same four adjacent paddocks as in Trial 1 to allow for easy stock and pasture management. None of these sheep were thought to be in lamb when the trial began. However, one sheep in the experimental group had a late and unexpected lamb (see case O15).

### 3.2.3.1 Sampling procedure, Trial 2

Over the course of Trial 2, samples were taken from all sheep weekly and each sheep was assessed and scored for footrot, as described below. Swabs were taken from the foot with the highest footrot score or, if no clinical signs were apparent, from the front right foot. If any lesions were present, these were sampled in addition to the skin-horn junction (a different regime from that used in Trial 1). Mouth and faecal swabs were also taken at this time.

### 3.2.4 Footrot scoring system

A footrot scoring system based on the one described by Egerton and Roberts (1971) was used. This system was modified and expanded to allow a single score to describe sheep without footrot, sheep with under-running footrot in more than one foot and to
clarify if under-running was present. Being able to describe under-running is important to a scoring system, since it is a defining sign of footrot (Beveridge 1941). Other authors modifications to Egerton's scoring system also reflect this, where scores that represent under-running are weighted to emphasise them during analysis (Whittington and Nicholls 1995).

### 3.2.4.1 Footrot scoring system used in Trials 1 and 2

Footrot pathology was described using foot scores throughout Trials 1 and 2. The various criteria to determine a score were: inflammation of inter-digital skin and/or damp inter-digital skin; "footrotty" smell; lesions; under-running and/or extensive hoof damage in one or more hooves. The foot score and the pathology it represents are described below, and the most important criteria differentiating the various foot scores are in bold.

<table>
<thead>
<tr>
<th>Description of pathology</th>
<th>Foot Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No interdigital inflammation, dry</td>
<td>1.0</td>
</tr>
<tr>
<td>Slight interdigital <strong>inflammation</strong> or <strong>dampness</strong></td>
<td>1.5</td>
</tr>
<tr>
<td>Inflammation and/or dampness</td>
<td>2.0</td>
</tr>
<tr>
<td>Inflammation, very damp, clammy, <strong>no smell</strong></td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Smells &quot;footrotty&quot;</strong>, very inflamed, usually damp, no lesions</td>
<td>3.0</td>
</tr>
<tr>
<td>&quot;Footrotty&quot; smell, initial <strong>lesions present</strong> on inside of inter-digital skin or hoof, and/or a small lesion on the outside of hoof, <strong>no under-running</strong></td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Under-running</strong> and/or <strong>extensive hoof damage</strong> and/or shedding of hoof may be beginning (note, <strong>disease only present on one foot</strong>)</td>
<td>4.0</td>
</tr>
<tr>
<td><strong>Two or three feet have under-running</strong> footrot</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>All four feet have under-running</strong> footrot</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Sheep were considered diseased if they showed signs of virulent footrot (foot score \( \geq 4.0 \)), benign footrot or inter-digital dermatitis (foot scores 3.0 and 3.5).
3.2.5 Classification of case studies using footrot pathology

Using classifications of footrot pathology, a conceptual framework was constructed (Figure 3.1). This allowed the case studies to be classified and organised as footrot pathologies developed and changed over the course of the trials.

**Figure 3.1. Conceptual framework of footrot pathology.** This conceptual framework describes the pathology seen as footrot develops. A footrot-diseased sheep may go through several cycles of virulent or benign pathologies before persisting as cryptic lesions or self-curing (Egerton 1983). This conceptual framework was used to classify pathologies observed in case studies.

The "virulent footrot" category of pathology was further subdivided into "new under-running footrot cases" and "chronic footrot" categories once the trial began, as different pathologies were observed between these two states. This subdivision of virulent footrot has also been mooted by other authors who noted that some cases are chronic and last for years, while others can develop and change rapidly over several weeks (Egerton 1983). Once analysis began, it became apparent that there were two more types of cases in the "disease free" category. As a result, "disease free" cases were subdivided into the categories "subclinical cases with slight hoof deformation or inflammation" and "subclinical infections" describing cases with no signs of disease, yet that still might have detectable *D. nodosus* or *F. necrophorum*. 
Chapter 3: Case study descriptions of footrot pathology: D. nodosus and F. necrophorum

3.2.6 Detection of *D. nodosus* and *F. necrophorum* from swabs taken from sheep

Once swabs were taken DNA was extracted and stored at 4°C. DNA extractions were used as a template for *fimA* and *lktA* PCRs, with extra BSA and MgCl$_2$ added, as described previously.

3.2.7 Collecting weather data for the two trials

Weather data were accessed using the National Climate Database (CliFlo) maintained by the National Institute of Weather and Atmospheric Research (NIWA). Data were taken from Station 17603, maintained by Plant & Food Research in Lincoln. Station 17603 is approximately 10.8 km from the trial site, with similar weather and topography. It was assumed that this station would provide weather data reflecting conditions consistent with the trial site.

3.2.8 Animal Ethics

Interventions used in Trials 1 and 2 were conducted under the auspices of the Lincoln University Animal Ethics Committee projects 155 and 199.

3.2.9 Comparison of control and experimental groups

Differences in the frequency of bacterial detections between the control and experimental groups were described using statistical analysis. This analysis used a generalised linear mixed model with a Poisson distribution and a logarithmic link function. The experimental groups were considered to be random terms and the treatments fixed terms. The generalised log-linear model was created and applied by Dr Richard Sedcole of Lincoln University using GenStat (version 12).

3.2.10 Analysis of bacterial prevalence and weather

To assess if wet weather or irrigation could be causing an increase in the frequency of bacterial detections within a group, a combination of descriptive statistics and modelling were used. Descriptive statistics (Excel, version 12.0.06) were used to identify observations lying two standard deviations from the mean of a binomial distribution at selected events (wet weather or irrigation). This was followed by the application of a generalised log-linear model to fit a binomial distribution to the data with a factor identifying selection events. The model removed a peak from the data set and compared the modified and original data sets. This was repeated for every peak, allowing the
significance of each peak to be described in the context of the entire data set. The generalised log-linear model was created and applied by Dr Richard Sedcole of Lincoln University using GenStat (version 12).

### 3.2.11 Analysis of bacterial detection and disease

Statistical analysis was carried out to describe mathematical relationships between detection of *D. nodosus*, *F. necrophorum* and disease presentation over time. This analysis comprised two stepwise regressions drawing Pearson correlations that were presented using path analysis (Li 1975). After incomplete data sets were excluded, a total 34 cases were analysed. The trial variables examined were, detection site (skin-horn junction or lesion), detection mean (positive detection was assigned a score of 1, lack of detection a score of 0), detection variance, foot score and foot score variance.

Both stepwise regressions removed variables except skin-horn junction *D. nodosus* variance, skin-horn junction *F. necrophorum* mean and either, lesion *D. nodosus* mean or lesion *F. necrophorum* variance. To maintain simplicity within the analysis, the variables lesion *D. nodosus* mean and lesion *F. necrophorum* variance were analysed separately, since other non-eliminated variables were significant (P>0.01). Analysis was performed by Dr Richard Sedcole of Lincoln University using Minitab (version 15) and GenStat (version 12).

### 3.3 Results and analysis

#### 3.3.1 Trial 1

During the spring of 2006 when Trial 1 was carried out, weather was generally warm with occasional intermittent storms followed by cooling (see Appendix C). Maximum temperatures were usually above 10°C and minimum temperatures above 0°C. A notable heavy rainfall event occurred on day -8, before the trial began. One sheep in the control group (O15) jumped two fences and ran with the experimental group for the last 9 days of the trial.
Table 3.2 Overview of the experimental group in Trial 1

<table>
<thead>
<tr>
<th>Pathology observed</th>
<th>Case</th>
<th>D. nodosus detection frequency</th>
<th>F. necrophorum detection frequency</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>skin-horn junction</td>
<td>lesion</td>
<td>skin-horn junction</td>
</tr>
<tr>
<td>Chronic footrot</td>
<td>G71 (footrot carrier)</td>
<td>N/A</td>
<td>8/23</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>B17 (footrot carrier)</td>
<td>N/A</td>
<td>5/23</td>
<td>N/A</td>
</tr>
<tr>
<td>Cryptic lesion</td>
<td>G49</td>
<td>3/19</td>
<td>4/4</td>
<td>3/19</td>
</tr>
<tr>
<td>Benign footrot and/or inter-digital dermatitis</td>
<td>G6</td>
<td>4/17</td>
<td>1/7</td>
<td>0/17</td>
</tr>
<tr>
<td></td>
<td>G77</td>
<td>0/23</td>
<td>N/A</td>
<td>2/23</td>
</tr>
<tr>
<td>Subclinical cases with slight hoof deformation or inflammation</td>
<td>G48</td>
<td>0/23</td>
<td>N/A</td>
<td>1/23</td>
</tr>
<tr>
<td>Subclinical infection</td>
<td>G18</td>
<td>0/22</td>
<td>N/A</td>
<td>2/22</td>
</tr>
<tr>
<td></td>
<td>B76</td>
<td>0/23</td>
<td>N/A</td>
<td>1/23</td>
</tr>
<tr>
<td></td>
<td>B21</td>
<td>1/23</td>
<td>N/A</td>
<td>0/23</td>
</tr>
<tr>
<td></td>
<td>O15</td>
<td>1/5</td>
<td>N/A</td>
<td>0/5</td>
</tr>
<tr>
<td>Disease free</td>
<td>B55</td>
<td>0/23</td>
<td>N/A</td>
<td>0/23</td>
</tr>
</tbody>
</table>

3.3.1.1 Case studies from the experimental group in Trial 1

Cases from the experimental group that showed signs of disease or had *D. nodosus* or *F. necrophorum* detected are presented as figures. The legend for these figures can be found below (Table 3.3).
Table 3.3. Figure legends

<table>
<thead>
<tr>
<th>Legend</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Trial 1</td>
</tr>
<tr>
<td>T2</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Exp</td>
<td>Experimental group</td>
</tr>
<tr>
<td>Con</td>
<td>Control Group</td>
</tr>
<tr>
<td></td>
<td>Foot score</td>
</tr>
</tbody>
</table>

- When and where samples were taken
- Detection of *D. nodosus*
- Detection of *F. necrophorum*
- Detection of *D. nodosus* and *F. necrophorum*

**Chronic footrot**

**Figure 3.2. Case G71.** This case was a footrot "carrier" that showed signs of chronic footrot.
Figure 3.3. Case B17. This case was a footrot "carrier" that showed signs of chronic footrot.

Cryptic lesion

Figure 3.4. Case G49. At the start of the trial, this case did not show signs of disease until a cryptic lesion opened on the front of the hoof. Later in the trial, on day 34, benign footrot symptoms were observed including formation of a new lesion on the skin-horn junction. Two data points (on days 4 and 35) had no foot score recorded. As a result this missing foot score was replaced by a mean of the preceding and following foot score.
Case G49 was found to have a cryptic footrot lesion. This cryptic lesion burst out from the front of the front, left foot on day 8, 16 days after wet weather. While this site was observed to briefly reopen on day 25, no signs of under-running were seen.

From day 25, the previously undiseased right front foot became slightly inflamed, occasionally smelt "footrotty", and both \textit{D. nodosus} and \textit{F. necrophorum} could be detected (Figure 3.4). However, no lesions or other signs of disease were visible. On day 34, a new lesion formed on the skin-horn junction of the front left foot and both \textit{D. nodosus} and \textit{F. necrophorum} were detected (Figure 3.4).

\textbf{Figure 3.5. Case G6.} Case G6 had sporadic signs of benign footrot during Trial 1. Near the end of the trial on day 34, a cryptic lesion was observed opening. This lesion opened, closed and re-opened over a week.

Case G6 was found to carry a cryptic lesion and showed signs of benign footrot after irrigation began on day 18 and \textit{D. nodosus} became detectable on day 22 (Figure 3.5). On day 34 a cryptic lesion opened on the outside of the hoof and both \textit{D. nodosus} and \textit{F. necrophorum} were detected.
Benign footrot and/or inter-digital dermatitis

Case G77 had inflamed feet which occasionally smelt "footrotty". F. necrophorum was detected on the skin-horn junction twice, first on day 8 and later on day 18 when irrigation began (Figure 3.6).

Figure 3.6. Case G77. This case's feet displayed slight inflammation and an occasional "footrotty" smell, but no under-running or hoof damage was observed.

Two cases (G6 and G49) showed signs of benign footrot or inter-digital dermatitis and were found to be carrying cryptic lesions. These are described above under "Cryptic lesions".
Subclinical cases with slight hoof deformation or inflammation

Figure 3.7. Case G48. This case had occasionally inflamed feet, but no other clinical signs were noted until day 48 of the trial when a slight "footrotty" smell was identified and *F. necrophorum* was detected. A data point on day 35 had no foot score recorded and the missing score was replaced by a mean of the preceding and following foot score.

Subclinical infection

During Trial 1, and three cases (G18, B76, B21) from the experimental group had no signs of disease but had *D. nodosus* or *F. necrophorum* detected on their feet (Figures 3.8, 3.9, 3.10).
Figure 3.8. Case G18. This case had no signs of disease, but *F. necrophorum* was detected. The data point on day 32 was missing, with no foot score recorded. As a result, the absent foot score was replaced by a mean of the preceding and following foot score.

Figure 3.9. Case B76. This case had no signs of disease and *F. necrophorum* was detected.
Figure 3.10. Case B21. This case had no signs of disease and *D. nodosus* was detected.

**Disease free**

One case (B55) from the experimental group appeared disease free and neither *D. nodosus* nor *F. necrophorum* were detected.

**Table 3.4 Overview of the control group in Trial 1**

<table>
<thead>
<tr>
<th>Pathology observed</th>
<th>Case</th>
<th><em>D. nodosus</em> detection frequency</th>
<th><em>F. necrophorum</em> detection frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subclinical infection</td>
<td>B66</td>
<td>2/23 N/A</td>
<td>1/23 N/A</td>
</tr>
<tr>
<td></td>
<td>B30</td>
<td>1/23 N/A</td>
<td>1/23 N/A</td>
</tr>
<tr>
<td></td>
<td>B47</td>
<td>1/23 N/A</td>
<td>1/23 N/A</td>
</tr>
<tr>
<td></td>
<td>O48</td>
<td>1/23 N/A</td>
<td>1/23 N/A</td>
</tr>
<tr>
<td></td>
<td>Y18</td>
<td>1/23 N/A</td>
<td>1/23 N/A</td>
</tr>
<tr>
<td></td>
<td>O15</td>
<td>1/18 N/A</td>
<td>0/18 N/A</td>
</tr>
<tr>
<td>Disease free</td>
<td>O12</td>
<td>0/22 N/A</td>
<td>0/22 N/A</td>
</tr>
<tr>
<td></td>
<td>G69</td>
<td>0/23 N/A</td>
<td>0/23 N/A</td>
</tr>
</tbody>
</table>
3.3.1.2 Case studies from the control group in Trial 1

No cases from the control group showed signs of disease, however, six cases had subclinical infections of *D. nodosus* or *F. necrophorum* detected and these have been presented as figures (Figures 3.11, 3.12, 3.13, 3.14, 3.15., 3.16)

**Subclinical infection**

![Figure 3.11. Case B66. This case had no signs of disease and both *D. nodosus* and *F. necrophorum* were detected.](image)

![Figure 3.12. Case B30. This case had no signs of disease and both *D. nodosus* and *F. necrophorum* were detected.](image)
Figure 3.13. Case B47. This case had no signs of disease and *F. necrophorum* was detected.

Figure 3.14. Case O48. This case had no signs of disease and *D. nodosus* was detected.
Figure 3.15. Case Y18. This case showed no signs of disease and both *D. nodosus* and *F. necrophorum* were detected.

Figure 3.16. Case O15. This case showed no signs of disease and *D. nodosus* could be detected. While this case was part of the control group, on day 39 it was found to have crossed two fences and joined the experimental group. To prevent contamination of the control group, this case was run with the experimental group for the reminder of the trial.

**Disease free**

Two cases from the control group appeared disease free and neither *D. nodosus* nor *F. necrophorum* could be detected.
3.3.2 Trial 2

Trial 2 was conducted during the spring of 2007. Maximum daily temperatures during the trial were above 10°C, while minimum temperatures occasionally dipped below 0°C (see Appendix D). Notable wet weather events occurred before the trial began on day -5 and during the trial between days 23 to 37 (see Appendix D). One sheep (G470) was lost from the trial on day 62.

Table 3.5 Overview of the experimental group in Trial 2

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Case</th>
<th>$D.\ nodosus$ detection frequency</th>
<th>$F.\ necrophorum$ detection frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>skin-horn junction</td>
<td>lesion</td>
</tr>
<tr>
<td>Chronic footrot</td>
<td>NT (footrot carrier)</td>
<td>16/18</td>
<td>13/18</td>
</tr>
<tr>
<td></td>
<td>B194 (footrot carrier)</td>
<td>13/18</td>
<td>9/18</td>
</tr>
<tr>
<td></td>
<td>Y296 (footrot carrier)</td>
<td>14/18</td>
<td>13/18</td>
</tr>
<tr>
<td>New under-running footrot</td>
<td>G75</td>
<td>2/18</td>
<td>10/27</td>
</tr>
<tr>
<td>Self curing footrot</td>
<td>R421 (footrot carrier)</td>
<td>2/18</td>
<td>1/6</td>
</tr>
<tr>
<td>Cryptic lesions</td>
<td>O19</td>
<td>1/18</td>
<td>4/9</td>
</tr>
<tr>
<td>Benign footrot and/or inter-digital dermatitis</td>
<td>G386</td>
<td>0/18</td>
<td>N/A</td>
</tr>
<tr>
<td>Subclinical infection</td>
<td>G393</td>
<td>1/18</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>R476</td>
<td>1/18</td>
<td>N/A</td>
</tr>
<tr>
<td>Disease free</td>
<td>G637</td>
<td>0/18</td>
<td>N/A</td>
</tr>
</tbody>
</table>
3.3.2.1 Case studies from the experimental group in Trial 2

Cases from the experimental group that showed signs of disease or had *D. nodosus* or *F. necrophorum* detected have been presented as figures, as described previously.

**Chronic footrot**

![Figure 3.17. Case NT. This case showed signs of chronic footrot throughout the trial.](image-url)
Figure 3.18. Case B194. This case showed signs of chronic footrot throughout the trial.

Figure 3.19. Case Y296. This case showed signs of chronic footrot throughout the trial.
New under-running footrot

Case G75 showed signs of four new lesions. Each lesion started as an external lesion and developed into under-running footrot before self-curing. As a result, an overview describing when and where samples were taken (Figure 3.20) and data from each individual lesion is presented (Figures 3.21, 3.22, 3.23).

Figure 3.20. Overview of case G75. This case had four separate lesions form, develop under-running and heal. The timing of when each lesion was sampled is shown. Note, during the development of the 1st lesion, an abscess-like pathology formed and was sampled separately.
**Case G75, 1st lesion (right claw sampled, days 6 - 82)**

During the first month of Trial 2, a new lesion appeared and developed into under-running footrot. Initially, a small (~5 mm diameter), damp, lesion was observed on the skin-horn junction before this lesion progressed into under-running footrot. This lesion also developed an abscess-like pathology that was at a separate site on the same claw. Both sites began to heal as the claw appeared to "self cure".

![T2 Exp G75, 1st lesion](Image)

**Figure 3.21. Case G75, 1st lesion.** During the first month of Trial 2, a new lesion formed on the skin-horn junction of the right claw. This lesion developed into under-running footrot and a distinct abscess-like pathology before healing. Before the lesion formed on the skin-horn junction, both wet weather and hoof weakness (hoof unusually pliant and soft) were observed.

**Case G75, 2nd lesion (left claw, sampled from days 48 - 103)**

A new lesion developed in the left claw of the same foot that had harboured the first lesion described above. During the first weeks of disease, extensive hoof damage was observed before the lesion healed. Note, during this time, two other lesions were active on
the same hoof and are described below in "Case G75, 3rd lesion" and "Case G75, 4th lesion".

**Figure 3.22. Case G75, 2nd lesion.** This case showed signs of a second lesion on the left claw with under-running and extensive hoof damage observed. This lesion healed over 2 weeks (days 69-82), before signs of a new lesion (3rd lesion) on the left claw, were noted on day 82.

**Case G75, 3rd lesion (left claw, sampled day 82)**

A new lesion was observed on day 82 in a different site on the left claw described above in "Case G75, 2nd lesion" (Figure 3.22) and both *D. nodosus* and *F. necrophorum* were detected (Figure 3.20). At this time, the diseased claw of "Case G75, 2nd lesion" was described as "well healed" with no signs of disease for two weeks. The site of the 3rd lesion was only sampled once due to another new lesion (4th lesion) forming on the adjacent right claw which was sampled preferentially for the rest of the trial.
Case G75, 4th lesion (right claw, sampled days 90 - 103)

Figure 3.23. Case G75, 4th lesion. This case showed signs of a new under-running lesion on day 90 before showing signs of healing on day 103. It should be noted, before clinical signs of this lesion were observed, this claw appeared free of signs of disease for three weeks since the 1st lesion (described above) had healed.

**Self curing footrot**

Case R421 began the trial with footrot before showing signs of self curing. Initially this case was described as showing signs of footrot including under-running, a sulphurous "footrotty" smell and extensive hoof damage. However, by day 42 no signs of under-running could be found and this case appeared to have healthy feet for most of the trial until day 69 when a single score of 3 (indicating a "footrotty" smell) was recorded.
Case R421. This case began the trial with under-running footrot and showed signs of self-curing, appearing to be free of disease by day 42.

Cryptic lesions

Case O19 appeared to have a cryptic lesion that developed into under-running footrot after irrigation began and this ewe gave birth to a late and unexpected lamb (Figure 3.25). This lesion began deep in the heel and grew rapidly out from this focus of disease (suggesting an undiagnosed cryptic lesion). Within two weeks, this foot showed signs of extensive hoof damage, putrefaction and under-running.
Figure 3.25. Case O19. This case developed under-running footrot from a suspected cryptic lesion after she gave birth to a late lamb and irrigation began. For the rest of the trial, this case showed signs of under-running accompanied by extensive hoof damage.

Benign footrot and/or inter-digital dermatitis

Case G386 showed signs of benign footrot or inter-digital dermatitis with feet intermittently described as inflamed, smelly or wet. However, no hoof lesions, under-running or hoof destruction was observed.
Figure 3.26. Case G386. This case showed sporadic signs of benign footrot or inter-digital dermatitis following wet weather or irrigation beginning.

Subclinical infection

Figure 3.27. Case G393. This case had no signs of disease, but subclinical infections of *D. nodosus* and *F. necrophorum* were detected.
Figure 3.28. Case R476. This case had no signs of disease, but a subclinical infection of *D. nodosus* was detected.

**Disease free**

One case from the experimental group appeared disease free with neither *D. nodosus* nor *F. necrophorum* detected.

**Table 3.6 Overview of the control group in Trial 2**

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Case</th>
<th><em>D. nodosus</em> detection frequency</th>
<th><em>F. necrophorum</em> detection frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>skin-horn junction lesion</td>
<td>skin-horn junction lesion</td>
</tr>
<tr>
<td>Cryptic lesions</td>
<td>R495</td>
<td>0/17 2/13</td>
<td>2/17 4/13</td>
</tr>
<tr>
<td>Subclinical cases with slight hoof deformation or inflammation</td>
<td>G507</td>
<td>1/17 1/3</td>
<td>0/17 0/3</td>
</tr>
<tr>
<td>Disease free</td>
<td>B792</td>
<td>0/17 N/A</td>
<td>0/17 N/A</td>
</tr>
<tr>
<td></td>
<td>B918</td>
<td>0/17 N/A</td>
<td>0/17 N/A</td>
</tr>
<tr>
<td></td>
<td>G546</td>
<td>0/17 N/A</td>
<td>0/17 N/A</td>
</tr>
<tr>
<td></td>
<td>R43</td>
<td>0/17 N/A</td>
<td>0/17 N/A</td>
</tr>
</tbody>
</table>
3.3.2.2 Case studies from control group in Trial 2

No cases from the control group showed signs of under-running footrot. However, two cases had signs of milder disease and *D. nodosus* or *F. necrophorum* was detected.

**Cryptic lesions**

**Figure 3.29. Case R495.** This case was found to be harbouring a cryptic, abscess-like lesion. This lesion was sampled over several weeks before it closed and the lesion surface was sampled until day 76. On day 90, a small damp lesion appeared on the skin-horn junction before it dried, formed a scab and appeared to heal.

Case R495 was found to be carrying a cryptic abscess-like lesion. This lesion was initially identified by slight hoof deformation and probing on day 20. Over the next 22 days, the lesion gradually healed and closed. This lesion did not appear to be virulent and did not act as a focus of new disease during the trial.
Near the end of the trial on day 90, a small (~5 mm in diameter), damp lesion appeared on the skin-horn junction on the same claw that contained the cryptic lesion described above and *F. necrophorum* was detected (Figure 3.29). However, this lesion failed to progress, instead it dried out and healed over several weeks without under-running or hoof damage being observed.

**Subclinical cases with slight hoof deformation or inflammation**

Case G507 had feet which were occasionally inflamed and front hooves that were deformed and cracked. On day 34 one of these cracks opened and was sampled. After irrigation began, this crack was sampled again and tested positive for *D. nodosus* (Figure 3.30). It should be noted that at this time this crack appeared to be in much better physical condition than it had four weeks earlier.

![T2 Con G507 Diagram](image)

**Figure 3.30. Case G507.** This case had deformed and cracked front feet. During the trial one of these cracks opened on day 34 and from days 62-69.

**Disease free**

Six cases from the control group appeared disease free and neither *D. nodosus* nor *F. necrophorum* were detected.
3.3.3 Comparison of the control and experimental groups

A statistical model was used to describe differences in bacterial detection frequency between the control and "treated" experimental groups. The effect of the "treatment" on the experimental groups was found to be significant ($P < 0.01$) with back transformed means of detections rates of 2.925 in the experimental groups versus 0.237 in the control groups. The output of this model can be found in Appendix E. The data used to create this model can be found below in Figures 3.32, 3.33, 3.35, 3.36.

3.3.4 Analysis of bacterial prevalence and weather

Descriptive statistics identified that the peak in *D. nodosus* detection on day 34 of Trial 1 (Figure 3.31) was an outlier from the binomial distribution. When these data were subjected to a generalised log-linear model, the peaks on day 13 ($P = 0.036$) and day 34 ($P = 0.009$) were significant. However, once these data was separated between experimental (Figure 3.32) and control (Figure 3.33) groups, only the peak observed on day 13 within the experimental group was still a significant outlier ($P = 0.013$).
Figure 3.32. Detection of bacteria from the experimental group in Trial 1. The frequency of detection of each bacterium is shown. Peaks which are significant outliers from these data are marked *.

Figure 3.33. Detection of bacteria from the control group in Trial 1. The frequency of detection of each bacterium is shown.
During Trial 2, *D. nodosus* and *F. necrophorum* were detected (Figure 3.34) in both the experimental (Figure 3.35) and control (Figure 3.36) groups. Statistical analysis found these data lay within the expected range of a fitted binomial distribution with no significant outliers present.

![Figure 3.34. Detection of bacteria from control and experimental groups in Trial 2. The frequency of detection of each bacterium is shown.](image)

![Figure 3.35. Detection of bacteria from the experimental group in Trial 2. The frequency of detection of each bacterium is shown.](image)
Figure 3.36. Detection of bacteria from the control group in Trial 2. The frequency of detection of each bacterium is shown.

### 3.3.4.1 Detection of *D. nodosus* and *F. necrophorum* from lesions during Trial 2

**T2, detection of *D. nodosus* from lesions**

Figure 3.37. Detection of *D. nodosus* from lesions during Trial 2. The frequency that *D. nodosus* was detected on lesions swabs is shown. While a peak (circled) of *D. nodosus* detection was observed on day 62, this peak was not a significant outlier.

During Trial 2, a peak in *D. nodosus* detection frequency was noted on day 62 in samples derived from lesions (Figure 3.37). Descriptive statistics identified that this peak
in *D. nodosus* detection was an outlier based on binomial distribution. However, when these data were subjected to a log-linear model using a factor identifying selection events, the peak on day 62 in *D. nodosus* detection was not (P=0.053) a significant outlier.

Two peaks of *F. necrophorum* detection from lesions were observed on days 42 and 62 (Figure 3.38). However, statistical analysis found that these peaks in *F. necrophorum* detection lay within the fitted binomial distribution and were not significant outliers.

### 3.3.5 Analysis of bacterial detection and disease

Data from 34 cases from the experimental and control groups were used to conduct Pearson correlations and presented using path analysis. The two variables, skin-horn junction *D. nodosus* variance and skin-horn junction *F. necrophorum* mean were highly correlated with foot score (P<0.01) (Figures 3.39, 3.40). Other variables which correlated with foot score were lesion *F. necrophorum* variance (P=0.034) and lesion *D. nodosus* mean (P= 0.027). Matrix plots between all variables can be found in Appendix F.
Figure 3.39. Path analysis of relationship between variables including "Lesion, *F. necrophorum variance". Stepwise regression eliminated the least significant variables relative to high foot scores. The path diagram has been rooted to foot score under the assumption that *D. nodosus* and *F. necrophorum* cause footrot. The "Residual" variable represents unknown factors that affect foot score. Correlations between variables are shown, with (P<0.01) marked ** and (0.01<P<0.05) marked *. 
Figure 3.40. Path analysis of relationship between variables including "Lesion, D. nodosus mean". Stepwise regression eliminated the least significant variables relative to high foot scores. The path diagram has been rooted to foot score under the assumption that D. nodosus and F. necrophorum cause footrot. The "Residual" variable represents unknown factors that affect foot score. Correlations between variables are shown, with (P<0.01) marked ** and (0.01<P<0.05) marked *.

These results have been presented as a conference paper (Bennett et al. 2011a), see Appendix B.

3.4 Discussion

Case studies describing footrot pathology, weather, detection of F. necrophorum and D. nodosus were classified using pathology. In addition, statistical analysis was undertaken to assign mathematical relationships between the prevalence of bacteria and disease severity over time. This combination of case studies and statistical analysis allowed qualitative questions to be explored such as "What role could these bacteria be playing in different pathologies?" or "How does the detection of bacteria relate to disease?"
In new, under-running footrot cases, *F. necrophorum* was detected 2.5 times more frequently than *D. nodosus*. This observation is consistent with, and supports, Egerton et al.’s (1969) work where *F. necrophorum* like cells were seen at the leading edge of developing under-running lesions. It is also noteworthy that when footrot began in these case studies, the first detection of *D. nodosus* coincided with the first observation of under-running. This supports both Egerton et al.’s (1969) and Roberts and Egerton’s (1969) conclusions that infection with *F. necrophorum* predisposes sheep to infection by *D. nodosus*, which then causes footrot.

Chronic footrot cases have been described by Egerton et al. (1983), who observed that some sheep were diseased with under-running footrot year after year and that these sheep had a low rate of self-curing (~10%). The microbiology of this subset of sheep has not been described, but given that they had under-running footrot, they may be assumed to have similar microbiology to other under-running footrot cases (Egerton et al. 1969). However, the results obtained for chronic footrot suggest that in the context of *F. necrophorum*, it is possible they may have a different microbiology to "new" under-running footrot cases. In chronic footrot cases, *D. nodosus* and *F. necrophorum* were either, detected at similar frequencies or *D. nodosus* was detected more frequently than *F. necrophorum* (over 5:1 in the case of Y296). This is in contrast to new under-running footrot cases were *F. necrophorum* was detected more frequently.

In this investigation, self-curing of footrot was observed in two cases (R421 and G75). In the case of R421, once self-curing occurred, *D. nodosus* could no longer be detected, but *F. necrophorum* continued to be detected. Case G75 also showed self-curing of the first lesion. However, this case it was observed that once the lesion cured, only *D. nodosus* was detected. The ongoing detection of *D. nodosus* after virulent footrot has "healed" has also been observed by Morgan et al. (1972), who found that after self-curing of footrot, *D. nodosus* could still be observed in disease free sheep in small foci of infection within the inter-digital skin. It is not known if the *D. nodosus* cells detected in this investigation were from such foci or were shed from footrot lesions as they healed. Alternatively, they may have persisted on the surface of the healing lesion, or were transmitted from another source of infection.
While the microbiology of cryptic footrot lesions is unknown they are described as a source of footrot that can be detected by observing aberrant hoof morphology (Egerton et al. 1989). This investigation confirmed that cryptic lesions do carry D. nodosus and this supports Egerton et al.’s (1989) description that they are a source of footrot. However, this investigation also found that F. necrophorum was present in all the cryptic lesions observed. It is not known if cryptic lesions are an important source of F. necrophorum in the field. While sheep faeces have been used to provide a F. necrophorum challenge (Egerton et al. 1969, Roberts and Egerton 1969), other literature (Smith and Thornton 1993a, Smith and Thornton 1993b) implies F. necrophorum is only intermittently shed in ruminant faeces.

In benign footrot pathologies, two patterns of bacterial prevalence were seen. Some sheep had signs of benign footrot, accompanied by detection of F. necrophorum alone. While other sheep with signs of benign footrot tested positive for both F. necrophorum and D. nodosus, and carried cryptic footrot lesions.

The detection of F. necrophorum alone from benign footrot pathologies matches Parsonson's observations that F. necrophorum-like cells were observable in ovine interdigital dermatitis cases (Parsonson et al. 1967). However, some of these case could also be the pathologically identical disease "benign footrot" (Egerton and Parsonson 1969) which is described as being able to be distinguished from ovine interdigital dermatitis by the presence of D. nodosus in smears. This chapter casts some doubt on the validity of this classification, since it was found that cases that had benign footrot pathologies accompanied by detection of D. nodosus were also carrying cryptic footrot lesions, a possible source of D. nodosus.

In several cases no signs of footrot were observed, but D. nodosus or F. necrophorum was detected. Moore et al. (2005) also found D. nodosus in healthy sheep and Morgan et al. (1972) observed microscopic foci of D. nodosus like-cells within the inter-digital skin. However, it is unknown if F. necrophorum can persist in a similar manner. Even if F. necrophorum is unable to persist on the feet of sheep without disease, other sources of F. necrophorum may exist, such as faeces (Egerton et al. 1969, Roberts and Egerton 1969, Smith and Thornton 1993a) or oral spaces, where it is found in humans (Aliyu et al. 2004) and cattle (Mackey 1968).
This investigation revealed peaks in the frequency at which *D. nodosus* or *F. necrophorum* were detected. To study if these peaks could be caused by wet weather or irrigation, a statistical analysis was carried out. This analysis found that peaks in *D. nodosus* detection observed after wet weather or irrigation were not statistically significant outliers from a normal distribution of data. Despite the non-significant findings of this study the observation of peaks in *D. nodosus* detection after irrigation began (Figures 3.34, 3.37) supports Graham and Egerton’s (1968) description of footrot being linked to warm, wet weather.

Further statistical analysis using regression and path analysis showed that increases in foot score were significantly correlated with detection of *F. necrophorum* and *D. nodosus*. This supports previous work describing both of these bacteria as causes of footrot (Roberts and Egerton 1969).

There are several issues that need to be considered when drawing conclusions from this study. The most important of these is the small numbers of cases observed (especially for each different pathology). It also needs to be acknowledged that individual sheep have different disease histories, genetics, nutritional status and immune responses. As a result, individual sheep could skew the results for reasons unknown. Other issues include the differences between the trials, the presence of subclinical infections in the control animals, the risk of "data dredging" (i.e. using observed trends in the data to suggest hypotheses rather than testing a hypothesis) and considering the limits of the statistical methods used. These issues are discussed in more detail below.

As mentioned above, Trial 1 and Trial 2 were not identical. Differences between trials include sampling rates and the presence or absence of lambs. During Trial 1, samples were taken three times a week while during Trial 2 samples were taken weekly. As a result it is possible that weekly sampling would not have detected a short lived peak in *D. nodosus* or *F. necrophorum* detection. While it is unknowable if this occurred, a rises and fall in *D. nodosus* detection over 7 days was observed twice during Trial 1 (from days 8-15 and 29-35 [Figure 3.31]). In contrast, in Trial 2 a similar rise and fall of detection frequencies was not observed.
This difference in sampling rates also reflects how often paddocks were visited and sheep were handled. It is possible handling of sheep was a source of contamination which affected the trials, since more cases of subclinical infections were observed in Trial 1 (n=9) than Trial 2 (n=2). If such subclinical infections were transmitted to the control group in Trial 1, this would be in spite of the precautions taken. These precautions include maintaining a separation between groups of at least one paddock at all times and visiting the control group before the experimental group. However, the possibility that contamination occurred as the result of *D. nodosus* persisting on boots, vehicle surfaces or foot wells in vehicles cannot be eliminated, given that shoe-bathing and sterile shower facilities were not available at the trial site and that *D. nodosus* is an aero-tolerant anaerobe (Myers et al. 2007).

Lambs were present during Trial 1 and absent from Trial 2. Lambs were born throughout the early stages of Trial 1 and removed on day 34. This has two potential effects on the trial. Firstly, the presence of lambs creates an artificially high stocking rate. Secondly, the removal of lambs created a disturbance to the experiment, as it required farm staff to enter the paddocks, draft and transport the lambs, creating the potential for inadvertent contamination. While it is unknowable if such contamination existed, it is conceivable it affected the trial since a significant (P=0.009) peak in *D. nodosus* detection was observed on day 34, when lambs were removed (Figure 3.31).

The presence of subclinical infections of *D. nodosus* in the control sheep is a concern as it undermines the effect of exposing animals to pathogens in the experimental group. However, the presence of *D. nodosus* in the control groups, does not necessarily invalidate this study since, individual cases were categorised by pathology and no under-running footrot was observed in the control groups, suggesting the *D. nodosus* was not coming from active disease within the flock. Other potential sources of *D. nodosus* include contamination by shepherds and equipment used to handle animals, animals crossing fences, unknown reservoirs or vectors, or persistent subclinical infections in the host.

In addition to experimental influences, during analysis of case studies, human bias in the form of "data dredging" may weaken conclusions. Data dredging occurs when trends and patterns are looked for in the data rather than testing a hypothesis. This does not make the trends and patterns observed necessarily invalid; rather it limits the weight that should
be placed on any conclusions. When conducted and assessed correctly, case studies can provide context and data on what may be happening in a whole system. This allows identification of whether a model is inconsistent with reality and why this may have occurred (Flyvbjerg 2006). As such, case studies are a useful observational tool that allows us to make conclusions about what may or may not be occurring and to identify areas for future work (Flyvbjerg 2006).

While step-wise regression and path analysis is useful for describing mathematical relationships between variables, care must be taken to be aware of the limitations and assumptions of this analysis. To be meaningful, path analysis assumes that the relationship between variables is causal. This creates an immediate investigator bias, because path analysis requires that the investigator defines an association between variables before the strength of that relationship is described (by a Pearson correlation). In addition, when drawing conclusions about such correlations, two variables can appear linked if they are both affected by a third external factor. Therefore, when reading a path analysis, while variables may appear to be correlated, this does not mean a causal relationship must exist. All such statistics can describe are the correlations or relative weightings of each variable under the assumptions made during the analysis.

In spite of the above limitations, this work appears to confirm the presence of *F. necrophorum* in developing footrot lesions. This is important given that Bergey's Manual of Systemic Bacteriology (Krieg and Holt 1989) states there is "considerable doubt" on the validity of any *F. necrophorum* identification made prior to 1970. This cast doubt on the work by Roberts and Egerton (1969) and Egerton et al. (1969) who identified that *F. necrophorum* is involved in footrot. These doubts are inflated given that much of Roberts and Egerton's (1969) and Egerton et al.'s (1969) work involved histological observations of *F. necrophorum*-like cells, a difficult prospect given the pleomorphic morphology of *F. necrophorum* (Krieg and Holt 1989).

In new cases showing signs of new under-running footrot, detection of *F. necrophorum* predated under-running, while *D. nodosus* was only detected once under-running began. This suggests that when under-running is present, either *D. nodosus* infects the skin-horn junction as an opportunistic coloniser of diseased feet or it is an etiological agent causing disease from the skin-horn junction.
In all cases of chronic footrot *D. nodosus* was frequently detected, suggesting it is required for disease to be maintained. However, this may not be true for *F. necrophorum* since it was not frequently detected in all cases, with one case (Y296) only having 5/36 swabs testing positive for *F. necrophorum*. In comparison, 27/36 swabs were positive for *D. nodosus*. It is unknowable whether the low rate of *F. necrophorum* detection was the result of single aberrant case, if *F. necrophorum* was present but as an un-described, undetectable strain or if *F. necrophorum* was not required for the maintenance of chronic footrot.

Two cases of healing of footrot lesions were observed. Although both cases had *F. necrophorum* detected during healing, in only one case (G75, 1\(^{st}\) lesion) was *D. nodosus* detected during healing. This contrasting pattern of *D. nodosus* detection highlights that healing lesions are variable environments that may be dominated by host responses rather than the presence or absence of a pathogen. Further complicating such observations is that healing lesions do not exist in isolation and it is possible that in case G75 while the 1\(^{st}\) lesion was healing, it was contaminated by *D. nodosus* shed from the adjacent diseased claw (G75, 2\(^{nd}\) lesion).

In all the cases where cryptic lesions became evident or opened, both *D. nodosus* and *F. necrophorum* could be detected. This is important, as it suggests that both *D. nodosus* and *F. necrophorum* persist together in cryptic lesions. This in turn implies that both *D. nodosus* and *F. necrophorum* could play an aetiological role in the persistence of cryptic lesions. While cryptic lesions are known to be a source of footrot (Egerton et al. 1989) and, by implication, a source of *D. nodosus*, it is unknown if cryptic lesions are an important source of *F. necrophorum* in the field given that it is found in other habitats such as the mouth (Jang and Hirsh 1994) or rumen (Tan et al. 1994b).

If *D. nodosus* and *F. necrophorum* do persist together in cryptic footrot lesions, a possible model of synergism can be envisaged, where both bacteria coexist to resist environmental and immune stresses. In such a model, the leukotoxin secreted by *F. necrophorum* plays a protective role allowing *D. nodosus* to escape phagocytosis (as demonstrated by Roberts and Egerton [1969]). In turn, *D. nodosus* may have a role in maintaining anaerobiosis and supplying *F. necrophorum* with amino acids from digested hoof proteins.
In benign footrot cases, either *F. necrophorum* alone was found or, if cryptic lesions were present, *D. nodosus* was also detected. If this pattern of co-detection of *D. nodosus* and cryptic lesions was repeatable, it implied that either, such animals were vulnerable to *D. nodosus* colonisation, or *D. nodosus* was shed from the cryptic lesions they carried. Furthermore, some benign footrot cases (e.g. G75 and G495) had small (~5mm) lesions which were both challenging to detect and dynamic since they would develop into other pathologies within days. One approach to describe such footrot pathologies and their dynamics is the use digital photography to document how they develop and change over the course of the disease.

During this study, *D. nodosus* was also detected in sheep not showing signs of disease that were not exposed to footrot. If such subclinical infections are common in New Zealand, they may act as sources of future disease and explain why footrot can be difficult to eradicate. There is the potential to use PCR-based diagnostics to study what risk subclinical infections of *D. nodosus* pose as a source of disease and how they are affected by preventative treatments, immune responses and host genetics. The hypothesis that host genetics affects subclinical *D. nodosus* populations is plausible since, in this study, not all sheep exposed to footrot are colonised by detectable numbers of *D. nodosus*. Moreover, host genetics has been shown to have a strong influence on footrot resistance (Outteridge *et al.* 1989, Litchfield *et al.* 1993, Escayg *et al.* 1997, Raadsma *et al.* 1999, Ennen *et al.* 2009). If a genetic predisposition to resist subclinical infection exists, it may represent an important, unknown mechanism explaining how some sheep are able to avoid footrot despite exposure.

When data from the case studies was pooled and aligned with wet weather events, no significant peaks in *D. nodosus* and *F. necrophorum* detection frequency could be observed. Despite these peaks failing to pass the statistical test of significance, this may be a reflection of the small study size and it is still possible that these bacteria do respond to wet weather. Potential mechanisms driving such a response could include existing bacterial populations tending to, be shed, colonise new hosts, or increase in numbers in response to wet weather.

In the case of *F. necrophorum*, it is conceivable that weather could induce a change in diet which results in more growth and shedding of *F. necrophorum* from the rumen.
While it is unknown if this occurs in sheep, in cattle an increase in *F. necrophorum* numbers in the rumen is attributed to a carbohydrate rich diet, resulting in rumen acidosis (Jensen *et al.* 1954) and a rise in lactate and lactic acid, the primary carbon source utilised by *F. necrophorum* (Tan *et al.* 1994b). However, *F. necrophorum* is rarely shed in faeces by cattle (Smith and Thornton 1993a) and mice models suggest *F. necrophorum* does not survive competition with intestinal microbes unless millions of cells are ingested or the "normal" micro-flora is disrupted by antibiotics (Smith and Thornton 1993b). Therefore, it seems likely that in sheep, *F. necrophorum* also resides in the rumen and is shed intermittently in faeces when numbers are high enough or the intestinal micro-flora is disturbed.

The regression analysis of the case studies revealed the following significant correlations: detection of *F. necrophorum* from the skin-horn junction; detection of *D. nodosus* from lesions; variance in *F. necrophorum* detection from lesions, and; variance in *D. nodosus* detection from the skin-horn junction. The correlation of variance in the detection of bacteria with high foot scores is notable since it suggests the link between the pathogens and disease is complex. This complexity is illustrated further by the large correlations between variables, suggesting variables either interact strongly or are influenced by an unknown factor outside the analysis.

Of the results from the regression analysis, the correlation of variance in *D. nodosus* detection with high foot score is particularly noteworthy since it suggests that when the hoof is diseased, *D. nodosus* populations "wax and wane" in numbers. There are several mechanisms that could explain this. For example, *D. nodosus* may exist in a "boom and bust" life cycle where populations quickly expand when nutrients are briefly available on the skin-horn junction. Such a brief availability of nutrients would be particularly growth-limiting to *D. nodosus* since it must import most of its amino acids, being unable to synthesise them metabolically (Myers *et al.* 2007). Once growth of *D. nodosus* halted, the "bust" in its numbers would be exacerbated since it is vulnerable to both phagocytosis (Roberts and Egerton 1969) and oxygen. Alternatively, it is also possible that the variance observed in *D. nodosus* detection is driven by environmental influences affecting how many cells can be sampled and detected using PCR. Examples of such environmental influences include: cells being washed on or off surfaces; the horn becoming softer or harder during disease, and; intermittent shedding of PCR inhibitors from putrefying tissue.
In summary, this investigation supports the contention that *F. necrophorum* acts as a precursor to, and is an inhabitant of, under-running footrot lesions. Both *D. nodosus* and *F. necrophorum* also appear to persist together in cryptic lesions while *F. necrophorum* alone could be found in benign footrot. *D. nodosus* was detected on the feet of disease free sheep not exposed to footrot. Statistical analyses indicated that both detection of bacteria and variance in detection were strongly correlated with disease, implying that the relationship between pathogens and footrot is complex.

The presence of *D. nodosus* in disease free sheep suggests it is highly persistent on the foot or is being transmitted from a subclinical source. One potential subclinical source is the host’s gastro-intestinal tract due to its proximity to the hoof and its anaerobic habitat. This possibility will be examined further in Chapter 4 which investigates whether *D. nodosus* can be detected in (or being shed from) anaerobic spaces of the gastro-intestinal tract such as the teeth, gut or rumen.
Chapter 4: Testing the gastro-intestinal tract of sheep for *D. nodosus*

4.1 Introduction

As described in Chapter 3, *D. nodosus* appears to persist in sheep when no signs of footrot are present. This could be explained by the presence of *D. nodosus* in inter-digital lesions (Moore *et al.* 2005) and small, subclinical foci of *D. nodosus* cells remaining within the inter-digital skin after footrot heals (Morgan *et al.* 1972). However, it is also possible that persistence of *D. nodosus* in healthy feet is due to the reintroduction of infection from an unknown reservoir. This chapter will explore the possibility that the gastro-intestinal tract is one such reservoir.

The gastro-intestinal tract of the sheep may be a candidate reservoir for *D. nodosus*, because it is an anaerobic habitat near the hoof. While *D. nodosus* has not been identified in the gastro-intestinal tract of sheep previously, this could be a reflection of the limitations of conventional microbial techniques. These techniques may have lacked the sensitivity or reliability to detect the organism, especially if it is present at low levels.

If *D. nodosus* was present within the gastro-intestinal tract, it would probably not be a permanent resident given it is possible for mobs of sheep to become free of virulent strains of *D. nodosus* and footrot (Egerton *et al.* 2004). Furthermore, faecal contamination has been used in pen trials to induce footrot (Egerton *et al.* 1969) suggesting that if *D. nodosus* was in faeces, it is likely to be shed infrequently, or in low numbers, since these trials do not report regular failures in their negative controls.

Despite these observations, the possibility that *D. nodosus* is present in the gastro-intestinal tract of sheep cannot be eliminated given the difficulty of culturing from such sites, the anaerobic nature of *D. nodosus* and the proximity of the gastro-intestinal tract to the hoof.

If *D. nodosus* is able to persist in the gastro-intestinal tract of a sheep without footrot, the consequences could be important for footrot quarantine programmes. Therefore, a
diagnostic PCR was used to investigate if *D. nodosus* could be detected in the gastro-intestinal tract of slaughtered sheep and in faecal or oral swabs, from live sheep.

### 4.2 Materials and methods

Two approaches were used in this study: a survey of the gastro-intestinal tract of slaughtered sheep, and the monitoring of live sheep over time. The survey of slaughtered sheep involved taking biopsies from the gastro-intestinal tract and samples or swabs from the contents of the gastro-intestinal tract. The monitoring of live sheep was performed during Trial 2 (described in Chapter 3) by taking rectal and oral swabs.

#### 4.2.1 Survey of the gastro-intestinal tract of slaughtered sheep

A total of 25 sheep with footrot or exposed to footrot were identified and slaughtered. A combination of oral scrapings, biopsies and gut content samples or swabs were taken, extracted and used as templates for *fimA* PCR. Following electrophoresis, if amplimers were present they were cut from the gel and purified using the MinElute™ Gel Extraction Kit (28604, Qiagen) according to the manufacturer's instructions. Following screening, amplimers were sequenced.

Two sets of jaws were collected from slaughtered sheep, and oral scrapings were taken from their periodontal spaces. DNA was extracted from the oral scrapings by using vortexing to suspend them in 500 µL of sterile TE (10 mM Tris, 1 mM EDTA, pH 8.0) before the addition of 50 µL of 10% SDS and 500 µL Tris-buffered phenol (pH 7.8) in chloroform (1:1). Suspensions were then frozen, thawed, extracted and the DNA precipitated by an equal volume of isopropanol before being re-suspended in dH$_2$O, as described previously. This was followed by *fimA* and *lktA* PCRs. Neither PCR contained BSA or extra MgCl$_2$ and the basic diagnostic reaction was performed as described previously.

Ten slaughtered sheep had biopsies taken from the rumen wall, caecum wall and a sample of the caecum contents. All samples were digested to create a lysate using the Wizard® SV Genomic DNA Purification kit (Promega) according to the manufacturer's instructions (McDonald 2005), with the addition of polyvinyl-pyrrolidine (PVP) and 2-$\beta$-mercaptoethanol (2-BME) to the nuclei lysis buffer. Tissue samples were then incubated with proteinase K for 18 hours at 50°C to create a lysate according to the...
manufacturer's instructions. As part of method development, each lysate was extracted using both a phenol-chloroform method (as described previously and the Wizard\textsuperscript{®} SV Genomic DNA Purification Kit (Promega) (used according to the manufacturer's instructions [McDonald 2005]). To assess for PCR inhibition, DNA extractions were serially diluted and used as a template for a \textit{fimA} PCR. This PCR did not contain BSA or extra MgCl\textsubscript{2}, and was performed as described previously.

Thirteen sheep were slaughtered and had gut contents samples or swabs taken. Of these sheep, five were from the Lincoln University research farm and samples of the contents of the rumen, duodenum, caecum) and colon were taken. The remaining eight sheep were from the experimental group of Trial 2 (described in Chapter 3). These eight sheep had swabs taken from the contents of their rumen, duodenum, caecum and colon as well as having their incisor-gum lines swabbed. DNA was extracted from these samples and swabs using a phenol-chloroform extraction, and a \textit{fimA} PCR containing BSA and extra MgCl\textsubscript{2} was performed as described previously.

### 4.2.2 Monitoring of the gastro-intestinal tract of sheep

Throughout Trial 2 (Chapter 3), all sheep had oral and rectal swabs taken weekly. Swabs were stored at -80°C before DNA was extracted as described previously. DNA extractions from individual rectal and oral swabs were pooled in groups of five and used as templates for a \textit{fimA} PCR containing BSA and extra MgCl\textsubscript{2}, performed in triplicate, as described previously. If a pool tested positive for \textit{fimA}, individual DNA extractions that formed the pool were re-tested in triplicate using the \textit{fimA} PCR. Potential \textit{fimA} amplimers were then purified and subjected to screening and sequencing (see below).

Once amplimers were visualised they were cut from the gel and purified. Gel fragments were suspended in 1000 \(\mu\text{L}\) of sterile dH\textsubscript{2}O by heating the suspension for 5 minutes at 70°C. When suspensions were used as a template for further PCR reactions, this created an effective dilution of 1:500-1000 that was appropriate for further PCR analysis (Sambrook \textit{et al.} 1989).
4.2.3 Screening and sequencing of potential fimA amplimers

To screen potential fimA amplimers and indentify which pairs of fimA primers could be used in the sequencing reaction, the purified, diluted gel fragments were used as templates for a repeat 30 cycle fimA PCR using the six possible combinations of the fimA primer pairs.

Amplimers were sequenced using an ABI Prism 3130xl Genetic Analyser with a 16 capillary 50 cm array installed and using Performance Optimized Polymer 7 sequencing reactions and the BigDye Terminator v3.1 Cycle Sequencing Kit (#4336917, Applied Bio Systems, Victoria, Australia). These were performed according to manufacturer's instructions. Post-sequencing clean up used the Agencourt CleanSEQ Sequencing Reaction Clean-up System (#APN 000121, Beckman Coulter).

4.2.4 Analysis

Sequence analysis was performed using DNAMAN (Version 4.0, Lynnon BioSoft, Vaudreuil, Canada. The NCBI GenBank (http://www.ncbi.nlm.nih.gov/) was searched using the BLAST algorithm to identify potential homologues to the sequences. The results were compared with previously described fimA sequences (Zhou and Hickford 2000a).

4.2.5 Assessment of PCR inhibitors in DNA extractions from rumen fluid and faecal swabs

To identify if PCR inhibition could have occurred in samples taken from the gastro-intestinal tract or faecal swabs, samples were spiked with F. necrophorum (an identified ovine footrot isolate supplied by Craig Trotter from Lincoln University) before undergoing DNA extraction, serial dilution and lktA PCR. Fresh rumen fluid and duplicate faecal swabs were taken immediately following slaughter and stored at -80°C. Following thawing on ice, 200 µL aliquots of rumen fluid and a faecal swab were spiked with 150 µL of a F. necrophorum plate scraping. A 200 µL of F. necrophorum plate scraping, 200 µL of rumen fluid and a faecal swab were retained as positive and negative controls. DNA was extracted from faecal swabs and gastro-intestinal samples, as described previously. F. necrophorum plate scrapings were extracted using the same protocol as the gastro-intestinal samples. DNA extractions were serially diluted to $10^{-3}$ in sterile dH2O and used as templates in a lktA PCR containing MgCl2 and BSA as described previously. PCR
products were visualised by electrophoresis using ethidium bromide staining and UV transillumination.

4.3 Results

4.3.1 Survey of the gastro-intestinal tract of slaughtered sheep

FimA was not detected from two slaughtered sheep that had scrapings taken from their periodontal spaces. However 2/10 samples had detectable lktA. None of the ten sheep who had biopsies taken from the rumen or caecum walls tested positive for fimA using PCR. While six potential amplimers were identified following dilution of extracted DNA, these amplimers were eliminated as fimA amplimers by pre-sequencing screening.

Figure 4.1. Screening of poor quality PCR amplicons from a 30 cycle fimA PCR using six possible combinations of fimA primer pairs. Screening of candidate amplicons was used to assess if they were suitable for sequencing and, if so, what primers could be used in the sequencing reaction. The amplicons seen here are typical of the ones produced from the gastro-intestinal survey and monitoring study. Lanes 1-40 show the products of a fimA PCR using a single set of up and down primers with negative (C-) and positive (C+) controls. In comparison to the positive controls, the poor quality of the candidate fimA amplicons is evident and sequencing confirmed that even the best quality of these "products" were likely to be PCR artefacts (see Appendix G). Note, a very faint band of the correct size to be a fimA amplimer was observed in Lane 8, but is not able to be seen in this photo.
Of the 13 sheep that had samples and swabs taken from their gastro-intestinal tract contents, 28 potential fimA amplimers were observed after DNA extraction and PCR. However, after screening only one sample produced two appropriately sized amplimers, which were submitted to sequencing. While these amplimers were of the correct size, the bands were blurry, suggesting they were of poor quality (see Figure 4.1 for a representative example).

4.3.2 Monitoring of the gastro-intestinal tract of sheep

Over the course of Trial 2, approximately 324 oral and 324 rectal swabs were taken from live sheep. After DNA extraction and PCR, 72 potential fimA amplimers were identified. Following screening, 19 potential fimA amplimers were submitted for sequencing. It should be noted that some of these amplimers were poor quality (lacking intensity or were blurry).

4.3.3 Sequencing and analysis of potential fimA amplimers

Of the 21 potential fimA amplimers submitted for sequencing, six were able to be sequenced. BLAST searching of NCBI GenBank found that these six potential fimA sequences were made up of small (~24 bp) repeats. Individually, these repeats were highly homologous with the fimA gene of D. nodosus, but a multiple alignment (Appendix G) using DNAMAN (Version 4.0, Lynnon BioSoft), showed that these sequences were not homologous with a set of complete fimA genes (Zhou and Hickford 2000a). Rather, the ~24 bp repeats matched the conserved regions of the fimA gene homologous to the u1, u2, d1, d2, and d3 primers.

4.3.4 Assessment of PCR inhibitors in DNA extractions from rumen fluid and faecal swabs

To test for the presence of PCR inhibitors in DNA extractions, aliquots of rumen fluid or faecal swabs were spiked with F. necrophorum cells, extracted, serially diluted and subjected to lktA PCR. F. necrophorum was able to be detected by PCR in both the spiked rumen fluid and faecal swabs while un-spiked rumen fluid and faecal swabs tested negative (Figure 4.2).
Chapter 4: Testing the gastro-intestinal tract of sheep for D. nodosus

4.2 Figure 4.2. LktA PCR of serially diluted DNA extractions from rumen fluid and faecal swabs with and without the addition of F. necrophorum. LktA PCR products are shown that have been generated from serially diluted DNA extractions of F. necrophorum (Lanes 1-4), rumen fluid spiked with F. necrophorum (Lanes 5-8), rumen fluid (9-12), faecal swabs spiked with F. necrophorum (Lanes 13-16) and faecal swabs (Lanes 17-20). PCR products from each serially diluted DNA extraction have been displayed in a set of four with dilutions reading left to right of $10^0$, $10^{-1}$, $10^{-2}$ and $10^{-3}$. Positive (C+) and negative (C-) PCR controls are also shown. DNA extraction, lktA PCR and electrophoresis using ethidium bromide staining and UV transillumination were performed as described above. Note, products in Lanes 13-20 are 50% v/v of those in Lanes 1-12.

4.4 Discussion

Several potential amplimers of the fimA gene of D. nodosus were generated from gastro-intestinal tract samples, oral swabs and rectal swabs. These potential fimA amplicons were analysed using a combination of PCR, sequencing, BLAST searches of GenBank and sequence alignments.

While the sequences of the potential fimA amplicons were homologous with the fimA gene of D. nodosus, in most cases, this homology comprised small, short sequences (usually ~24 bp) repeated throughout the length of the amplicon rather than complete sequences. This lack of homology between the amplicon and the complete fimA gene was confirmed by performing a multiple alignment of the sequenced amplicons with a library of published fimA sequences (Zhou and Hickford 2000a). These results suggested that these amplimers were not derived from the fimA gene of D. nodosus, but were instead PCR artefacts that may have been created by primer interactions. As such, it would appear that the fimA gene of D. nodosus was not detectable in the samples studied. This finding is consistent with previous descriptions of D. nodosus, as being found only on the feet of...
The apparent absence of \textit{D. nodosus} in the gastro-intestinal tract of sheep supports the use of ovine faeces as a source of \textit{F. necrophorum} for footrot challenges as they are, arguably, free of \textit{D. nodosus} (Egerton et al. 1969). Furthermore, these findings support current footrot eradication and quarantine programmes, which assume once a sheep is free of footrot and cryptic lesions in the hooves, it is no longer a source of disease (Egerton et al. 1989, Egerton et al. 2002, Egerton et al. 2004).

The observation that PCR can create artefacts as a result of inadvertent amplification demonstrates that care needs to be taken when interpreting PCR results. This is particularly true when primers are poor matches for target sequences, if marker sequences are carried by other species and when samples are taken from environments such as the gastro-intestinal tract that contain a diverse range of microbes and large amounts of eukaryotic and prokaryotic DNA (Lin et al. 1997). Such abundant and diverse DNA in a sample creates issues for PCR since it can provide many possible targets for amplification increasing the risk that primers amplify the “wrong” target or bind to targets in a non-specific fashion.

The methods used in this chapter have been successfully used on a variety of material including, lesion material (Zhou and Hickford 2000a), foot swabs (Chapters 2 and 3) faecal swabs and rumen fluid. However, in such environments, there is an inherent danger that PCR inhibitors can be co-extracted with DNA and affect a diagnostic. To assess if this could have occurred in faecal and rumen samples, the \textit{lktA} PCR and \textit{F. necrophorum} were used since they were appropriate indicators of PCR inhibition and both were available. Despite a lack of PCR inhibition in this test, since individual samples can vary considerably, it is conceivable that some samples could have inhibited the PCRs used. This issue is compounded by that when samples from the first 12 slaughtered sheep were analysed, the \textit{fimA} PCR with BSA was not developed, validated or able to be retrospectively applied to these samples. As a result this highlights that conclusions must be framed in terms of the detection or lack of detection of an organism, rather than the presence or absence of an organism.
While it is impossible to prove a negative statement such as "*D. nodosus* is not present in the gastro-intestinal tract", in this investigation *D. nodosus* was not detected in gastro-intestinal tract of sheep. This suggests that either, *D. nodosus* was not in the gastro-intestinal tract of the sheep studied, or if it was present, numbers were too low or the population too short-lived, to be detected by the methods used.
Chapter 5: Detection of *D. nodosus* and *F. necrophorum* in lame cattle from dairy farms in New Zealand

5.1 Introduction

The detection of *D. nodosus* and *F. necrophorum* in sheep with footrot was a catalyst for an investigation to determine the prevalence of *D. nodosus* and *F. necrophorum* in lame dairy cattle. This knowledge would be important to the dairy industry as well as for ovine footrot management, especially if it could identify or eliminate potential sources of infection.

*D. nodosus* and *F. necrophorum* have both been described as bovine hoof pathogens in Australia (Egerton and Parsonson 1966) and Brazil (Cruz *et al.* 2005). However, bovine strains of *D. nodosus* have previously been described as benign in sheep (Richards *et al.* 1980) and field experiments failed to induce virulent footrot in sheep (Laing and Egerton 1978, Wilkinson *et al.* 1970). Unlike ovine footrot, which is a contagious bacterial disease, lameness in dairy cattle results from the complex interplay of multiple factors such as the environment, management practices, nutrition, genetics and pathogens (Choquette-Levy *et al.* 1985, Chesterton *et al.* 1989).

Lameness is costly, with direct impacts on production, fertility and welfare, as well as an increase in farm labour costs (Westwood *et al.* 2003). The estimated cost of lameness in Australia and New Zealand is $150-$850 per cow, per season, (Jubb and Malmo 1991, Tacoma *et al.* 2004). Moreover, in dairy herds in New Zealand the incidence of lameness is increasing (Chesterton *et al.* 1989, Tranter and Morris 1991, Gibbs and Laporte 2006). The prevalence of lameness is also variable between herds with reported levels of 5-50% of a herd being lame at any one time (Tranter and Morris 1991, Gibbs and Laporte 2006). In New Zealand, lameness rates are seasonal and increase during lactation, with the highest rates observed 5-6 months into lactation (January and February in a typical New Zealand dairy production calendar).

In New Zealand links have been made between lameness and wet conditions (Chesterton 1989) and/or warm ambient temperatures, hoof damage and distances walked by the cattle (José Laporte, Lincoln University, pers. comm.). Chesterton *et al.* (1989) also
showed that track maintenance and farmers moving cows to milking in an impatient manner were predictors of lameness risk in New Zealand. While the bacteriology of lameness in New Zealand has not been studied, overseas histological studies have identified *Treponema*, *Fusobacterium* and *Campylobacter* species invading the hooves of lame cattle in Brazilian feedlots (Cruz et al. 2005). *F. necrophorum* has also been shown to cause bovine footrot if accompanied by *Prevotella melaninogenica* (*P. melaninogenica*) (Berg and Loan 1975), while *D. nodosus* has been implicated in bovine footrot outbreaks in Australia (Egerton and Parsonson 1966).

In New Zealand, little is known about the bacteriology of lameness in pastoral dairy herds, or how frequently either *F. necrophorum* or *D. nodosus* occur in this production system. As a result, an investigation was undertaken to determine the prevalence of *D. nodosus* and *F. necrophorum* in lame cattle in New Zealand dairy herds. Hoof-scrapings were taken from lame cattle by farmers and tested for the presence of *D. nodosus* and *F. necrophorum* using the PCR diagnostic tools described previously.

### 5.2 Materials and methods

#### 5.2.1 Sampling protocol for taking hoof-scrapings from lame dairy cattle

During the September 2005 - May 2006 season, sampling packs were sent to 21 New Zealand dairy farmers. Farmers received instructions (Appendix H) to take hoof-scrapings of pus, crumbling horn, or a small piece of horn (3 mm size). Scrapings were collected from approximately 1 in 10 lame dairy cattle using sterile tubes with built in scrapers (LBS 3805, Biolab, Victoria, Australia). Samples were returned to the laboratory by post and stored at -80°C until required for analysis.

#### 5.2.2 DNA extraction from hoof-scrapings from lame cattle

Following thawing at room temperature, DNA was extracted from hoof-scrapings using the phenol-chloroform based method, as described previously.

#### 5.2.3 PCR amplification of *D. nodosus* and *F. necrophorum*

To test for the presence of *D. nodosus* and *F. necrophorum*, *fimA* and *lktA* PCRs were carried out, as described previously. All PCR products were visualised using ethidium bromide staining and UV transillumination. If large amounts of genomic DNA
were present on the gel, or if inhibition of the PCR was suspected, the PCR was repeated using DNA diluted in sterile dH_{2}O to either 1/10 or 1/100 of the original concentration, or until a sharp band could be resolved or a negative result obtained.

Higher success rates for PCR amplification were obtained using BSA and MgCl\textsubscript{2} in PCR reactions, so the same approach was employed with PCR amplification of DNA extracted from cattle hoof-scrapings (Figure 5.3). Where possible, each DNA extraction was subsequently re-tested for the presence of \textit{D. nodosus} and \textit{F. necrophorum} using \textit{fimA} and \textit{lktA} PCRs containing an additional 400 ng/µL BSA (New England Biolabs) and 2.5 mM of MgCl\textsubscript{2}. If a DNA sample had evaporated during storage, it was re-suspended in 50 µL of sterile dH\textsubscript{2}O before re-testing. Of the 148 DNA extractions originally tested, 147 were re-tested using PCRs with additional BSA and MgCl\textsubscript{2}.

5.3 Results

5.3.1 Prevalence of \textit{D. nodosus} and \textit{F. necrophorum} in hoof-scrapings from lame dairy cattle

A total of 148 samples from lame dairy cattle were received and analysed. After DNA extraction and PCR testing, 79 samples tested positive for the \textit{lktA} gene of \textit{F. necrophorum} and seven samples tested positive for the \textit{fimA} gene of \textit{D. nodosus}. Of these, 21/79 \textit{lktA} amplimers and 4/7 \textit{fimA} amplimers were only retrieved when BSA and extra MgCl\textsubscript{2} were used in the diagnostic PCRs (Figure 5.3).

Typical results are shown in Figures 5.1 and 5.2.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure51.png}
\caption{\textit{LktA} PCR products generated from hoof-scrapings from lame dairy cattle. Lanes 1-4 were generated from hoof-scrapings, Lanes 1 and 3 are positive for \textit{lktA} while Lanes 2 and 4 are negative, other lanes show the negative (C-) and positive controls (C+) using \textit{F. necrophorum} genomic DNA isolated from type strain NCTC no.10575.}
\end{figure}
Chapter 5: Detection of D. nodosus and F. necrophorum in lame cattle from dairy farms in New Zealand

5.4 Discussion

In this study, 148 hoof-scrapings were taken from lame dairy cows and 79 of these tested positive for the lktA gene of F. necrophorum while seven tested positive for the fimA gene of D. nodosus. This suggests that in New Zealand dairy cattle, it is possible that F. necrophorum is a cause of lameness. In contrast, while D. nodosus was present on the hooves of lame dairy cattle, its low prevalence suggests that it is not a widespread cause of lameness. However, the results of this investigation need to be considered carefully, since no samples were able to be collected from healthy cattle so no comparison between healthy
and lame cattle can be made. As a result, no definitive conclusions about the causes or epidemiology of lameness can be drawn from this study, however, some hypotheses can be eliminated and areas of future work identified.

The possibility that *F. necrophorum* is cause of lameness in New Zealand pastoral dairy farms is supported by several overseas studies. In Brazil, *F. necrophorum* was detected in 41% of bovine digital dermatitis lesions and if administered together *F. necrophorum* and *P. melaninogenica* can cause bovine footrot (Berg and Loan 1975). *F. necrophorum* has also been shown to play a role in lameness in cattle feedlots (Cruz et al. 2005) and *F. necrophorum* vaccination reduced lameness rates in cattle in Canadian feedlots (Checkley et al. 2005). If *F. necrophorum* is causing lameness in New Zealand dairy cattle, vaccination against *F. necrophorum* should reduce the prevalence of lameness. However, if *F. necrophorum* is not a cause of lameness in New Zealand dairy cattle, it suggests that it is either an opportunistic coloniser of damaged hooves or a widespread benign organism. This chapter was unable to assess if *F. necrophorum* was a widespread benign organism since it was considered inappropriate for farmers to collect samples from and potentially damage the hooves of healthy dairy cattle in a commercial farming system.

The detection of *F. necrophorum* in lame dairy cattle may also have important implications for other animal diseases that *F. necrophorum* has been described as a cause of such as necrobacillosis (Nagaraja et al. 2005, Agerholm et al. 2007), calf diphtheria (Mackey 1968) and ovine footrot (Roberts and Egerton 1969). It is unknown if the incidence and spread of these diseases is influenced by the presence of *F. necrophorum* on the hooves of lame dairy cattle. However, even if *F. necrophorum* does not directly influence other diseases, it is still possible that *F. necrophorum* is affecting cow health through the expression of its leukotoxin gene. This would require the leukotoxin gene to be expressed, secreted and be able to kill white blood cells of the cow. If the immune system of a dairy cow is negatively impacted as a secondary consequence of lameness, the impact on herd production and health should be considered.

In regard to the detection of *D. nodosus* in lame dairy cattle, despite the small number of hoof-scrapings testing positive for *D. nodosus* (7/148), some conclusions can still be drawn. While *D. nodosus* would appear to infect a proportion of lame dairy cattle in
New Zealand, it would seem unlikely to be a widespread cause of lameness in dairy cattle given its low prevalence. Of course, it is conceivable that the sampling method failed to collect the organism from all the lame hooves (e.g. if the bacterium was sequestered in a hoof). The detection of *D. nodosus* in this investigation is still notable given that the lame dairy cattle studied were from specialised dairy farms and would be expected to have little contact with sheep carrying footrot. This suggests that *D. nodosus* either, regularly re-infects lame dairy cattle from an unknown reservoir, or is able to persist in cattle within a dairy herd. Given that *D. nodosus* is described in some lameness disorders of cattle, such as bovine footrot (Egerton and Parsonson 1966, Thorley *et al.* 1977) and digital dermatitis (Blowy and Sharp 1988), this gives credence to the notion that *D. nodosus* is able to persist within a dairy herd.

The detection of *D. nodosus* in lame dairy cattle also has implications for sheep production systems, since *D. nodosus* has been transmitted from cattle to sheep (Wilkinson *et al.* 1970). While Wilkinson *et al.*’s (1970) trial only resulted in benign footrot in sheep, virulent footrot has been induced in sheep by inoculation with lesion material from diseased cattle hooves (Egerton and Parsonson 1966). If lame dairy cattle are able to act as a reservoir for *D. nodosus* strains that are both transmittable and virulent in sheep, this source needs to be considered in the management of ovine footrot control and quarantine programmes.

Typically, New Zealand dairy herds do not have contact with sheep during the milking season when lameness rates are high. However, during winter, New Zealand dairy herds may be moved off-farm onto "run-off" properties which also graze sheep, creating the opportunity for inter-specific transmission of *F. necrophorum* and *D. nodosus*. While the occurrence and impact of *D. nodosus* transmission between dairy cattle and sheep is unknown, it may be low on winter "run-off" properties, given footrot outbreaks tend not to occur in cold weather (Graham and Egerton 1968).

In summary, little is known about the effects *D. nodosus* or *F. necrophorum* on lameness in New Zealand dairy cattle or whether these dairy cattle could be a source of footrot in sheep. This raises questions for dairy production and management, such as "Do lame cattle need to be quarantined from sheep?" and "Should dairy cattle and sheep be
Chapter 5: Detection of D. nodosus and F. necrophorum in lame cattle from dairy farms in New Zealand

...separated from each other on run-off properties?”. One approach to address these questions is to identify if the *F. necrophorum* strains found in cattle are similar to those found in ovine footrot. This is investigated in Chapter 6, where sequence variation of the *lktA* gene is examined to identify if different variants of *lktA* (and by implication, different strains of *F. necrophorum*) are present in different hosts or associated with different pathologies.
Chapter 6: Variation in *Fusobacterium* leukotoxin genes in relation to different hosts and pathologies

6.1 Introduction

*D. nodosus* has been described as having "extensive, genetic diversity" (Myers et al. 2007) and a wide range of virulence (Stewart et al. 1986). In contrast, little is known about the genetic diversity of *F. necrophorum* and what role it may play in ovine footrot, despite its apparent role in disease (Roberts and Egerton 1969).

Roberts and Egerton’s (1969) study used an isolate of *F. necrophorum* and other authors described ovine strains found in foot abscesses as biovar AB (Corner et al. 1996). However, *F. necrophorum* biovar AB has not been classified and it is unclear how it relates to the current classification of *F. necrophorum* (Citron 2002), *F. necrophorum* sub sp. *necrophorum* (formerly biovar A), *F. necrophorum* sub sp. *funduliforme* (formerly biovar B) and *F. varium* (formerly biovar C). In addition, these different sub-species tend to be identified from species other than sheep. For example, *F. necrophorum* sub sp. *necrophorum* tends to be isolated from cattle, while *F. necrophorum* sub sp. *funduliforme* tends to be isolated from humans (Tadepalli et al. 2008a, Tadepalli et al. 2008b).

The sub-species of *F. necrophorum* are described with differences in the leukotoxin gene (*lktA*) promoter length; the distance of promoter from the structural genes; the sequence of the promoter and its strength (Zhang et al. 2006); the level of expression of leukotoxin (Okwumabua et al. 1996, Narayanan et al. 1997); the potency of the leukotoxin (Narayanan et al. 1997) and the structural gene sequence and length (Narayanan et al. 2001, Tadepalli et al. 2008a, Tadepalli et al. 2008b). The only other bacterial species known to carry this leukotoxin is *F. equinum* (Tadepalli et al. 2008c), a bacterium that is considered phenotypically similar to, but genetically distinct from *F. necrophorum* (Dorsch 2001). Despite *F. equinum* being shown to carry and secrete a leukotoxin gene product, no sequence information about the leukotoxin gene has been able to be generated (Tadepalli 2008c). As a result study of *F. equinum* was also required to determine if it could lead to false positives in the *lktA* PCR used previously.
In summary, there is little information about the strains, species and genetic variants of *F. necrophorum* that carry the *lktA* gene and that may be associated with ovine footrot, or similar diseases in other hosts. Therefore, variation in the *lktA* gene of *F. necrophorum* and *F. equinum* was investigated in sheep, goats and cattle.

### 6.2 Material and methods

PCR of the *lktA* gene of *F. necrophorum* was undertaken from various clinical samples and the PCR products were screened and sequenced in two separate experiments. The first experiment used samples from sheep, goats and cattle with footrot and was performed by a collaborator, Dr Huitong Zhou. It is described in a jointly authored paper in Appendix B. In the second experiment, a comparison of *lktA* PCR products generated from samples taken from lame dairy cattle and goats and sheep with footrot was performed by G. N. Bennett. Both these experiments are described below.

#### 6.2.1 Experiment 1: Comparing *lktA* amplimers from cattle, sheep and goats with footrot

##### 6.2.1.1 Sampling procedures and processing

Swabs were used to sample exudates from the axial skin-horn junction of hooves of nine cattle, 15 sheep and six goats with footrot. The end of each swab was broken off into a sterile 1.5 mL tube containing 0.7 mL PBS with 20 mM Na₂EDTA (adjusted to pH 8.0), before being frozen at -20°C until DNA extraction. DNA was extracted from the swabs, as previously described (Zhou and Hickford 2000a).

##### 6.2.1.2 *LktA* PCR amplification

An *lktA* PCR was designed using the primers *lktA-up* (5’-aatcggagttagtagttcgtg-3’) and *lktA-dn* (5’-ctttggtaactgcactgc-3’). Amplification was performed in a 20 µL reaction containing 1 µL of extracted DNA, 0.25 µM of each primer (synthesised by Integrate DNA Technologies, Coralville, IA), 150 µM dNTPs (Eppendorf, Hamburg, Germany), 3.5 mM MgCl₂, 500 ng/µL bovine serum albumin (Sigma, St Louis, MI), 0.5 U *Taq* DNA polymerase (Qiagen) and 1 × reaction buffer as supplied. The PCR reaction was carried out in a Mastercycler EP thermocycler (Eppendorf) with an initial denaturation at 94°C for 2 min, followed by 32 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 40 s, with a final
extension step at 72°C for 5 min. PCR products were visualised using ethidium bromide staining and UV transillumination.

6.2.1.3 Validation of the lktA PCR

The specificity of the lktA PCR was validated by testing it against DNA extracted from type strains of *F. pseudonecrophorum* (ATTC 51644), *F. varium* (ATTC 8501), *F. necrophorum* sub sp. *funduliforme* (ATTC 51357), *F. necrophorum* sub sp. *necrophorum* (NCTC 10575), *F. nucleatum* sub sp. *nucleatum* (ATCC 25586) and *F. equinum* (NCTC 13176). *D. nodosus* (strain A198) was also included as this bacterium is associated with footrot. DNA from all bacterial cultures except *F. equinum* was extracted from cells by boiling for 10 min in 0.8% (v/v) Triton X-100 solution and centrifugation at 12 000 ×g. A 1µL aliquot of the supernatant was used as a template for PCR amplification. *F. equinum* DNA was supplied by Professor Julian Rood’s research group (Monash University, Vic, Australia) who obtained the original type strain from Dr Jacqueline Norris (University of Sydney, NSW, Australia).

6.2.1.4 Comparison of lktA amplicons using single-strand conformational polymorphism

Single-strand conformational polymorphism (SSCP) was used to compare the different lktA amplicons (Figure 6.1). A 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95°C for 5 min, samples were cooled on wet ice and loaded on 16 cm x 18 cm, 12% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 300 V for 18 h at 5°C in 0.5 x TBE buffer. Gels were visualised using a modified silver-staining method (Sanguinetti *et al.* 1994).

6.2.1.5 Cloning and characterisation of representative lktA amplimers

DNA samples representative of different SSCP patterns were selected for amplification using *Pwo* SuperYield DNA polymerase (Roche Applied Science, Mannheim, Germany) and the methods described above. Amplicons were ligated into the pCR® 4 Blunt-TOPO® vector (Invitrogen) and then a 2 µL aliquot of the ligation mixture was used to transform competent *E. coli* cells (One Shot® INVαF’, Invitrogen), following
6.2.1.6 Sequencing of the cloned lktA DNA

Plasmid DNA was extracted from overnight cultures of the original clones using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced in both directions using the M13-forward and reverse primers at the Waikato DNA Sequencing Facility, University of Waikato, Hamilton, New Zealand. Identical sequences obtained from at least three separate clones that produced identical PCR-SSCP patterns were then subjected to further sequence analysis.

6.2.2 Experiment 2: Comparing lktA amplimers from lame cattle and sheep and goats with footrot

Once the method for characterisation of lktA PCR products using SSCP had been developed and validated, it was used to compare a selection of lktA PCR products generated from lame dairy cattle, sheep with footrot and goats with footrot. These were screened using the SSCP typing system, as described above.

6.2.3 Identifying the F. equinum leukotoxin sequence

As part of the lktA PCR validation, a portion of the lktA gene of F. equinum was sequenced to determine if F. equinum did contain an lktA gene and if F. equinum DNA was able to be amplified with the lktA PCR described previously in section 2.2.1.3.

Identifying if F. equinum contained an lktA gene was delayed by environmental protection regulations preventing the importation of live bacteria into New Zealand. These difficulties were overcome by importing F. equinum DNA extracted from the reference strain (NCTC 13176). This F. equinum DNA was extracted by Dr Ruth Kennan and Professor Julian Rood (Monash University, Vic, Australia) who, in turn, obtained the reference strain from Dr Jacqueline Norris (University of Sydney, NSW, Australia).
6.2.3.1 Initial PCR of \textit{F. equinum}

The initial work involving PCR design and sequencing of \textit{lktA} amplimers was performed by a collaborator, Dr Huitong Zhou. For a complete description of this work see Appendix B.

Testing of \textit{F. equinum} DNA using the \textit{lktA} PCR failed to produce an amplicon. However, after further primer refinement, two primers were identified that were able to amplify \textit{lktA} like sequences from \textit{F. equinum}. These primers were, 5'-acagcagattctaagcaagg-3' and 5'-gaaacaactccagatactcc-3'. Amplification was performed using 35 cycles, with an annealing temperature of 57°C and without the addition of BSA. Other than these changes, this \textit{lktA} PCR was performed as described above.

6.2.3.2 Sequencing of amplimers of the \textit{lktA} gene of \textit{F. equinum}

Amplimers of the \textit{lktA} gene of \textit{F. equinum} were cloned and sequenced, as described above.

6.2.4 Detection of \textit{F. equinum} in footrot lesions

Once the leukotoxin sequence of \textit{F. equinum} became available, this sequence was used to design a species-specific PCR able to amplify \textit{F. equinum} \textit{lktA} sequences, but not \textit{lktA} sequences, found in \textit{F. necrophorum} sub sp. \textit{necrophorum} or \textit{F. necrophorum} sub sp. \textit{funduliforme}. This method was used to test if \textit{F. equinum} \textit{lktA} sequences could be detected in a selection of bovine and ovine footrot samples.

6.2.4.1 \textit{F. equinum} \textit{lktA} PCR conditions

The \textit{F. equinum}-specific \textit{lktA} amplification used two new primers, 5'-gtttctgtgaatcaactatcc-3' and 5'-ccgattccaacaattccgc-3'. Amplification was performed using 40 cycles with an annealing temperature of 59°C. Other than these changes, this PCR was performed, as described above, to amplify the \textit{lktA} gene \textit{Fusobacterium necrophorum}.

6.2.4.2 Sequencing and cloning of \textit{F. equinum} \textit{lktA} amplimers from footrot swabs

\textit{LktA} amplicons from \textit{F. equinum} were amplified from swabs retrieved from the hooves of sheep with footrot, and cloned and sequenced, as described above.
6.2.5 Comparative sequence analysis of \textit{lktA} sequences using multiple alignment and phylogenetic analyses

Unless otherwise stated, the BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov) databases to find and compare sequences. If a figure describing a multiple alignment or phylogenetic tree has been derived from a publication, it is described when the figure is first mentioned in the text and a reference made to the appendix where the paper is described. Such figures were generated by using DNAMAN (Version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada) to perform sequence alignments, translations and comparisons.

Multiple alignments and phylogenetic trees not derived from papers were generated using DNAMAN (Version 4.0, Lynnon BioSoft) to perform sequence alignments, translations and comparisons. In these cases, phylogenetic trees were constructed on the basis of genetic distances estimated by the Neighbour-Joining method (Saitou 1987). The reliability of the trees was estimated by bootstrap confidence values (Felsenstein 1985), using 1000 bootstrap replications.

6.3 Results

6.3.1 Experiment 1: Comparing \textit{lktA} amplimers from cattle, sheep and goats with footrot

The \textit{lktA} PCR was found to only produce amplicons of the correct size with \textit{F. necrophorum} sub sp. \textit{necrophorum} (NCTC 10575) or sub sp. \textit{funduliforme} (ATCC 51357). After validation of this \textit{lktA} PCR, it was used to assess footrot samples and 9/9 cattle, 5/6 goats and 13/14 sheep tested positive for the \textit{lktA} gene. These \textit{lktA} amplicons were typed into variants using SSCP (Figure 6.1). Variant A tended to be found in cattle while variant C tended to be found in goats and sheep. These results are summarised in Table 6.1.
Chapter 6: Variation in Fusobacterium leukotoxin genes in relation to different hosts and pathologies

Figure 6.1 SSCP of the four lktA variants amplified using PCR. The SSCP patterns of the four variants (A, B, C and D) identified in this study are shown.

Table 6.1. LktA variants found on cattle, sheep and goats with footrot

<table>
<thead>
<tr>
<th>Leukotoxin variant</th>
<th>Host</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>8/9</td>
<td></td>
<td>1/9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>2/13</td>
<td>11/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td>1/5</td>
<td>3/5</td>
<td>1/5</td>
<td></td>
</tr>
</tbody>
</table>

These results have been published (Zhou et al. 2009a), see Appendix B.

6.3.2 Experiment 2: Comparing lktA amplimers from lame cattle and sheep and goats with footrot

Of the lktA amplicons initially screened using SSCP, 32/59 cattle samples, 15/19 sheep samples and 9/9 goat samples could be successfully typed using SSCP. Those that could not be typed had no visible bands or patterns. LktA amplicons from cattle were most often variant A (28/32) while those from sheep and goat were most often variant C (21/24). Only 1/56 of the samples analysed contained a detectable mixture of two variants. These results are presented below in Table 6.2.
Table 6.2. *LktA* types found on lame cattle and sheep and goats with footrot

<table>
<thead>
<tr>
<th>Leukotoxin variant</th>
<th>Host</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>A+C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>28/32</td>
<td>3/32</td>
<td></td>
<td></td>
<td>1/32</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>2/15</td>
<td>13/15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td>1/9</td>
<td>8/9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These results have been prepared for publication and are undergoing internal review (Bennett et al. 2011b), see Appendix B.

6.3.3 Combined data from Experiment 1 and Experiment 2

By combining the data from the Experiment 1 and Experiment 2, a larger, more comprehensive data set was constructed (Table 6.3). This comprised 83 different *lktA* amplimers derived from cattle, goats and sheep typed into four variants (Table 6.3). These data showed that variant A tended to be found in lame cattle, variant C tended to be found in sheep with footrot and goats and variants B and D were relatively rare. Furthermore, infections with multiple variants of *F. necrophorum* appear to be infrequent with only 1/83 samples having two detectable variants in the same sample at the same time.

Table 6.3. Combined results from Experiment 1 and Experiment 2 comparing the *lktA* variants found on cattle, sheep and goats

<table>
<thead>
<tr>
<th>Leukotoxin variant</th>
<th>Host</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>A+C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>36/41</td>
<td></td>
<td>4/41</td>
<td></td>
<td>1/41</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td></td>
<td>4/28</td>
<td>24/28</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td>1/14</td>
<td>1/14</td>
<td>11/14</td>
<td>1/14</td>
<td></td>
</tr>
</tbody>
</table>
6.3.4 Comparative sequence analysis of \textit{lktA} variants A, B, C and D

Comparative sequence analysis using the NCBI GenBank database and DNAMAN (Version 5.2.10, Lynnon BioSoft) revealed that variant A was identical to the type strain of \textit{F. necrophorum} sub sp. \textit{necrophorum}. While variants B, C and D were found to be similar, but not identical, to other known sequences of the \textit{F. necrophorum} leukotoxin gene. This comparison was made using a multiple alignment (Appendix I) and this multiple alignment is an excerpt taken from a co-authored paper (Zhou \textit{et al.} 2009a) that can be found in Appendix B.

6.3.5 Sequence generated from the \textit{lktA} gene of \textit{F. equinum}

A 407 bp DNA sequence was able to be amplified from \textit{F. equinum} DNA using leukotoxin-specific primers. This sequence was significantly different from other sequences in the NCBI GenBank, but was 67.8\% and 68.1\% homologous with leukotoxin sequences from \textit{F. necrophorum} sub sp. \textit{necrophorum} and \textit{F. necrophorum} sub sp. \textit{funduliforme} respectively. However, at the amino acid level, blocks of sequence conservation were observed (Appendix J). Note, this multiple alignment has been extracted from a co-authored publication (Zhou \textit{et al.} 2009b), found in Appendix B.

6.3.6 Detection of \textit{F. equinum} on footrot swabs

Fourteen footrot samples from cattle and sheep were tested for the presence of the \textit{lktA} \textit{F. equinum} sequence. \textit{F. equinum} \textit{lktA} sequences were detected in 6/14 of these samples (see Figure 6.2).

![Figure 6.2. \textit{F. equinum} specific \textit{lktA} PCR used on footrot swabs.](image)

Lanes 1-3 show type strains of \textit{F. equinum} (Lane 1), \textit{F. necrophorum} sub sp. \textit{necrophorum} (Lane 2), and \textit{F. necrophorum} sub sp. \textit{funduliforme} (Lane 3). Lane 4 shows an ovine footrot lesion and Lane 5 shows a bovine footrot lesion. Note \textit{D. nodosus} was detected previously from both footrot samples using a \textit{fimA} PCR (Zhou and Hickford, 2000a).
These results have been published (Zhou et al. 2009c, see Appendix B).

6.3.7 Comparative sequence analysis of \textit{lktA} sequences from \textit{F. equinum}, \textit{F. necrophorum} reference strains and \textit{F. necrophorum} variants A, B, C and D

A multiple sequence alignment was used to compare \textit{lktA} variants A, B, C, and D, a portion of the \textit{lktA} gene from \textit{F. necrophorum} sub sp. necrophorum, a portion of the \textit{lktA} gene from \textit{F. necrophorum} sub sp. funduliforme and a portion of the \textit{lktA} gene from \textit{F. equinum}. This multiple alignment can be found in Appendix K.

Following this multiple alignment, a phylogenetic tree was constructed comparing \textit{lktA} sequences of type strains, variants A, B, C, D and the \textit{F. equinum} \textit{lktA} sequence (Figure 6.3). The phylogenetic tree illustrated that the \textit{lktA} variant B sequence was similar to the \textit{lktA} sequences of the type strains \textit{F. necrophorum} sub sp. necrophorum (Fnn), \textit{F. necrophorum} sub sp. funduliforme (Fnf), while variants C and D were distinct from the \textit{lktA} sequences of the type strains Fnn and Fnf. The \textit{F. equinum} \textit{lktA} sequence appeared to be genetically distinct from other \textit{lktA} sequences studied here.

![Figure 6.3. Phylogenetic tree comparing \textit{lktA} type strain sequences and new variants.](attachment:image.png)

A comparison was made of type strains, \textit{F. equinum} and \textit{lktA} variants found in cattle, sheep and goats. The number at the forks show bootstrap confidence levels. \textit{F. necrophorum} sub sp. necrophorum (Fnn) is represented by "Variant A, (Fnn)". Fnn was found to have an identical \textit{lktA} sequence to variant A and, consequently, Fnn and variant A are combined in this figure. \textit{F. necrophorum} sub sp. funduliforme (Fnf) variants B, C and D and \textit{F. equinum} \textit{lktA} sequences are also shown. This phylogenetic tree was created using DNAMAN (Version 4.0, Lynnon BioSoft), the Neighbour-Joining method (Saitou and Nei 1987) and 1000 bootstrap replications to estimate confidence levels.
6.4 Discussion

In this chapter, genetic diversity in the lktA genes of *F. necrophorum* and *F. equinum* was studied. A combination of PCR, SSCP and sequencing identified four variants of the lktA gene of *F. necrophorum* (A, B, C and D). Variant A was found to match the lktA gene of the type strain of *F. necrophorum* sub sp. *necrophorum*, while the other three lktA variants (B, C and D) have not been previously described. It was also observed that variant A was found most often in lame cattle and cattle with footrot, while variant C was found most often in sheep and goats with footrot. Furthermore, the majority of lktA variants were monoclonal with only a single lktA variant detectable (82/83 samples). A previously un-described lktA-like gene sequence was found in *F. equinum*. This sequence was detected in both bovine and ovine footrot samples.

If the new *F. necrophorum* lktA variants described here are representative of the genome of *F. necrophorum*, it suggests there is greater variation in this organism than hitherto thought. If this is true, given the two sub-species of *F. necrophorum* appear more closely related than variants C and D, it is possible these variants represent un-described sub-species of *F. necrophorum*. In the context of *F. necrophorum*, other authors have noted that specific sub-species tend to be isolated from specific hosts such humans and cattle (Nagaraja et al. 2005, Tadepalli et al. 2008a), and here we report that different variants of lktA tend to be found in cattle, sheep and goats.

Previous work (Emery et al. 1985) in sheep and cattle, demonstrated that distinctive *F. necrophorum* biotypes tended to be isolated from pathologies in sheep and cattle. Biovar B (*F. necrophorum* sub sp. *funduliforme*) was isolated from the inter-digital lesions of cattle, while biovar AB was found in foot abscesses of both sheep and cattle. Furthermore, *F. necrophorum* biovar AB was demonstrated to induce abscesses in sheep following devitalisation of tissue (Corner et al. 1996). This finding is somewhat at odds with the conjecture that specific strains of *F. necrophorum* are found in specific host species, but it does suggest that specific strains may be found in specific pathologies. Either of these explanations could produce some the results observed in this chapter, given that sheep or goats with footrot and lame cattle differ in both pathology and species.
Chapter 6: Variation in Fusobacterium leukotoxin genes in relation to different hosts and pathologies

If these lktA variants represent strains of *F. necrophorum* that tend to be found in specific hosts, this does not necessarily mean that these strains are host-specific. Rather, it is possible the observed results could be explained by specific strains being found associated with particular pathologies. This would be a reflection that variant C of lktA was found pre-dominantly in sheep and goats with footrot and variant A was found in cattle diagnosed with lameness (a general syndrome rather than a specific disease).

Even if the novel variants described in this chapter do not represent new sub-species, given their genetic distance from type strains of *F. necrophorum*, they may behave and function quite differently from the type strains. Such differences in expression are not unexpected considering that even the relatively closely related sub-species *F. necrophorum* sub sp. *necrophorum* and *F. necrophorum* sub sp. *funduliforme* have considerable differences in leukotoxin sequence, expression and function (Tadepalli *et al.* 2008b).

To identify how important this work is to understanding ovine footrot will require further isolation and characterisation of the *F. necrophorum* strains found in ovine footrot and a study of the strains that carry the lktA variants observed. Once isolates have been purified, analysis of 16sRNA genes and the genome could be carried out to clarify the phylogeny of *F. necrophorum* and identify if these ovine strains represent a separate sub-species. In addition, this might lead to a description of a *F. necrophorum* type strain found in sheep. This could be particularly relevant to ovine footrot, since our current understanding of *F. necrophorum* biology is, for the most part, based on human and cattle *F. necrophorum* isolates. Previous studies comparing the leukotoxin of *F. necrophorum* strains have identified substantial differences between sub-species of *F. necrophorum*. If ovine *F. necrophorum* strains are a different sub-species, their lktA could be useful to study, since leukotoxin variation has only been described in *F. necrophorum* sub sp. *necrophorum*, *F. necrophorum* sub sp. *funduliforme* (Oelke *et al.* 2005) and *F. equinum* (Tadepalli *et al.* 2008c).

The observation that variant C of lktA was found most commonly in both sheep and goats supports the contention that caprine and ovine footrot have similar bacteriologies (Claxton and O’Grady 1986). However, when comparing these caprine and ovine footrot samples with those from lame dairy cows care is needed. This is because lameness in dairy
cattle is considered a general syndrome, with many different causes cited (Choquette-Levy et al. 1985, Chesterton et al. 1989), while footrot is a disease caused by a *D. nodosus* infection with a well defined pathology.

Infections involving more than one detectable *lktA* variant of *F. necrophorum* were rare in this study (only 1/83 of the samples contained two variants of *F. necrophorum lktA*). Similar trends have been noted by other authors who found that 97.3% of *Fusobacterium* isolates from animal samples contained only one species of *Fusobacterium* (Jang and Hirsh 1994). This was in contrast to infections caused by other anaerobes such as *Bacteroides* where a mixture of species tended to be found (Jang and Hirsch 1991), or in ovine footrot where mixed strain infections of *D. nodosus* comprised the majority of cases (Claxton et al. 1983, Zhou and Hickford 2000a). It is of course conceivable, given PCR-based detection methods that the amplification of one sequence from a given organism was at the expense of another sequence from a different organism, although this would be difficult to prove.

There are several possible mechanisms that could produce a monoclonal *F. necrophorum* infection in specific hosts. These include, different strains of *F. necrophorum* may be adapted to colonise specific hosts, hosts may be exposed to only one specific variant of *F. necrophorum* as a result of farm management practices, *F. necrophorum* may have a "boom and bust" life history where infrequent colonisation events are followed by rapid growth and die-off and *F. necrophorum* may undergo intensive intra-specific competition or strain dominance mechanics. Strain dominance has been observed in other bacterial species inhabiting specialised niches in animals such *Vibrio fischeri* (*V. Fisheri*) in squid, (Nishiguchi et al. 1998). In this case, native symbiotic strains of *V. fisheri* gain a 20-fold numerical advantage, against "non-native" strains over 48 hours (Nishiguchi et al. 1998). If such mechanics occur in footrot, how they affect the disease, or other species of *Fusobacteria*, such as *F. equinum*, is unknown.

Drawing inferences about which *F. necrophorum* strains are present in different hosts with or without pathology is difficult since only 4% of the *F. necrophorum lktA* gene was studied here, rather than the whole organism. Since the whole organism was not studied, it is harder to make assumptions about how these strains or sub-species are, or are
not, genetically related. While it is possible that the \textit{lktA} variants identified in this study represent a new sub-species of \textit{F. necrophorum}, this would only be true if the variation observed in \textit{lktA} is representative of the variation in the rest of the genome. To determine this, will require the isolation and further characterisation of the \textit{F. necrophorum} strains found in ovine footrot lesions.

SSCP was used in preference to sequencing since it was appropriate and cost effective. However some of the \textit{lktA} amplimers in Experiment 2 were unable to be typed using SSCP. This occurred most often in the \textit{lktA} amplimers derived from lame dairy cattle (54.2\% unable to be typed using SSCP). Not surprisingly, these amplimers were also the oldest (having been stored for a considerable time). In comparison, when using the fresher amplimers from sheep, 79\% of amplimers could be typed and 100\% of the freshest amplimers from goats could be typed. This suggests that degradation of amplimers occurred during storage, likely due to degradation of DNA by \textit{Taq} DNA polymerase, contamination by bacteria, fungi or DNAase enzymes. If this occurred it is likely that neither SSCP nor sequencing could have identified these \textit{lktA} amplimers.

In this study, sequences from an \textit{lktA}-like gene were generated from \textit{F. equinum}. Phylogenetic tree analysis showed that \textit{F. equinum \textit{lktA}} sequences were genetically distinct from the \textit{F. necrophorum \textit{lktA}} sequences and \textit{lktA} variants studied (Figure 6.3). This is not surprising given that \textit{F. equinum} has been described as a new species based on the analysis of its 16sRNA sequence, which was substantially different to \textit{F. necrophorum} (Dorsch \textit{et al.} 2001). Tadepalli \textit{et al.} (2008c) revealed that \textit{F. equinum} secreted a large functional leukotoxin similar to the \textit{F. necrophorum} leukotoxin, but was unable to generate a sequence for the \textit{F. equinum} leukotoxin. This is probably explained by the sequence differences observed in the \textit{lktA} gene sequence revealed in this study. Despite the large genetic differences seen, at the amino acid level blocks of sequence homology were conserved between \textit{F. necrophorum} and \textit{F. equinum} here. This supports Tadepalli’s conclusion that \textit{F. equinum} carries an expressed, functional leukotoxin (Tadepalli \textit{et al.} 2008c).

The sequences data from the \textit{F. equinum \textit{lktA}} gene enabled species-specific primers to be designed, allowing detection of the \textit{F. equinum \textit{lktA}} sequence in footrot samples from
sheep and cattle. While the detection of *F. equinum* in footrot has not been reported previously, it may be explained by that if *F. equinum* had been isolated from footrot, it is likely to have been classified as *F. necrophorum*, since *F. equinum* and *F. necrophorum* are phenotypically similar (Dorsch *et al.* 2001). The detection of *F. equinum lktA* in footrot lesions suggests that either, *F. equinum* is present in footrot, or that *F. equinum* has acted as a donor of the *lktA* gene to another organism that subsequently spread to sheep and cattle with footrot. It is possible such recombination events could have occurred between bacterial populations in cattle, sheep and horses since these hosts have similar habitats. To answer the question "Does the *lktA* sequence of *F. equinum* indicate the presence of *F. equinum*?" would require isolation and characterisation of the bacteria carrying the *F. equinum lktA* sequences detected in footrot lesions in this study.

Identification of a portion of the *lktA* sequence of *F. equinum* provides an opportunity to sequence and characterise the *lkt* operon of *F. equinum* using "chromosome walking" from the identified *lktA*-like sequence. These details may provide useful information given that considerable variation in leukotoxin genes has been observed between strains and sub-species of *F. necrophorum* with differences in structural gene size, promoter sequence and expression levels seen (Okwumabua *et al.* 1996, Narayanan *et al.* 1997, Narayanan *et al.* 2001, Zhang *et al.* 2006, Tadepalli *et al.* 2008a, Tadepalli *et al.* 2008b).

In summary, this chapter describes variation found in the *lktA* gene of *F. necrophorum* and *F. equinum*. This included a description of four variants of the *lktA* gene, some of which may be found in a "monoclonal" state in specific hosts or pathologies. In addition, part of *lktA*-like gene of *F. equinum* is described for the first time and a portion of this sequence was detected in footrot lesions from both cattle and sheep.
Chapter 7: Summary of findings and future directions

This thesis investigated the presence of \textit{F. necrophorum} and \textit{D. nodosus} in lame dairy cattle, and sheep and goats with footrot. It has previously been reported that \textit{F. necrophorum} is required to be present for \textit{D. nodosus} to initiate footrot in sheep in pen trials (Roberts and Egerton 1969). This thesis confirmed that \textit{F. necrophorum} is both associated and correlated with disease in the field and moreover, that it is found in the most destructive under-running lesions as well as persisting in cryptic footrot lesions. It was also observed that footrot pathologies were dynamic and variable. It is therefore recommended that this work should be repeated using digital photography to document how lesions develop and change over the course of the disease.

\textit{F. necrophorum}'s likely involvement in ovine footrot suggests there is an opportunity for a new approach to footrot control using vaccination. Bovine \textit{F. necrophorum} vaccines are already commercially available, such as Fusogard\textsuperscript{TM} (Novartis Animal Health) and Centurion\textsuperscript{TM} (Merck). For these vaccines to be used to combat ovine footrot, they need to be tested to determine if they generate an immune response in sheep and if this immune response is protective. This is particularly important in light of the genetic differences between the ovine \textit{F. necrophorum} strains described in this thesis and the bovine strains used in vaccines.

While \textit{D. nodosus} was found to be associated and correlated with disease it was also detected on the feet of sheep without footrot. This suggests that part of the lifecycle of \textit{D. nodosus} could involve the hooves of sheep without disease. If true, this provides an opportunity to intervene in footrot by treating subclinical infections using foot-bathing, probiotics or biocontrol agents. To be effective, such treatments would need to reduce infection rates, the transmission of \textit{D. nodosus}, or prevent the establishment of new infections.

No evidence was found that \textit{D. nodosus} was present in the gastro-intestinal tract of the sheep. While this cannot prove \textit{D. nodosus} is absent from this site; if it had been present in this study, it was in numbers below the detection limit of the methods used or was only carried by a small proportion of animals and could not be sampled successfully.
In this thesis *F. necrophorum* was found to be prevalent on the hooves of lame dairy cattle, suggesting it could play a role in lameness. To address this, a comparison of lame dairy cattle with healthy dairy cattle is required to ascertain whether *F. necrophorum* is associated with lameness in New Zealand dairy herds or if it is actually widespread on all dairy cattle. In contrast, while *D. nodosus* was detected on lame dairy cattle, it was only found in a small percentage of animals (4.7%). This suggests it is unlikely to be a major cause of lameness. It is unknown whether *D. nodosus* in lame dairy cattle may act as a source of ovine footrot and this possibility may need to be considered in the management of dairy run-off properties that also graze sheep.

Given the variation described in a portion of the *lktA* gene detected on sheep with footrot, there is potential to isolate and describe genetic variation in the *F. necrophorum* strain presumed to be carrying this gene. This would both clarify the taxonomic status of the ovine strains of *F. necrophorum* and identify if the current *F. necrophorum* vaccines could fail in sheep due to genetic differences between the vaccine and ovine strains.

This thesis reports that *F. necrophorum* is associated with under-running footrot in the field and that it is found in both destructive and cryptic footrot pathologies. Furthermore, a potentially, un-described, genetically distinct, footrot or sheep specific variant of *F. necrophorum* was identified. Given these results and the broad range of footrot pathology and virulence observed in the field, it is conceivable that *F. necrophorum* is critically important in pathogenesis of footrot. Proof of this will require further characterisation of both the organism and co-culture with *D. nodosus in-vitro* and *in-vivo* to describe its interactions with footrot.


References


Dewhirst F.E., Paster B.J., La Fontaine S., Rood J.I., 1990. Transfer of *Kingella indologenes* (Snell and Lapage 1976) to the genus *Suttonella* gen. nov. as *Suttonella indologenes* comb. nov.; transfer of *Bacteroides nodosus* (Beveridge 1941) to the genus *Dichelobacter* gen. nov. as *Dichelobacter nodosus* comb. nov.; and assignment of the genera *Cardiobacterium*, *Dichelobacter*, and *Suttonella* to *Cardiobacteriaceae* fam. nov. in the gamma division of *Proteobacteria* on the basis of 16S rRNA sequence comparisons. Int. J. Syst. Bacteriol. 40, 426-33.


References


References


Appendices
Appendix A. Letter to farmers

1 January 1900

Fred Dagg
Private Bag
Fairlie

Dear Fred,

I’m studying footrot with Jon Hickford as part of my PhD at Lincoln University. Currently only one bacterium, *Dichelobacter nodosus* is described as causing footrot, but in small scale lab trials this bacteria has been shown to be unable to cause footrot without the presence of a second bacterium, *Fusobacterium necrophorum*. It is unknown what is actually happening on sheep’s hooves in a normal farming environment when footrot is present.

To find out what is happening in the field and if *Fusobacterium necrophorum* is actually associated with footrot outside of a “lab setting” we are asking that you take a hoof swab from 3 sheep with footrot, 3 sheep without, attach the appropriate label (provided with sampling tubes) and return the swab to us. While these samples do not need to be taken by any specific deadline, it would be appreciated if you please take samples the next time an outbreak of footrot occurs.

To take samples, you need to take 3 footrot swabs from under-running footrot lesions which I will test for the presence of *Fusobacterium necrophorum* and *Dichelobacter nodosus*. Under-running lesions occur when footrot attacks the soft horn, followed by the hard horn within the hoof (see diagram below); eventually the whole hoof can be attacked and shed.

For a comparison, 3 swabs from healthy sheep with no symptoms of footrot are requested. The swabs can be sent back to me for no charge with the attached free post envelopes and will be collated into a much larger study involving a significant number of farmers.
Thank you for your co-operation. If you wish to receive preliminary results from the footrot swabs you send us or other preliminary results from my research regarding the behaviour of the bacteria involved in footrot, either include this letter with your swabs or contact me directly. If you would like more information, have any queries or questions, please feel free to contact me on Ph 03 325 3838 extension 8143 or by email at ‘bennetg2@lincoln.ac.nz’.

Yours Faithfully

Grant Bennett
Appendix B. Publications arising from this thesis


Short communication—Clinical microbiology

Convenient anaerobic techniques, science from the supermarket shelf

G.N. Bennett, J.G.H. Hickford*, H. Zhou

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Abstract

We describe the application and evaluation of a widely available commercial jar as an anaerobic container suitable for the growth of a wide variety of anaerobes. A system for generating stable anaerobiosis was developed by combining standard anaerobic environment generators with Click-Clack jars produced by Click-Clack Ltd. (http://www.clickclack.com). This system was simple, reliable, and reduced capital outlay on anaerobic jars by at least an order of magnitude.

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Keywords: Anaerobic; Jar; Click-Clack; Anaerobiosis; Technique

Traditionally anaerobic jars have been expensive, cumbersome and could be technically difficult to use. The capital cost of jars has inhibited both the initiation of anaerobic research and the quantity of anaerobic cultures able to be grown at any one time. To address these issues, a reliable system for generating anaerobiosis was developed by combining oxygen-depleting envelopes with Click-Clack jars produced by Click-Clack Ltd., Palmerston North, New Zealand (http://www.clickclack.com). The Click-Clack jars are part of the ‘Stack and Seal’ range, and are made from a base cylinder of polystyrene, a diaphragm of low-density polyethylene and a polypropylene lid.

Ten anaerobic bacterial strains from a variety of species were used to test the anaerobic system developed in this study. These bacterial strains were: *Dichelobacter nodosus* strain A198, *Clostridium perfringens* (ATCC1 no. 13124), *Fusobacterium necrophorum* subsp. necrophorum (NCTC2 no.10575), *Fusobacterium nucleatum* subsp. nucleatum (ATCC no. 25586), *Prevotella buccae* (ATCC no. 33574), *Porphyromonas levii* (ATCC no. 29147), *Peptostreptococcus anaerobius* (ATCC no. 27337), *Bacteroides fragilis* (ATCC no. 25285), *Bacteroides vulgatus* (ATCC no. 8482) and *Clostridium sordelli* (ATCC no. 9714). All strains were obtained from the New Zealand Culture Collection (Institute of Environmental Science and Research Ltd., Porirua, New Zealand) and identified either by their ATCC or NCTC numbers, except for the *D. nodosus* strain A198 which was acquired from Professor Julian Rood, Monash University, NSW, Australia. The identity of bacterial species was confirmed using RapID ANA II (Remel Inc., Lenexa, KS), Gram stain and morphology [1].

The *D. nodosus* strain A198 was cultured on plates containing 3% (w/v) Eugon broth (Becton Dickinson Labware, NJ), 5.1% (w/v) anaerobic agar (Merck, Darmstadt, Germany) and 10% (v/v) defibrinated sheep blood (Invitrogen, Carlsbad, CA). Freeze-dried cultures of other bacterial strains were dissolved in sterile thioglycollate broth (Merck) and cultured on pre-made Wilkins–Chalgren plates (Oxoid, Hampshire, UK). Plates were incubated at 37 °C in Click-Clack jars (production

Abbreviations: ATCC, American-type culture collection; NCTC, National collection of type cultures

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1American type culture collection

2National collection of type cultures
code 306005, Click-Clack Ltd.) and anerobiosis was generated using AnaeroGen™ oxygen-scavenging envelopes (Oxoid) and indicated by either resazurin (Oxoid) or methylene blue indicator strips (Becton Dickinson Labware). All bacterial strains were able to grow and form colonies in Click-Clack jars.

To evaluate relative efficacy of this anaerobic system, bacterial colonies from *F. necrophorum* subsp. *necrophorum*, *F. nucleatum* subsp. *nucleatum*, *P. buccae*, *P. anaerobius*, *C. perfringens* and *B. fragilis* were diluted, grown and quantified in both Click-Clack and metal jars. Colonies were suspended in sterile thioglycollate broth and serially diluted. Two sets of plates were inoculated with 100 μL aliquots; one set was incubated in Click-Clack jars and the other was in metal jars. This was performed in triplicate. Plates were prepared by combining Wilkins-Chalgren anaerobe broth (Oxoid) with 1% (w/v) agar (Germantown Co., Manakau, New Zealand), filtered water and adding 5% (v/v) defibrinated sheep blood post-autoclaving. AnaeroGen™ oxygen-scavenging envelopes were used in both jars to generate anaerobiosis. Methylene blue indicators were used to monitor anaerobiosis in metal jars, but in Click-Clack jars either resazurin or methylene blue indicator strips were used. After 4 days of incubation at 37°C, colonies were counted, and errors in plate counts were calculated by dividing the biggest difference between any two data points in a data set by two. The plate counts between both systems were comparable (Table 1). This demonstrated that a variety of anaerobes grew equally well in either Click-Clack jars or standard metal pressure jars. Initial testing also suggests that several other cylindrical Click-Clack jars are suitable as anaerobic jars, in particular the polycarbonate ‘Accent’ range and some of the ‘Design+’ range.

The combination of AnaeroGen™ envelopes with Click-Clack jars allowed a reliable anaerobic system to be developed, and an example of this system is shown in Fig. 1. Anaerobiosis was stable, as indicated by the complete reduction of a resazurin indicator strip within 5 h at 37°C. A long term of anaerobiosis can be maintained for weeks in Click-Clack jars. However, anaerobiosis may fail if the seals are physically blocked, the jars are malformed, or at temperatures below 20°C. By applying vacuum grease (silicon grease) (Beckman Instruments Inc., Fullerton, CA) to the jar’s seal, a great improvement was observed in the ability of these jars to maintain an anaerobic atmosphere under adverse conditions. The small cost of US$ 3–5/jar, ease of use, reliability, durability and functionality of these containers as anaerobic jars warrants their use as an alternative anaerobic culture system in both research and clinical diagnostic laboratories.

Table 1

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Mean plate count of plates grown in Click-Clack jars</th>
<th>Mean plate count of plates grown in metal jars</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. perfringens</em></td>
<td>$9.36 \times 10^7 \pm 0.9 \times 10^7$</td>
<td>$6.90 \times 10^7 \pm 1.35 \times 10^7$</td>
</tr>
<tr>
<td><em>P. buccae</em></td>
<td>$3.0 \times 10^6 \pm 0.1 \times 10^6$</td>
<td>$3.67 \times 10^6 \pm 0.3 \times 10^6$</td>
</tr>
<tr>
<td><em>P. anaerobius</em></td>
<td>$3.36 \times 10^7 \pm 0.15 \times 10^7$</td>
<td>$2.90 \times 10^7 \pm 0.75 \times 10^7$</td>
</tr>
<tr>
<td><em>B. fragilis</em></td>
<td>$1.60 \times 10^7 \pm 0.25 \times 10^7$</td>
<td>$1.63 \times 10^7 \pm 0.35 \times 10^7$</td>
</tr>
<tr>
<td><em>F. nucleatum</em> subsp. <em>nucleatum</em></td>
<td>$4.50 \times 10^7 \pm 1.1 \times 10^7$</td>
<td>$4.50 \times 10^7 \pm 1.1 \times 10^7$</td>
</tr>
<tr>
<td><em>F. necrophorum</em> subsp. <em>necrophorum</em></td>
<td>$3.30 \times 10^8 \pm 0.4 \times 10^8$</td>
<td>$3.37 \times 10^8 \pm 0.4 \times 10^8$</td>
</tr>
</tbody>
</table>
Acknowledgements

We would like to thank Karl Gately and Maree J. Clapham whose early work allowed the development of this culture system.

References

**Report on air-tightness of the series 5000 rectangular jars**

Report on Air-Tightness of the Series 5000 Rectangular Jars

By Grant Bennett, Lincoln University, February 2008

**Executive summary**
The ‘series 5000’ rectangular jars are airtight and able to maintain an anaerobic (oxygen free) environment.

**Results Summary**
3 series 5000 rectangular jars of different sizes were received and tested for air tightness. At 37°C all 3 jars maintained an air seal and an oxygen free environment. At room temperature and lower, the largest of the jars failed while the medium and smaller sized jars maintained their air seals up to -20°C.

**Methodology**
Each jar was tested for their ability to maintain an anaerobic oxygen free environment under a variety of temperatures for at least a 24 hour period. Oxygen scavenging envelopes were used to remove oxygen from the each jar (marketed as AnaeroPack®·Anaero-3.5L, produced by Mitsubishi Gas Chemical Co., Inc.). Methyl blue and resazurin indicator strips showed if any oxygen was still present within a jar. If a strip is white, it indicates no oxygen is present (reduced), if it is coloured it indicates oxygen is in the atmosphere or surface that the strip is touching (oxidised). Please note that resazurin is far more sensitive to oxygen than methyl blue and consequently takes much longer to turn white in most anaerobic systems.

Each jar contained indicators and an oxygen scavenging envelope; air tightness was tested at 37°C, ~20°C (room temperature), 4°C and ~-20°C (freezer); each test was run for 18-24 hours sequentially till a jar failed; testing started at the warmest temperature. 3 series 5000 jars were tested, a 4.3L jar, a 0.95L jar and a 0.42L jar.

**Results**
All 3 jars (4.2L, 0.95L and 0.42L) tested were able to hold a seal at 37°C; at room temperature, initially all 3 jars stayed anaerobic, but the largest of the jars (4.2L) started to fail after 18 hours; once placed in a fridge at 4°C the largest 4.2L jar failed completely, the 0.95L jar and 0.42 L Jar maintained their seals over 24 hours; in a freezer (approximately -20°C) the medium 0.95L and 0.42L jars still maintained their seal and stayed oxygen free.

**Conclusions and Recommendations**
The 3 series 5000 jars tested had good air seals at 37°C and should be suitable for growing anaerobes at this temperature. Further the 0.95L and 0.42 L jars are able to maintain an anaerobic environment in a wide range of temperatures, this could make them very useful for collecting and maintaining field or clinical samples.

These jars can be stacked inside each other-this allows the inner plastic jar, its seal and lid to be completely de- oxygenated before use (very useful if growing sensitive microbes). This allows a double layer system with an outer jar and an inner jar to be used, providing
backup against possible leaks and acts as a double seal (essential when transporting known pathogens).

**Raw Data**

**Jars placed in incubator**

**Large 4.3L jar, at 37°C**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2</td>
<td>Methyl Blue mostly reduced, Resazurin not reduced.</td>
</tr>
<tr>
<td>+5</td>
<td>Methyl Blue completely reduced, Resazurin mostly reduced.</td>
</tr>
<tr>
<td>+12</td>
<td>Methyl Blue, Resazurin completely reduced.</td>
</tr>
<tr>
<td>+72</td>
<td>Methyl Blue, Resazurin completely reduced.</td>
</tr>
</tbody>
</table>

**Medium 0.95L jar, at 37°C**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2</td>
<td>Methyl Blue mostly reduced, Resazurin slightly reduced</td>
</tr>
<tr>
<td>+5</td>
<td>Methyl Blue, Resazurin completely reduced</td>
</tr>
<tr>
<td>+12</td>
<td>Methyl Blue, Resazurin completely reduced</td>
</tr>
<tr>
<td>+72</td>
<td>Methyl Blue, Resazurin completely reduced</td>
</tr>
</tbody>
</table>

**Small Jar, 0.42L at 37°C**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2</td>
<td>Methyl Blue mostly reduced, Resazurin not reduced.</td>
</tr>
<tr>
<td>+5</td>
<td>Methyl Blue mostly reduced, Resazurin mostly reduced.</td>
</tr>
<tr>
<td>+12</td>
<td>Methyl Blue, Resazurin completely reduced</td>
</tr>
<tr>
<td>+72</td>
<td>Methyl Blue, Resazurin completely reduced</td>
</tr>
</tbody>
</table>

**Jars were placed at room temperature and observed**

**Large 4.3L jar, at room temperature (~20°C)**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2</td>
<td>Methyl Blue, Resazurin completely reduced.</td>
</tr>
<tr>
<td>+24</td>
<td>Methyl Blue, Resazurin slightly oxidised (Jar failing)</td>
</tr>
</tbody>
</table>

**Medium 0.95L jar, at room temperature (~20°C)**
<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2-24 hours</td>
<td>Methyl Blue, Resazurin completely reduced.</td>
</tr>
</tbody>
</table>

Small 0.42L jar, at room temperature (~20°C)

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2-24 hours</td>
<td>Methyl Blue, Resazurin completely reduced.</td>
</tr>
</tbody>
</table>

Jars were placed in fridge

Large 4.2L Jar, at 4°C

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1</td>
<td>Methyl Blue and Resazurin clearly oxidising <em>(Jar failing)</em></td>
</tr>
<tr>
<td>+4-24</td>
<td>Methyl Blue, Resazurin completely oxidised <em>(Jar failed)</em></td>
</tr>
</tbody>
</table>

Medium 0.95L jar, at 4°C

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2-24 hours</td>
<td>Methyl Blue, Resazurin completely reduced.</td>
</tr>
</tbody>
</table>

Small 0.42L jar, at 4°C

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2-24 hours</td>
<td>Methyl Blue and Resazurin completely reduced.</td>
</tr>
</tbody>
</table>

Jars placed in freezer (~20°C)

Large 4.2L Jar, at ~20°C

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>Jar failed, testing finished</td>
</tr>
</tbody>
</table>

Medium 0.95L jar, at ~20°C

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2-24 hours</td>
<td>Methyl Blue, Resazurin completely reduced.</td>
</tr>
</tbody>
</table>

Small 0.42L jar, at ~20°C

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2-24 hours</td>
<td>Methyl Blue and Resazurin completely reduced.</td>
</tr>
</tbody>
</table>

Backup Testing
To double check that anaerobiosis had been maintained, jars where incubated at 37°C and the gas scavenging envelopes tested to see if they still able to react with oxygen. If they can not react, it shows that the jar has leaked—this is a backup test to the indicators. The gas scavenging envelopes were also tested to ensure they function at -20°C.

Notes on Jar failure and references
The failure of the larger 4.2L jar could be for a number of reasons. The anaerobic sachets generate a slight negative atmospheric pressure inside the jar possibly causing flexing of the walls of the larger jar. Larger jars also have a bigger surface area to maintain a seal, therefore the seal may be less physically robust. The larger jar has a capacity of 4.3L, this is in theory slightly too large for a single 3.5L anaerobic sachet, but an anaerobic environment was still able to be established and maintained. The main consequence of using a large jar could be that there was very little buffering capacity compared to a smaller jar. This would only affect the results if any leaks in the small jars were small and short lived. A slow leak in a small jar should still quickly exhaust the buffering capacity of the anaerobic sachet.

If more information is required on anaerobic sachets or the costs of anaerobic culture I refer you to the article below. It details and tests 3 different culture systems and mentions costs in its last paragraph.


Veterinary anaerobes and diseases

*Dichelobacter nodosus*, *Fusobacterium necrophorum* and the epidemiology of footrot

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*Dichelobacter nodosus*
Footrot
Lameness
Sheep

**A B S T R A C T**

Footrot is a debilitating disease of sheep resulting in lameness, production losses and suffering. To study the basic bacteriology of the disease, a survey was initiated across commercial farms and non-commercial research flocks to compare the bacteriology of symptomatic footrot infected sheep with healthy asymptomatic sheep. Of the 80 farmers initially contacted, 14 collected hoof swabs and returned the swabs by post. Following DNA extraction, species-specific PCR was used to identify if *Dichelobacter nodosus* (*D. nodosus*) or *Fusobacterium necrophorum* (*F. necrophorum*) were present on each swab. Of the 42 swabs taken from symptomatic footrot infected sheep, 17 were positive for both *F. necrophorum* and *D. nodosus*, two were positive for *F. necrophorum* only, two for *D. nodosus* only and 23 swabs were negative for both species present on each swab.

**1. Introduction**

Ovine footrot is a highly contagious disease that results in lameness, production loss and suffering. The primary pathogen was first identified as *Dichelobacter nodosus* (*D. nodosus*), formally known as *Bacteroides nodosus* and *Fusiformis nodosus* by Beveridge in 1941 [1].

*D. nodosus* is however unable to fulfil Koch’s postulates of disease as it is unable to replicate the symptoms of disease on its own as a second pathogen *Fusobacterium necrophorum* (*F. necrophorum*) is reported to be required to induce footrot symptoms in sheep during small pen trials [2]. Strict application of Koch’s postulates to footrot would mean that the disease causing agent must be able to be isolated from a diseased host, be culturable on media independently of the host and if another un-diseased host must be able to be isolated from a diseased host, be culturable on media independently of the host. Despite failing to meet Koch’s postulates, *D. nodosus* is commonly considered the primary pathogen causing footrot since elimination and quarantine of virulent strains of *D. nodosus* appear to prevent footrot [3], vaccination against *D. nodosus* reduces disease prevalence [4] and *D. nodosus* is found within foot-rot lesions [5].

*D. nodosus* is a rod shaped, gram negative, obligate anaerobe bacterium that has proteases and keratinases that are able to dissolve sheep hooves. The central role that these enzymes play in the biology of *D. nodosus* and footrot is demonstrated by how they are still used today as a measure of strain virulence and historically dried hoof powder was considered a requirement for the reliable culture of *D. nodosus* [6].

*F. necrophorum* has been considered as a secondary pathogen in ovine footrot. It is a gram-negative anaerobic bacterium associated with many different diseases and disorders in both animals and humans. These range from Lemierre’s syndrome in humans [7–9] to calf diphtheria [10] as reported by Ref. [11–13], ovine footrot [5], bovine rumenitis-hepatic abscesses complex [14,15], abscesses in animals [16], bovine hoof abscesses, toe abscesses and various soft tissue infections in the hooves of both cattle and sheep [17–21].

The etiology of ovine footrot is complex, involving infection by multiple bacterial species [15] modulated by environmental conditions [22,23], host genetics, host immunity [24], nutrition and stocking rates. Some management practices affect footrot allowing disease control through quarantine, selective breeding, foot paring combined with zinc sulphate foot baths, vaccination [4,24] and...
antibiotic use. However, using these practices, establishing and maintaining quarantine are expensive, difficult and there is no guarantee that footrot may not become re-established at a later date.

Due to the difficulty of reliably isolating and anaerobically culturing *D. nodosus* and *F. necrophorum*, a PCR-based strategy was used to detect the microbes thought to be associated with the disease. PCR is able to detect non-viable cells, dead cells, live cells and difficult to culture cells, and when combined with specific primers [25] is a very precise way of ascertaining the presence of specific genetic material. It should be noted that PCR cannot prove the absence of genetic material, so care must be taken as to what conclusions are drawn from such results.

2. Materials and methods

2.1. Footrot samples

Of the eighty sheep farmers who were initially contacted, fourteen returned swabs from both/either healthy symptomatic sheep and/or symptomatic sheep with severe under-running footrot. These farmers had received instructions to take swabs from the skin–horn junction on the axial wall of the hoof and to return these swabs in tubes by post. Twelve sheep with severe footrot from the Lincoln university research farm were swabbed and swabs were processed in an identical manner to farmer collected swabs. Once received, swabs were stored at 2.1.4 °C until processed.

2.2. DNA extraction

DNA was extracted from swabs using a previously published protocol with minor modifications [25]. Briefly swabs were placed in sterile 1.5 ml tubes with 400 μl of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and shaken for 20 s. The swab was removed and 40 μl of 10% SDS was added with 220 μl of Tris-buffered phenol (pH 7.8) and 220 μl of chloroform. Tubes were shaken to lyse cells and frozen overnight at −20 °C. After thawing, suspensions were briefly mixed by inverting and centrifuged at 5000 × g for 5 min. The aqueous layer was aliquoted into a new tube and precipitated with 40 μl of 3 M Sodium Acetate (adjusted to pH 5.2) and 500 μl of ice cold isopropanol. Precipitated DNA was centrifuged at 14 500 × g for 15 min and the supernatant removed. The DNA pellet was air dried before being suspended in 50 μl of sterile dH2O. The DNA in solution was stored at 4 °C until used.

2.3. PCR amplification

The *lktA* gene encoding the leukotoxin of *F. necrophorum* was amplified using PCR primers lkt-up (5′-acaatcggagtagtaggttc-3′) and lkt-dn (5′-atttgtaactgccactgc-3′). The PCR was performed in an iCycler (Bio-Rad, CA, USA) with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s. A final extension of 5 min at 72 °C was performed.

The *fimA* gene of *D. nodosus* was amplified using the method described previously [25]. The thermal profile consisted of denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 50 s, with a final extension step at 72 °C for 5 min.

PCR products from both the *lktA* PCR and *fimA* PCR were separated electrophoretically, at 10 V/cm on a 1 × TBE (89 mM Tris–borate, 89 mM boric acid, 2 mM Na2EDTA [pH 8.9]) gel containing 1.0% agarose and 0.5 μg/ml of ethidium bromide and visualised using a transilluminator. If high concentrations of genomic DNA were visible on the gel, extracted DNA was diluted 1/10 and the PCR repeated to improve PCR consistency. If high quantities of genomic DNA were still seen at this concentration, the DNA was further diluted to 1/100 and the PCR repeated.

2.4. Statistical analysis

Statistical analysis of results was performed using a log-linear model and Poisson errors (GenStat version 10, 2007, Lawes Agricultural Trust, Rothamsted).

3. Results

Of the 50 swabs taken and received from healthy asymptomatic sheep, one was positive for *F. necrophorum*, none were positive for *D. nodosus* and 49 were negative for both *F. necrophorum* and *D. nodosus*. Forty-two swabs were taken and received from footrot infected sheep with under-running footrot. Of these, two were positive for *F. necrophorum*, two were positive for *D. nodosus*, 17 were positive for both *F. necrophorum* and *D. nodosus* and 23 were negative for both *F. necrophorum* and *D. nodosus*. Statistical analysis showed that *D. nodosus* and *F. necrophorum* are significantly linked to footrot (p < 0.001), and that these organisms are found together at a significantly higher rate than would be expected by a random assortment (p < 0.025).

The *fimA* and *lktA* PCRs from footrot swabs were found to work more reliably with the addition of 400 ng/μl of BSA and additional MgCl₂ to final concentration of 5.25 mM (Fig. 1). The *fimA* PCR was also improved by reducing the primer concentration to 100 nM (Fig. 1).

4. Discussion

This survey shows that in a pastoral farming system *D. nodosus* and *F. necrophorum* tend to be found on the feet of symptomatic sheep with under-running footrot compared to healthy asymptomatic sheep. We also showed that *D. nodosus* and *F. necrophorum* occur together at a significantly higher rate than if they distributed...
randomly. This demonstrates that not only are these bacteria both associated with under-running footrot, but they are also associating together, which suggests that they may both be involved in causing footrot.

The polymerase chain reaction is difficult to use with extremely dirty or contaminated samples. In this respect, hoof scrapings from footrot infected sheep are usually contaminated and dirty by their nature. However chlorof orm–phenol extraction of DNA combined with PCRs containing BSA greatly improved the consistency and success of these PCRs. BSA’s mode of action is to have a large binding capacity for phenolics and pushes the DNA-phenolic and enzyme-phenolic equilibrium in favour of BSA-phenolic complexes and unbound DNA and enzymes [26,27]. When using BSA in a PCR, much more MgCl2 needs to be added, since BSA also binds magnesium preferentially and this can inhibit PCR.

It is still conceivable that the approach described here detects an artificially low rate of D. nodosus and F. necrophorum in sheep diagnosed with under-running footrot than is actually present. Numerous things could conspire to either limit the amount of bacterial material collected, the amount of target genome extracted or inhibit the amplification of genome even if present. Accordingly PCR detection methods for bacteria from environmental samples are typically qualitative and care is needed to insure that a diagnostic PCR is equally sensitive for all samples especially if PCR inhibitors are present.

Assuming that DNA extractions and PCR protocols are working well, failure to detect D. nodosus and F. necrophorum on the surface of all sheep hooves infected with under-running footrot all the time, does not eliminate D. nodosus and F. necrophorum as causative agents of footrot, especially considering their anaerobic nature. Once D. nodosus and F. necrophorum are established on a hoof surface and causing disease, the possible fate of these pathogens is varied. They could spread to other sites of infection on other hosts; persist together on the hoof surface; colonise an asymptomatic reservoir and/or one or both species could be removed from the hoof surface either by oxidative pressure, the host’s immune system or other unfavourable environmental conditions. Even if these bacteria die out on the surface of the foot, they may still be able to persist in pockets of infection inside the hoof and cause disease. This contention is supported by the observation that F. necrophorum is generally considered to have a wide range of anaerobic habitats and pathologies in a variety of hosts [5,16].

In our research, F. necrophorum has been detected on swabs taken from the oral cavity of sheep (results not shown). This suggests that F. necrophorum can be transmitted to and from the mouth of sheep to the paddock, as yet by an un-described pathway.

The widespread detection of F. necrophorum with D. nodosus together, supports the hypothesis that footrot results from a synergistic interaction between these two organisms [5]. If F. necrophorum is not directly involved in causing footrot, it is certainly widespread amongst footrot positive sheep and with detectable D. nodosus. It would therefore appear adep at colonising the feet of footrot infected sheep. Widespread colonisation of footrot infected hooves with F. necrophorum could have serious consequences for animal health and welfare since F. necrophorum is a pathogen in its own right and possess a potent leukotoxin that could adversely affect a host’s immune system. A simple test to differentiate F. necrophorum as a causative agent of footrot or merely as an opportunistic pathogen that colonises footrot lesions would be to vaccinate sheep against the F. necrophorum using the truncated leukotoxin vaccine shown to be active against F. necrophorum in cattle [28,29].

Whether F. necrophorum is one of the factors contributing to footrot infection, or if it is merely an opportunistic infection that colonises footrot infected sheep feet, it is clear that it is associated with footrot. This means that both D. nodosus and F. necrophorum activity needs to be considered when making decisions regarding the long-term management of footrot and its effects.

Acknowledgements

Thank you to the participating New Zealand sheep farmers and the Lincoln University Gene-Marker Laboratory. This work has been funded by the Struthers Scholarship, the Ingleby Company Limited Pastoral Scholarship and the Hellaby Indigenous Grasslands Research Trust.

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Detection of both *Fusobacterium necrophorum* (*F. necrophorum*) and *Dichelobacter nodosus* (*D. nodosus*) has been shown to be highly associated with under-running footrot in sheep [1]. Footrot in goats is considered a similar disease to sheep and *D. nodosus* has been reported as an agent causing footrot in goats [2]. Using a previously described PCR diagnostic method [1] we undertook an experiment to see if *F. necrophorum* was associated with footrot in New Zealand goats, as has been shown in sheep [1].

Swabs were taken from the skin-horn junction of healthy goats (*n* = 20) and goats with under-running footrot (*n* = 24). Once returned, swabs were stored at −80 °C, until DNA was extracted and a single *fimA* and *ltkA* PCR was performed as previously described [1].

Neither *D. nodosus* nor *F. necrophorum* was detected on the 20 swabs taken from healthy goats. In contrast, 62.5% (15/24) of swabs from goats with under-running footrot tested positive for *D. nodosus* and 33.3% (8/24) swabs were positive for *F. necrophorum*. When *F. necrophorum* was detected on a swab, *D. nodosus* was also detected on 87.5% (7/8) of the swabs. *D. nodosus* was detected on 53.3% (8/15) of swabs without any *F. necrophorum* being detectable.

A previously described [1] log linear model was used to analyse this data. Detection of *D. nodosus* was found to be highly associated with under-running footrot (*P* < 0.0005), as was detection of *F. necrophorum* (*P* < 0.0002). *D. nodosus* and *F. necrophorum* also tended to be detected together, rather than singularly (*P* < 0.039).

These findings are similar to those presented previously for sheep with footrot [1]. However, it is notable that a large portion of swabs were negative for both *D. nodosus* and *F. necrophorum*. This would suggest either that the PCRs used lacked sensitivity and/or were inhibited by other compounds extracted from the swab, or that the sampling procedure was not contacting the organisms present. We consider the latter argument more likely, given that the skin–horn junction of the hoof was sampled rather than footrot lesions, which are variable in both location and accessibility. While sampling of this region was considered to be more consistent in practice, the swabs used may have failed to come in contact with the expected microflora.

It was also observed that there was a high number of swabs with only *D. nodosus* being detectable (8/15) compared to *F. necrophorum* (1/8). This was in contrast with footrot in sheep where *D. nodosus* and *F. necrophorum* were detected in similar proportions using similar PCR technologies [1]. This supports the argument that the failure to detect the organisms on some hooves is a function of the sampling procedure used, or that footrot is actually a different disease in goats.

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**References**


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Detection of *Fusobacterium necrophorum* and *Dichelobacter nodosus* in lame cattle on dairy farms in New Zealand

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**Abstract**

Lameness in the dairy industry in New Zealand causes a problem in lost production, animal welfare and associated costs. To understand what bacteria may be present on the hooves of lame dairy cattle in this grass-fed system, samples were scraped from lame dairy cows and examined for the presence of *Fusobacterium necrophorum* (*F. necrophorum*) and *Dichelobacter nodosus* (*D. nodosus*) using the polymerase chain reaction (PCR). The PCR primers were designed to detect the presence of the lktA gene, which encodes a leukotoxin unique to *F. necrophorum*, and the fimA gene of *D. nodosus*. A total of 148 hoof scrapings were collected by farm staff over the period September 2005 to May 2006. *F. necrophorum* was detected in 79/148 of the samples, while *D. nodosus* was detected in 7/148 of the samples. The frequent finding of *F. necrophorum* within dairy herds in New Zealand is noteworthy and the occasional finding of *D. nodosus* on some dairy cattle suggests a possible role in both ovine and bovine hoof pathology.

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The fimA fragment (approximately 450 bp) of D. nodosus was amplified using the method described previously (Zhou and Hickford, 2000). The thermal profile consisted of denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 50 s, with a final extension step at 72 °C for 5 min.

To reduce the effects of PCR inhibitors, each PCR was repeated with the addition of 400 ng/μl Bovine Serum Albumin (BSA) (New England Biolabs) and an extra 2.5 mM of MgCl₂.

PCR products from both the lktA and fimA genes were separated electrophoretically in 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide, and visualised using a transilluminator (Figs. 1 and 2). In the case of field samples; if high concentrations of genomic DNA were visible on the gel, the PCR was repeated using a 1/10 concentration of extracted DNA solution. If high quantities of genomic DNA were still visualised at this concentration, the DNA was further diluted again to 1/100 and the PCR repeated. This dilution of genomic DNA improved the quality and size consistency of the amplicons visualised as bands in the electrophoretic gel.

Of the 148 hoof scrapings examined, a total of 79 (53%) samples tested positive for the presence of the lktA gene of F. necrophorum and seven (5%) tested positive for the fimA gene of D. nodosus.

The detection of F. necrophorum on a proportion of the lame dairy cattle and D. nodosus at a lower rate, suggests that D. nodosus is not a major agent of lameness in New Zealand dairy cattle, while F. necrophorum possibly could be. This is consistent with the finding the vaccination against F. necrophorum reduces lameness rates in some production systems (Checkley et al., 2005), although this work was undertaken in Canadian feedlot cattle. We accept that this survey is not representative, as samples from healthy cattle could not be taken and the positive results for D. nodosus are low in number (7 positives out of 148 samples).

The presence of F. necrophorum in hoof scrapings from lame cattle leads us to hypothesise that it may be associated with lameness, present on the hoof for a transitory period, or that it is associated with the hooves of all New Zealand dairy cattle, and has not been detected previously. This has possible implications when managing herd health, since lame cattle could act as a source of F. necrophorum and potentially contribute to its spread and the incidence of other stock diseases. The wide-spread detection of the lktA gene on lame cattle also suggests that these cattle could have immunocompromised hooves but only if detection of the lktA gene is associated with expression and secretion of leukotoxin and a subsequent leukotoxic effect.

The detection of D. nodosus on the hooves of lame dairy cattle may be significant since this bacterium is typically described as a pathogen found in the footrot lesions of sheep (Moore et al., 2005), although it has been isolated from cattle (Berg and Loan, 1975; Laing and Egerton, 1978). Transmission of D. nodosus from cattle to sheep has been demonstrated (Wilkinson et al., 1970; Laing and Egerton, 1978), and benign footrot symptoms have been reported as being able to be induced in sheep by cow to sheep transmission (Wilkinson et al., 1970; Laing and Egerton, 1978) and virulent footrot symptoms by direct inoculation with lesion material (Egerton and Parsonson, 1966). However, detecting this bacterium on some lame cattle may not indicate that the lameness is caused by D. nodosus, but it does confirm that D. nodosus is present and able to persist on cattle hooves, even if the farm is a specialised dairy farm which does not stock sheep.

This is important in the New Zealand context as in its pasture-based dairy production systems sheep are not typically present on the farm. Any contact with sheep would usually occur during the winter months, when cows are dried off and moved to “run off” land on properties that may graze sheep. Even in that circumstance, the winter months are not usually associated with footrot outbreaks (Graham and Egerton, 1968).

The results may also suggest that other vectors apart from sheep may be involved in transmission of D. nodosus to dairy cattle, or that once D. nodosus is present within a herd of dairy cattle it is able to persist undetected for long periods of time.

In conclusion, F. necrophorum is frequently found on the hooves of lame dairy cattle in New Zealand and may be associated with lameness. This suggests that a leukotoxic activity may be present on dairy cattle hooves and that the presence of lame cattle should be considered when managing other diseases associated with F. necrophorum. D. nodosus may be present and persistent in lame cattle in New Zealand, which may have ramifications for both the management of cattle lameness and for ovine footrot quarantine and/or eradication programs, especially if the strains of D. nodosus carried by cattle are virulent in sheep. The presence of F. necrophorum in lame dairy herds may be important in ovine footrot management as F. necrophorum is thought to be involved in this disease as well.

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References


Variation in *Fusobacterium necrophorum* strains present on the hooves of footrot infected sheep, goats and cattle

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1. Introduction

Footrot is a contagious, debilitating hoof disease of ruminants, particularly of sheep, goats and cattle. The infection appears to be the result of the synergistic action of particular bacterial species, of which *Dichelobacter nodosus* (*D. nodosus*) and *Fusobacterium necrophorum* (*F. necrophorum*) are thought to be involved in the etiology of this disease. While a lot is known about the genetic diversity of *D. nodosus*, very little is known about variation in *F. necrophorum*, especially as regards its role in footrot. We used PCR in conjunction with SSCP and sequencing to analyse swabs collected from the hooves of sheep, goats and cattle with symptomatic footrot for the presence of a portion of the *lktA* gene of *F. necrophorum*. Out of 29 samples tested, 27 had amplifiable *lktA* sequences and within these we found four different variants of the *lktA* gene. Eight of the nine samples from cattle were positive for a variant that matched the type strain of *F. necrophorum* subsp. *necrophorum*. Of the 14 samples from sheep, 13 were positive for *lktA*, but none of these matched the known type strains, and 11/13 of the *lktA* sequences were identical. This sequence was distinct to those of the type strains. None of the footrot infections carried multiple variants of *lktA*, suggesting that only one strain of *F. necrophorum* is present in each case. This is in contrast to *D. nodosus* in footrot infections, which have been demonstrated to have up to seven strains infecting a single hoof.

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Little is known about the role of *F. necrophorum* in footrot infections, and no studies have described the subspecies or strains of *F. necrophorum* present in footrot lesions. As a result of the slow growth-rate of many anaerobes and frequently mixed flora in footrot infections, PCR analysis provides a rapid and reliable alternative for the detection and amplification of *F. necrophorum*. In this paper, we used PCR on hoof samples from footrot infected sheep, goats and cattle to amplify the *F. necrophorum* lktA gene, and report the identification of four novel lktA sequences.

2. Materials and methods

2.1. Bacterial strains and footrot infected samples

Bacterial strains from the following related species/subspecies were used to test the specificity of lktA PCR amplification: *F. pseudonecrophorum* [American Type Culture Collection (ATCC) no. 51644], *F. varium* (ATCC no. 8501), *F. necrophorum* subspecies *funduliforme* (ATCC no. 51353), *F. necrophorum* subsp. *necrophorum* [National type culture collection (NTCC) no. 10575], *F. nucleatum* subsp. *nucleatum* (ATCC no. 25586) and *F. equinum* (NTCC no. 13176). *D. nodosus* (strain 198) was also included as the bacterium is associated with footrot infections.

Sterile cotton swabs were used to collect exudate from the axial skin-horn junction of the infected hooves of 14 sheep, 6 goats and 9 cattle, from farms across New Zealand. The ends of these swabs were fractured off into 1.5 ml tubes containing 0.7 ml of phosphate buffered saline (PBS) and 20 mM Na₂EDTA (pH 8.0). The tubes were stored at −20 °C until the DNA extraction procedure could be carried out.

2.2. DNA extraction

DNA from all bacterial cultures except *F. equinum* was extracted from cells by boiling for 10 min in 0.8% (v/v) Triton X-100 solution and following centrifugation at 13,000 × g, a 1-μl aliquot of the supernatant was used as a template for PCR amplification.

DNA from lesion material collected on swabs was extracted using a method developed by Zhou and Hickford (2000). Briefly, after thawing on ice, swabs were agitated before removal to disperse the lesion material into the buffer. Following centrifugation of the resulting suspension at 13,000 × g for 1 min, the pellets were washed and suspended in 200–500 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Each sample was then mixed with 1/10 volume of 10% sodium dodecylsulphate (SDS) and an equal volume of phenol/chloroform (1:1) added. Tubes were shaken vigorously for 20 s to lyse the bacterial cells and then placed at −20 °C for 10 min. The lysates were centrifuged at 13,000 × g for 4 min and the aqueous phase containing the DNA was collected. The DNA was recovered by ethanol precipitation and resuspended in 50–150 μl of TE buffer, depending on an estimate of the original amount of lesion material.

2.3. PCR primers and amplification

Two published lktA sequences [GenBank accession numbers AF312861 (Narayanan et al., 2001) and AY972049 (Zhang et al., 2006)] were used to design PCR primers for amplifying a 401-bp lktA fragment spanning from nucleotides 6336–6736 (coordinates given relative to the start of the lktA coding sequence) and that did not show close homology to any other known sequences (GenBank nucleotide, accessed October 2007). These primers were 5’-atctggtagtaggtgcctg-3’ and 5’-cttggtaactgcctgc-3’, and they were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Amplification was performed in a 20-μl reaction containing 1 μl of extracted DNA, 0.25 μM of each primer, 150 μM dNTP’s (Eppendorf, Hamburg, Germany), 3.5 mM MgCl₂, 500 ng/μl bovine serum albumin (BSA) (Sigma, St. Louis, MI, USA), 0.5 U Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× reaction buffer supplied. Amplification was carried out in a Mastercycler EP thermocycler (Eppendorf), and the thermal profile consisted of denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s, with a final extension step at 72 °C for 5 min. PCR amplicons were visualized by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na₂EDTA), containing 200 ng/ml ethidium bromide.

The presence of *D. nodosus* was detected by PCR as described previously (Zhou and Hickford, 2000).

2.4. Single-strand conformational polymorphism analysis

A 0.7-μl aliquot of each amplicon was mixed with 7 μl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), and after denaturation at 95 °C for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 12% acrylamide:bisacrylamide (37.5:1) (Bio-Rad Laboratories, Hercules, CA, USA) gels. Electrophoresis was performed using Protein II xi cells (Bio-Rad), at 300 V for 18 h at 5 °C in 0.5× TBE buffer, and gels were silver-stained.

2.5. Cloning of PCR amplicons and screening of clones

DNA samples representative of different SSCP patterns were selected for amplification using Pwo SuperYield DNA polymerase (Roche Applied Science, Mannheim, Germany) and using the conditions described previously. Amplicons were ligated into the pCR4 Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA), and a 2-μl aliquot of the ligation mixture was used to transform competent *Escherichia coli* cells (One Shot INVαF′, Invitrogen), following the manufacturer’s instructions. Between 10 and 15 insert positive colonies for each transformation were picked and incubated overnight in Terrific broth (Invitrogen) at 37 °C, in a shaking rotary incubator (225 rpm).

Clones were screened using a clonal PCR–SSCP approach as described previously (Zhou and Hickford, 2008), and only those clones for which the SSCP patterns matched those of the corresponding genomic DNA were selected for DNA sequencing.
2.6. DNA sequencing and sequence analysis

Plasmid DNA was extracted from overnight cultures of the original clones using a QIAprep Spin Miniprep kit (Qiagen) and was sequenced in both directions using the M13-forward and reverse primers at the Waikato DNA Sequencing Facility, University of Waikato, Hamilton, New Zealand. Identical sequences obtained from at least three separate clones that produced identical PCR-SSCP patterns were subjected to further sequence analysis.

Sequence alignments, translations and comparisons were carried out using DNAMAN (Version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada). The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) databases for homologous sequences.

A neighbor-joining phylogenetic tree was constructed on the basis of genetic distances, estimated by the Kimura (1980) two-parameter method, using MEGA (version 3.1; Kumar et al., 2004; http://www.megasoftware.net/). The reliability of the trees was estimated by bootstrap confidence values (Felsenstein, 1985), and 1000 bootstrap replications were used.

3. Results

Of the bacterial species and subspecies tested, only F. necrophorum subsp. necrophorum and F. necrophorum subsp. funduliforme generated PCR amplicons of the expected size (approximately 400 bp). Cloning and sequencing of these amplicons indicated that they were identical to the published lktA sequences from F. necrophorum subsp. necrophorum (Narayanan et al., 2001) and F. necrophorum subsp. funduliforme (Zhang et al., 2006), respectively. No PCR amplicons were observed with the other bacterial species tested.

The hoof samples were all confirmed to have the presence of D. nodosus using a PCR technique as described previously (Zhou and Hickford, 2000).

PCR amplification using the lktA primers revealed that 13 of the 14 hoof samples from sheep, 5 of the 6 scrapings from goats and all of the nine samples from cattle, generated PCR amplicons of the expected size (approximately 400 bp). These amplicons exhibited polymorphism upon SSCP analysis and in total four unique patterns could be detected (Fig. 1).

Cloning and sequencing of PCR amplicons representative of these SSCP patterns revealed four different nucleotide sequences (Fig. 2). One sequence (corresponding to the SSCP pattern A) was identical to the published F. necrophorum subsp. necrophorum lktA sequence (GenBank accession number AF312861), while the remaining three were novel, but the highest sequence homology upon BLAST searching was to this lktA sequence and the F. necrophorum subsp. funduliforme lktA sequence (GenBank accession number AY972049). These newly identified lktA sequences B–D were deposited into the GenBank with accession numbers FJ230830–FJ230832, respectively. No lktA sequences identical to the published subsp. funduliforme (GenBank accession number AY972049) were found in this study.

There were two, three and two different sequences of the lktA gene detected in sheep, goats and cattle, respectively (Table 1). However, only one lktA sequence was detected for individual animals. Sequence A (identical to lktA of subsp. necrophorum) was predominantly found in cattle, whereas in sheep and goats, sequence C was most commonly detected (Table 1).

Phylogenetic analysis of these newly identified lktA sequences B–D and the lktA sequences of F. necrophorum subsp. necrophorum and subsp. funduliforme, revealed that sequences C and D grouped together, but were separated from the cluster of F. necrophorum subsp. necrophorum and funduliforme. Sequence B was more similar to F. necrophorum subsp. necrophorum and funduliforme than to sequences C and D (Fig. 3).

4. Discussion

This paper describes the detection of the F. necrophorum lktA gene on hoof samples taken from footrot infected sheep, goats and cattle and the identification of four different lktA sequences. This suggests that F. necrophorum is frequently, if not always present, yet genetically diverse on the hooves of lame animals.

The presence of a single band of PCR amplicons with the expected size for both F. necrophorum subsp. necrophorum and subsp. funduliforme using the lktA primers and absence of amplicons for other related bacterial species, suggests that the PCR amplification of the lktA gene was highly specific under the conditions described.

The almost absolute detection of F. necrophorum in footrot lesions, suggests a role for this bacterium in footrot infection, supporting the contentions of Beveridge (1941) and Roberts and Egerton (1969). No F. necrophorum subsp. funduliforme was detected from these animals, which is consistent with it either being less pathogenic (Smith and Thornton, 1993), or tending to be found in human disease only (Riordan, 2007).

Although there were four different variants of F. necrophorum present in footrot lesions, there was quite
surprisingly no evidence of mixed-strain infection for *F. necrophorum* in sheep, goats and cattle. This is in contrast to the essential causative agent of footrot: *D. nodosus*, for which mixed-strain infections are common (Zhou and Hickford, 2000) and there may be up to seven strains present on a single hoof (Zhou et al., 2001). The detection of only a single variant from any one sample would seem to suggest that *F. necrophorum* variants are either very specialised; are under strong inter-specific competition; only colonise footrot samples in very small numbers (and therefore lack strain diversity); or a combination of these factors. This can only be revealed upon further detailed investigation.

Different host species also appeared to be infected by different variants of *F. necrophorum*. This would seem to suggest that either a host-specific effect occurs, with one variant favouring one host, or that host populations are only exposed to specific strains in the environment, as a consequence of some farm or management-specific effect.

Although the newly identified *lktA* sequences were homologous to the previously reported *F. necrophorum* sequences (Narayanan et al., 2001; Zhang et al., 2006), they were at some genetic distance from these *F. necrophorum* sequences (Fig. 3). It is possible that the variants detected in this study may represent different species of *Fusobacterium*, rather than different strains of *F. necrophorum*. A similar phenomenon has been previously reported for "*F.
Fusobacterium necrophorum” strains isolated from horses, with the reclassification of *F. equinum* as a new species based on phylogenetic analysis of the 16S rRNA gene, DNA–DNA hybridization and phenotypic characterisation (Dorsch et al., 2001). These analyses will assist the classification of these variants, but it also will require isolation of individual variants, a difficult and time-consuming process.

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References


Tadepalli et al. (2008) claimed recently that *Fusobacterium equinum* (*F. equinum*) possessed a functional leukotoxin (*lktA*) gene based on protein expression and Southern hybridisation analyses. However, efforts to amplify the *lktA* sequence from this species have failed and the identity of the *lktA* homologue awaits confirmation. Here we report the identification of a 407-bp DNA sequence amplified from the type strain of *F. equinum* (NTCC 13176) using PCR primers that were based on published *Fusobacterium lktA* sequences (GenBank accession numbers AF312861 and AY972049). This sequence did not show significant similarity to any NCBI GenBank sequence upon BLAST searching, but there was approximately 67.8% and 68.1% sequence homology found between this sequence (excluded primer binding regions) and the *lktA* sequences from *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme*, respectively. What is more, at the predicted amino acid sequence level, blocks of sequence conservation could be identified across the amplified region (Fig. 1). This suggests that the sequence identified here represent the *F. equinum* *lktA* gene and that this gene is functional, supporting the findings of Tadepalli et al. (2008) that *F. equinum* carries a functional *lktA* gene. However, the *F. equinum* *lktA* nucleotide sequence reported here appears to have low sequence homology to the *F. necrophorum lktA* gene, and this may explain why previous attempts to amplify the *lktA* gene from this species using *F. necrophorum* primers have failed (Tadepalli et al., 2008). This sequence was deposited into the GenBank with accession number EU836325.

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**Reference**


**Fig. 1.** The putative leukotoxin amino acid sequence from *F. equinum* aligned with the homologous sequences from *F. necrophorum* subsp. *funduliforme* (labelled as Fnf) and *F. necrophorum* subsp. *necrophorum* (labelled as Fnn). Conserved regions between the species are marked as hyphens.
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Letter to the Editor

Detection of *Fusobacterium equinum* on footrot infected hooves of sheep and cattle

**Article Info**

**Keywords:**
- *Fusobacterium equinum*
- Leukotoxin (lktA) gene
- Footrot
- Sheep
- Cattle

*Fusobacterium equinum* (*F. equinum*), an anaerobic bacterium originally isolated from horse oral flora, is phenotypically similar to *Fusobacterium necrophorum* (*F. necrophorum*) [Dorsch et al., 2001], and therefore may not be easily identified by biochemical methods. The identification of a unique lktA sequence in the *F. equinum* genome (Zhou et al., in press), provides an opportunity to detect this bacterium by PCR, without the need for bacterial culture and isolation. Based on published lktA sequences, we designed two PCR primers that would specifically amplify the lktA sequence from *F. equinum* and not from *F. necrophorum* subsp. *necrophorum* or subsp. *funduliforme* (Fig. 1). Interestingly, a PCR amplicon with the same size as that generated from *F. equinum* was produced from footrot lesion material collected from sheep and cattle (Fig. 1), suggesting that *F. equinum* was present on the footrot infected hooves of sheep and cattle. The presence of this bacterium was confirmed by cloning and sequencing of the PCR amplicons, which allowed the isolation of a DNA sequence that was identical to the *F. equinum* lktA sequence (GenBank accession number EU836325). This is the first report of *F. equinum* being detected on the hooves of sheep and cattle.

The detection of *F. equinum* in footrot lesion is interesting, but not surprising, given that it phenotypically resembles *F. necrophorum* and may have therefore historically been mistyped as *F. necrophorum* species previously. It also suggests that *F. equinum* may be involved in footrot infection. Further investigation is currently underway to address this.

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(J.G.H. Hickford)
Undetected lktA genes within *Fusobacterium necrophorum*

In a recent issue of this journal, Ludlam *et al.* (2009) outlined a PCR-based study on the basis of which they claimed that the lktA gene was not a universal virulence factor within strains of *Fusobacterium necrophorum* isolated from human and animal clinical cases. The authors also claimed that the detection of lktA was unevenly distributed across human and animal cases, between the two subspecies of *F. necrophorum* (subspecies *necrophorum* and subspecies *funduliforme*, hereafter referred to as *Fnn* and *Fnf*, respectively) and between the subspecies in isolates of bovine origin. We consider these unsafe conclusions as we are not convinced that the authors would have been able to detect the wide variety of lktA gene sequences that appear to be present within *F. necrophorum* with the PCR conditions they employed.

There are two reasons why we believe this is so, although these reasons are not completely unrelated. When using PCR on novel genetic material, it cannot be assumed that the primers will match the unknown target sequence, as even a single nucleotide mismatch at the 3’ end of either primer could completely stop amplification. As a result, the only conclusions that should ever be drawn from such diagnostic PCRs should be framed in terms of amplification or non-amplification, and not sequence presence or absence. To state absolutely that a sequence is not present is unjustifiable.

Accordingly in our opinion the title of their paper ‘lktA-encoded leukotoxin is not a universal virulence factor in invasive *Fusobacterium necrophorum* infections in animals and man’ (Ludlam *et al.*, 2009) overstates the case on the evidence presented. This inability to prove the absence of something is not unique to PCR-based diagnostics, as most diagnostic techniques cannot definitively prove the absence of something. As a result when drawing conclusions from such diagnostic approaches, the wording used needs to reflect what can, and cannot, be proven.

We acknowledge that Ludlam *et al.* (2009) attempted to address the dangers of false negatives by using a positive control in the form of two geographically distant isolates of *F. necrophorum* that have had their lktA genes described (GenBank accession nos AF312861 and DQ672338). However, both of these isolates were from cattle, and recent studies suggest that the host may play a stronger influence on the genetic variation of lktA in *F. necrophorum* than geography, with sheep, goats and pigs tending to carry variants of lktA that are different to those found in cattle (Zhou *et al.*, 2009a, 2010). Additionally, the *Fnn* lktA sequence isolated from cattle in the USA (GenBank accession no. AF312861) and the partial sequence isolated from cattle in New Zealand (Zhou *et al.*, 2009a) are identical.

When we aligned the primer and probe sequences reported by Ludlam *et al.* (2009) with the published *F. necrophorum* lktA sequences (GenBank accession nos AF312861, AY972049 and FJ230830–FJ230832) in a region where variation has been reported previously (Zhou *et al.*, 2009a), mismatches were found between their sequences and some of the *F. necrophorum* lktA sequences, including three from cloned DNA derived from uncultured strains of *F. necrophorum* (Fig. 1). In particular, there are at least 1, 3 and 1 nucleotide mismatches in the forward, reverse and probe-binding regions, respectively, when compared to *F. necrophorum* variants C and D, the former being the most common *F. necrophorum* variant found in sheep, goats and pigs (Zhou *et al.*, 2009a, 2010). Mismatches in these regions might prevent these variants from being amplified and hence these variants of the lktA gene may go undetected. Therefore, it is also possible that the primers and probes reported by Ludlam *et al.* (2009) might not detect all *F.

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<th>(a) LT2 forward:</th>
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<td>Fnn:</td>
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<th>(b) LT2 reverse:</th>
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<td>Fnn:</td>
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<tr>
<th>(c) LT2 probe:</th>
<th>AAGCTGAAAGTTACAGCCC</th>
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Fig. 1. Alignments of the primer and probe sequences reported by Ludlam *et al.* (2009) with *F. necrophorum* lktA sequences: (a) the LT2 forward primer, (b) the LT2 reverse primer and (c) the LT2 probe binding region. Dashes represent nucleotide identical to the top (primer/probe) sequence, and unknown nucleotide sequences are shown by dots. All sequences are presented 5’ to 3’, and the reverse complementary sequence of LT2 reverse primer is shown. The GenBank accession numbers for the *F. necrophorum* lktA sequences are AF312861 (*Fnn*), AY972049 (*Fnf*), FJ230830 (variant B), FJ230831 (variant C) and FJ230832 (variant D).
necrophorum variants, which may explain why they found only two sequences corresponding to the reported isolates of Fnn and Fnf.

Interestingly, although we have previously reported a total of four variants of F. necrophorum from sheep, goats, cattle and pigs, there was no evidence for the presence of Fnf in these animal species (Zhou et al., 2009a, 2010), while in the paper by Ludlam et al. (2009) Fnf appears to be common in animals, and even dominant over Fnn in some animal species such as cattle and sheep.

The issue of substantive variation in lktA genes preventing PCR techniques from being utilized to detect fusobacteria has been noted by other authors (Tadepalli et al., 2008). They reported Fusobacterium equinum (a bacterium phenotypically similar to F. necrophorum) produces a leukotoxin of equivalent size and potency to that of F. necrophorum, and that it is encoded by an lktA gene (shown to be present using probes and Southern hybridization), but that no amplicons were produced from the nominal lktA gene of F. equinum using PCR, probably because of sequence differences between the lktA genes of F. equinum and the two F. necrophorum subspecies. A portion of the lktA gene of F. equinum was only recently sequenced (Zhou et al., 2009b) and this confirmed that the gene was substantively different at the DNA sequence level from both Fnn and Fnf.

While we agree with the conclusion drawn by Ludlam et al. (2009) that vaccines against the lktA gene of Fnn may be unsuitable to be used against all strains, we do not agree with their reasoning and the assumptions that their conclusions are based on. Rather than lktA vaccines being likely to fail due to lktA not being universally present in all F. necrophorum strains, we consider it a possibility that vaccines could fail due to genetic differences observed in the lktA genes found in the various F. necrophorum strains or other F. necrophorum-like bacteria.

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Authors’ reply to ‘Undetected lktA genes within Fusobacterium necrophorum?’: presence or absence of an amplicon – the cornerstone of molecular diagnostics

Bennett et al. (2010) raise some interesting points and present some intriguing sequence data for the leukotoxin gene (lktA) of Fusobacterium necrophorum, drawn from work recently published by them, indicating three novel variants in the lktA gene (Zhou et al., 2009a). Bennett et al. (2010) speculate from these data that the conclusions drawn in our previous communication (Ludlam et al., 2009), in which we reported our failure to detect the gene employing PCR in a significant proportion of the two subspecies of F. necrophorum (subspecies necrophorum and funduliforme) recovered from infections in humans and animals (Ludlam et al., 2009), may be unsound.

Whilst we accept that a limitation of PCR-based diagnostics can be the failure to detect a novel variant of a pathogen, we cannot accept the assertion of Bennett et al. (2010) that the only conclusion that should ever be drawn from negative diagnostic PCR results is simply non-amplification of the sequence in question, rather than absence of the gene, or host organism. Following rigorous commercial and in-house test development and validation of PCR-based diagnostics of the sort we reported, diagnosis and management of infectious diseases is now routinely based on both positive and negative PCR results. Indeed, we note that the authors themselves ruled out the presence of F. necrophorum subspecies funduliforme in material from the lesions of the animal hooves that they examined on precisely this basis; i.e. a negative PCR result for lktA (Zhou et al., 2009a).

We were aware of the possible existence of sequence variation and went to great lengths to ensure that putative variants of the sort described by Zhou et al. (2009a) would not pass undetected. Whilst Bennett et al. (2010) focus on the LT2 primer set and TaqMan probe that emphasize their novel sequence data, they do not mention the other two primer sets (LT1 and LT3) that we employed, which targeted entirely different regions of the three published entire lktA gene sequences (F. necrophorum subspecies necrophorum, AF312861 and DQ672338; F. necrophorum subspecies funduliforme, AY972049). Our LT1 primer set is perfectly matched for all three known entire lktA gene sequences, while the LT3 forward primer contained just a single mismatch with F. necrophorum subspecies funduliforme AY972049 at the 5’ end of the primer and the LT3 reverse primer was perfectly matched for all three. All three primers sets amplified the lktA gene from the two.
Review

Ovine footrot: New approaches to an old disease

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ABSTRACT

Footrot is a bacterial disease that has substantial economic and welfare impacts in sheep and can be difficult to manage. Research is focussed on reducing the impact that footrot has on farmers and their flocks and better understanding the aetiology of the disease. Key areas of current research include, developing better vaccines, deploying tailored vaccines in a specific and targeted fashion on individual farms, analysing and developing better farm management practices to suit specific sheep farming environments, elucidating the virulence genes and bacterial population dynamics that drive footrot and using genetic testing in combination with selective breeding to produce stock that are more resilient to disease.

Footrot aetiology: an update

Towards a better understanding of *D. nodosus* virulence

The role of *F. necrophorum* in footrot

Changes in footrot treatment and management

The use of foot-paring and antibiotic treatment

Footrot eradication: the NSW Footrot Strategic Plan

Vaccination as part of footrot eradication and control strategies

Long term genetic improvement as a footrot management tool

Summary

References

Footrot is a contagious hoof disease of sheep and other ungulates and begins as an interdigital dermatitis, which is followed by formation of lesions on the interdigital wall of the hoof and subsequent separation of the hard horn from the foot (called under-running). The essential transmissible agent of the disease is the bacterium *Dichelobacter nodosus* (*D. nodosus*), although the role of other infective agents in the onset of disease is not fully understood.

Historically, footrot was reported to have been prevalent within English sheep in the 18th century and in France it was recognised as a contagious disease by the 19th century (*Stewart, 1989*). It was also identified on sheep farms in the United States of America, Italy, Germany and Australia by the early 19th century. On some Australian sheep farms, the impact of footrot was severe, with many deaths being recorded (*Stewart, 1989*). We believe the seminal work “Footrot and Foot Abscess of Ruminants” (*Egerton et al., 1989*) remains the definitive history of footrot.

Footrot can result in poor feed intake, losses in production, a reduction in wool strength and in the worst
cases; death from a combination of starvation, thirst and other systemic bacterial infections that occur in sheep that spend prolonged periods recumbent (Stewart, 1989). The cost of the disease can be substantial with estimates of £24 million per annum in the United Kingdom (Nieuwhof and Bishop, 2005) and Aus $42.6 million in New South Wales before a state-level footrot eradication plan was undertaken (Egerton et al., 2004).

Despite having been known about and researched for over 200 years, and with a comprehensive synopsis (Egerton et al., 1989) written about the disease, footrot remains a problem world-wide. However, there are several promising areas of footrot research and development that may provide new tools and approaches for better management or eradication of the disease. These include the development of specific vaccines, a markedly increased knowledge of the genetics of D. nodosus, the development and evaluation of new footrot management strategies customised to particular environments and the development of new genetic testing and selective breeding tools, which would create stock that are less likely to be infected and are less affected once they have footrot.

1. Footrot aetiology: an update

D. nodosus, the infectious agent that causes footrot, is a gram-negative, anaerobic bacterium (Beveridge, 1941). A second gram-negative anaerobic bacterium Fusobacterium necrophorum (F. necrophorum) is also required for D. nodosus to successfully initiate an infection in pen trials (Roberts and Egerton, 1969). These two bacteria are highly associated ($p < 0.001$) with footrot in the field (Bennett et al., 2009) and while D. nodosus may form multi-strain infections, Hill et al. (2010) have recently reported that the median number of serogroups per affected hoof is one, although it ranged from one to four. This observation is supported by the findings of Buller et al. (2010), although Zhou and Hickford (2000) report up to seven different D. nodosus strains on a single hoof. Zhou et al. (2009a) have reported that F. necrophorum tends to be found as a monoclonal infection, although only a small number of hooves were studied.

The complexity of the bacteriology of footrot lesions is further complicated by the genetics of the virulence of D. nodosus, which are intricate and potentially involve mobile genetic factors, including extra-chromosomally derived virulence islands with phage and plasmid-like forms (Cheetham et al., 2008). The bacterial complexity of the disease is further complicated by the effect of variation in temperature and rainfall on disease presentation (Graham and Egerton, 1968), by variation in host genetics (Emery et al., 1984), by variation in stocking rate (Stewart, 1989) and by the use of different farming practices such as hoof-trimming or paring (Wassink et al., 2003a).

Temperatures above 10°C appear to be required for footrot transmission to occur, while consistent rainfall over several weeks seems to be required for a footrot outbreak, rather than a single heavy rainfall event that only lasts a short period of time (Graham and Egerton, 1968). It has been proposed that wet weather affects footrot susceptibility, either by inducing physical changes in the hoof that make it more vulnerable to attack (Graham and Egerton, 1968), or by changing the biology of the pathogens that cause footrot.

Despite being anaerobic, D. nodosus is able to survive on plates exposed to air for up to 10 days (Myers et al., 2007) and both footrot and specifically D. nodosus can be transmitted between stock via soil contact (Stewart, 1989). As a result of being able to be transmitted via soil contact, stocking rates are likely to affect how quickly D. nodosus is transmitted through a mob (Stewart, 1989; Wassink et al., 2003a). Footrot is also difficult to manage, since as well as D. nodosus being able to survive on and be transmitted via soil, it can also persist for months as a sub-clinical infection within the inter-digital skin, or in small cryptic lesions within the hoof (Stewart, 1989).

Footrot displays a wide range of virulence and the disease has been categorised as virulent, benign or intermediate (Stewart et al., 1986). The virulence of a specific outbreak is driven by how a specific population or populations of D. nodosus interact with the host and the various factors that affect those infections. Virulent footrot is characterised by destruction of the horn and typically involves erosion of the skin-horn junction that penetrates the hoof, causing de-lamination because of under-running. In contrast, benign footrot causes inflammation of the inter-digital skin with inter-digital dermatitis, but no under-running or de-lamination is observed (Stewart et al., 1986). While intermediate footrot can cause under-running in some cases, it is observed to be much less widespread and transmittable within a flock, even in favourable environmental conditions. It is diagnosed by the isolation and identification of D. nodosus strains with intermediate virulence factors (Stewart et al., 1986). While the environment affects the transmission of footrot, one study has proposed that there is no basis for suggesting that intermediate footrot diagnosed in an unfavourable environment will cause virulent footrot if the disease spreads to sheep in a more favourable environment (Abbott and Egerton, 2003a), and this suggests that virulence is still determined primarily by D. nodosus and/or any other transmissible agent that moves with it between sheep.

1.1. Towards a better understanding of D. nodosus virulence

The genome of D. nodosus has been sequenced recently (Myers et al., 2007) and this is a major step forward in understanding footrot biology, as well as providing a platform that should allow a better understanding of the behaviour and virulence of the bacterium. D. nodosus has a wide range of virulence with strains classified as virulent, benign, or intermediate (Stewart et al., 1986). The virulence of D. nodosus isolates can be tested in vitro by testing for the presence, activity and stability of key virulence factors such as proteases or fimbriae-mediated motility (Stewart et al., 1986). Other virulence tests have also been developed that detect genetic elements associated with virulence, such as intA (previously known as vap) and vrl (Cheetham et al., 2006).

Despite both intA and vrl being associated with virulence in D. nodosus, these elements do not encode...
any known virulence factors. Rather, it is thought they may play a regulatory role by being inserted into, or near, global regulators of gene expression (Myers et al., 2007). If intA and vrl do affect global gene expression and regulation, their potential impact may be larger in D. nodosus than would normally be expected, since D. nodosus has a unusually small genome (1.4 MB) of which only 3% is devoted to regulation (versus the 8% and 8.4% that E. coli and P. aeruginosa utilise respectively) (Myers et al., 2007).

Recent research by Tanjung et al. (2009) illustrated this relationship by demonstrating that intD (a variant of intA) is associated with a benign phenotype of D. nodosus. The behaviour of the genetic elements of D. nodosus also appear to involve phage, since they carry genes homologous with phage regulatory genes, are found in phage insertion sites and share genes with the integrated D. nodosus phage, DinoH1 (Cheetham et al., 2008).

While the genetic elements of D. nodosus are not fully understood, it is clear that the complex intra- and inter-chromosomal behaviour of these elements may have a significant effect on the virulence of D. nodosus, footrot and the evolution of D. nodosus. The potential role horizontal gene transfer plays in D. nodosus biology is highlighted by the observation that up to 20% of its genome appears to have been derived from other organisms (Myers et al., 2007). Given the degree to which the genome of D. nodosus may have been derived from horizontal gene transfer and its tendency to form multi-strain infections, this suggests strain to strain transformations could be a driver of genetic variation in this bacterium. Such inter-strain transformations have the potential to be particularly important in D. nodosus pathogenicity since it can undergo sero-conversion following transformation and is serologically diverse with ten serogroups (Myers et al. 2007). The serogroups of D. nodosus are subdivided into serotypes and new variation in these serotypes is still being found with recent work on two novel German isolates of D. nodosus describing a variant of serotype B1 and a new serotype within serogroup H (Zhou et al., 2009b).

D. nodosus has several other well described virulence factors such as its fimbriae, proteases and outer-membrane proteins. The fimbriae of D. nodosus are required for binding to epithelial cells, providing twitching motility are involved in the uptake of extra-cellular DNA and are part of a secretion system able to export extra-cellular proteases involved in the uptake of extra-cellular DNA and are part of a secretion system able to export extra-cellular proteases by D. nodosus (formally biovar A) and F. necrophorum sub-species funduliforme (formally biovar B) (Nagaraja et al., 2005).

These sub-species of F. necrophorum tend to be found in particular hosts (Nagaraja et al., 2005) with F. necrophorum sub-species funduliforme tending to be found in animals (particularly cattle). When Zhou et al. (2009a) compared the F. necrophorum specific, leukotoxin gene (lktA) sequences from two known sub-species of F. necrophorum to the leukotoxin sequences detected in infected sheep and goats (variant C) and cattle (variant A), they observed that while the cattle variant was identical to F. necrophorum sub-species necrophorum, the variant commonly detected on sheep and goats with footrot was undescribed and appeared to be distinct from both F. necrophorum sub-species necrophorum and F. necrophorum sub-species funduliforme. Zhou et al. (2009a) also detected other un-described variants (variants B and D), but these were only detected in a minority (3/27) of cases. These new leukotoxin variants found in footrot may also represent a vaccine target given that it is now possible to use the leukotoxin as part of a vaccine effective against F. necrophorum in cattle (Saginala et al., 1997).

The presence of previously un-described variants of F. necrophorum in footrot lesions of sheep would suggest our understanding of the biology of footrot is in its infancy. Similarly, other bacteria not expected to be found in footrot lesions may be particularly problematic for footrot testing labs, because phenotypically this bacterium resembles D. nodosus (Zhou et al., 2009d). It is unknown if these bacteria have been previously mis-identified as D. nodosus and if their existence has been “clouding” the virulent, intermediate and benign descriptions of D. nodosus. In conclusion, the role that bacteria other than D. nodosus may play in footrot and how they modulate or affect the virulence of this complex disease is still poorly understood, but represents a potential direction for future footrot research.
2. Changes in footrot treatment and management

A variety of different footrot management and treatment approaches are utilised world-wide and these reflect the large number of differences in sheep production systems. In short, variation in management and treatment reflects variation in stocking rate (of importance with a contagious disease), the size of flocks, the cost of labour for labour-intensive management practices, the cost and availability of remedies and the acceptability of the various management and treatment regimes in different markets. The various approaches to footrot treatment and management are comprehensively covered by Egerton et al. (1989) and in the following section we will only touch on developments that have followed that influential work.

2.1. The use of foot-paring and antibiotic treatment

Foot-paring (also known as foot or hoof-trimming) is used to remove diseased tissue and promote a good foot conformation (Stewart, 1989). However it has become apparent that care needs to be taken not to over-use foot-paring, as it has been proposed that such practice may damage hooves and make sheep lame (Hosie, 2004), by giving footrot a site to enter the hoof and become established (Wassink et al., 2003a). What-is-more, in one study (Skerman et al., 1983), it was revealed that paring did not influence the final curative effect of foot-bathing in either formalin or zinc-surfactant, thus further questioning the efficacy of the practice.

Whether to pare the hooves of sheep with footrot has been under debate recently in the United Kingdom, since a survey of farmer management practices illustrated that routine foot-paring was associated with increased footrot prevalence (Wassink et al., 2003a). After some debate and following criticism (Abbott et al., 2003b), Wassink et al. (2003b) presented a follow-up study of 80 of the farmers from the original survey and revealed a large portion (77%) of them had not changed their foot-paring practices for over five years, which they suggested was evidence that the original epidemiological associations were much stronger than they first appeared.

To study if routine foot-paring could be a direct cause of footrot, Green et al. (2007) conducted a study that suggested that in a farming situation, footrot appeared to increase in prevalence following routine foot-paring. While this does not definitively prove that routine foot-paring causes footrot, it does suggest that excessive use of paring might be a risk factor that increases footrot prevalence and as such Green et al. (2007) suggested, the practice may need to be used with care. This suggestion is strongly supported by a study that concludes that if foot-paring on day 1 or 6 of diagnosis was stopped and parenteral antibacterials were instead used, then over 1 million sheep per annum lame with footrot in the United Kingdom would recover more rapidly and with benefits to productivity (Kaler et al., 2010).

While this might be seen as a solution to footrot, we see limitations to the widespread use of parenteral antibiotics including the observation that they appear to be most effective if sheep are held in dry conditions for 24 h following treatment (Egerton et al., 1968) and that sheep cannot be sold for human consumption until the antibiotic withdrawal period has expired (Jordan et al., 1996). Additionally, because foot-paring and the use of parenteral antibiotics are labour-intensive procedures, this will reduce their utility in many sheep production systems.

2.2. Footrot eradication: the NSW Footrot Strategic Plan

In New South Wales, a concerted program (the “NSW Footrot Strategic Plan”) to eradicate virulent footrot has been implemented. It has used a combination of treatment, vaccination, culling, and strict quarantine to eradicate virulent footrot from farms and maintain those farms free of footrot. This eradication programme has required strong and effective regulatory support and conscientious farmer involvement to ensure eradication of footrot on their properties (Egerton et al., 2004). The programme has enabled eradication of virulent footrot on individual properties throughout the state and a recent Government press release reveals that less than 1% of flocks in New South Wales now have virulent footrot1. In comparison it is claimed in the press release (see footnote 1) that before the programme began, virulent footrot could be found in 15% of sheep flocks. All of the currently affected flocks are under Government-imposed quarantine and farmers are only able to sell their sheep for slaughter.

The benefits of the New South Wales program have been substantial, with losses and costs attributable to footrot dropping from Aus $42.6 million per annum (Egerton et al., 2004) to Aus $500,000 per annum, with the main ongoing cost being disease surveillance (see footnote 1).

It should be noted that such programmes can be challenging to implement and the New South Wales programme is the result of over 20 years of concerted and costly effort. It was greatly facilitated by the periodically dry climate of New South Wales that allowed footrot to be eradicated from individual farms during periods of low transmission, and when the expense, time and effort spent on eradication would be most effective (Egerton et al., 2004).

In contrast with Australia, the United Kingdom does not have predictable periods of dry weather with low footrot transmission rates and so the Australian eradication protocols may be impracticable to use in the United Kingdom. While individual cases of footrot can be treated with antibiotics, vaccination, foot-paring and foot-bathing in zinc sulphate, the labour and material costs of these approaches suggest that a more sustainable long-term footrot control strategy may be needed in the United Kingdom where footrot is widespread and the climate favours regular transmission (Green and George, 2008). In a review of the potential strategies that could be viable in such a challenging environment such as the United Kingdom, Green and George (2008) outlined two possible long-term footrot management scenarios, elimination or eradication of footrot, as undertaken in New South Wales.

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or an approach that reduces the impact of footrot, but does not eliminate it.

For an extensive United Kingdom eradication program to be successful, Green and George (2008) recommend that culling of sheep with footrot must become an accepted practice. However, United Kingdom farmers are unable to transport sheep with footrot to slaughter, hence a regulatory barrier probably hinders this approach. Farmers would also need to work collectively with their neighbours to strategically eliminate footrot and once eliminated, prevent reintroduction by only purchasing footrot free sheep (such stock may be difficult to source in the United Kingdom). Eradication would also require strong, effective fencing to be maintained, substantial industry support and protocols developed to suit local United Kingdom conditions (Green and George, 2008).

Such a “locally-tailored” approach is also likely to be required for footrot eradication to be implemented in other temperate sheep growing countries such as New Zealand, Uruguay and Argentina.

An alternative to the eradication of footrot was also proposed by Green and George (2008). Their idea was to create a state of endemic stability where footrot occurs at a low rate, with a low impact on production and animal welfare. They suggested that this may be possible by lowering footrot transmission rates through the implementation of improved farm management practices. Management practices which may have this kind of effect include the rapid isolation and treatment of affected sheep, reducing stocking rates to decrease transmission, avoiding hoof damage by not using foot-paring, using improved vaccination technologies and increasing the sheep’s natural resistance to footrot by genetic selection (Green and George, 2008). However, even if endemic stability is achieved, it may subsequently fail if the local environment becomes more favourable for footrot transmission due to a change in climatic conditions or reversion to inappropriate management practices. This is a particularly pressing issue in the United Kingdom, given that its climate is conducive to footrot transmission.

2.3. Vaccination as part of footrot eradication and control strategies

Vaccines against *D. nodosus* have been shown to be effective at treating and preventing footrot (Liardet et al., 1989). The ability of these vaccines to prevent footrot on a large scale is probably limited, through a combination of antigenic competition reducing the effectiveness of vaccination against multiple serogroups (Schwartzkoff et al., 1993) and the propensity for multi-strain infections to occur (Zhou and Hickford, 2000). As a result, current vaccine research is focussed on: (1) the development of new “universal vaccines” using a “reverse vaccinology” approach, whereby genome sequencing is used to identify potential antigens from *D. nodosus* that are not hampered by antigenic competition and can prevent infection by multiple serogroups of *D. nodosus* (Myers et al., 2007), or (2) developing monovalent vaccines that are used strategically in specific footrot outbreaks (Egerton et al., 2002; Dhungyel et al., 2006; Dhungyel et al., 2008).

Strategic use of a monovalent vaccine requires that only one serogroup of virulent *D. nodosus* strains is causing footrot. This necessitates the isolation, virulence testing and identification of this serogroup followed by application of a monoclonal vaccine against this serogroup to control footrot. Such an approach has been successfully used in several parts of the world including Nepal (Egerton et al., 2002), Bhutan (Gurung et al., 2006) and Australia (Dhungyel et al., 2008).

The Nepalese programme was conducted at a national level and took advantage of the fact that only a single virulent serogroup of *D. nodosus* was present in the country as the result of a single importation of sheep with footrot (Egerton et al., 2002). The annual migration of flocks between alpine and low-land pastures also created an opportunity for a diagnosis and movement control system to be implemented in the national flock of 25,000 sheep (Egerton et al., 2002). As a result, a monovalent vaccine active against only the virulent serogroup of *D. nodosus* was able to be deployed to vaccinate the entire Nepalese flock, and once non-responding animals were identified and culled, virulent footrot was eradicated from Nepal (Egerton et al., 2002).

At the farm level, similar specific vaccination programs have been successfully conducted in Australia (Dhungyel et al., 2008) and Bhutan (Gurung et al., 2006). In Australia, a pilot study was conducted in two separate flocks, each infected with a single virulent or intermediate serogroup of *D. nodosus* (Dhungyel et al., 2008). No other virulent or intermediate *D. nodosus* serogroups could be found within these flocks, which allowed a specific monovalent vaccine to be deployed. This resulted in a marked reduction in the prevalence of footrot from pre-vaccination (44% and 8.5% prevalence) to 5–6 months after vaccination (no cases and 0.3% prevalence respectively). Follow-up monitoring was conducted at 16 or 18 months post vaccination respectively and no footrot cases could be detected. Dhungyel et al. (2008) noted that a strict quarantine programme had to be instituted to prevent re-infection of the flocks and that if lambs are born to infected ewes, they may also need to be vaccinated if climatic conditions favour footrot transmission.

Unlike the Australian programme, the program in Bhutan was conducted in a climate where the warmest temperatures are accompanied by a high, monthly, monsoon rainfall (over 100 cm of rain per month). Despite such a climate favouring footrot transmission, virulent footrot was able to be eradicated in this Bhutanese flock using a specific vaccination program (Gurung et al., 2006). The virulent *D. nodosus* serogroup present was able to be identified and a monovalent vaccine was applied over a period of two years leading to footrot eradication and follow-up examinations were performed for two years pre- and post-monsoon and no cases of footrot where subsequently identified.

A recent report (Dhungyel and Whittington, 2009) concluded that bivalent recombinant fimbrial antigens are also effective vaccines against virulent footrot, and without detrimental impact on the humoral immune response, although vaccines with four antigens present were less effective. In this same study it was shown that an inter-
vaccination interval of 3 months can be applied between two different bivalent vaccines without detrimental impact on the humoral immune responses to the various fimbrial antigens of *D. nodosus*. This suggests that vaccination against multi-strain infections may be possible without antigen competition compromising the vaccine and have wider application in vaccination against diseases caused by multivalent or multi-strain microbes.

In combination, the Australian and Bhutanese experiences suggest that it may be possible to eliminate virulent *D. nodosus* serogroups from individual farms, regions and possibly countries, thus providing an improved footrot management tool.

### 2.4. Long term genetic improvement as a footrot management tool

Footrot resistance has been demonstrated to be a heritable trait with some breeds such as Merino sheep being particularly susceptible to footrot, while others such as Romney are more resistant (Emery et al., 1984). Footrot resistance or vulnerability to disease in sheep has also been linked to variation in MHC II genes and forms the basis of a commercially available gene test developed in New Zealand (Bishop and Morris, 2007). The New Zealand gene test underpins selective breeding programmes that aim to reduce the impact of footrot on individual farms by creating more resistant stock that are not afflicted as often and more resilient stock that have less severe footrot (Bishop and Morris, 2007). The New Zealand programme has been assisted by a sheep industry that has contracted in recent years, allowing a strong culling pressure to be applied to flocks and in combination with an industry that has been driven to become more professional, efficient and effective. However the use of gene tests outside the breeds and populations they where developed in may be inappropriate. The debate around this subject has been hindered by the commercial interests that are involved in supplying advice and recommendations, elucidating the virulence genes and bacterial population dynamics that drive footrot and using genetic testing in combination with selective breeding to produce stock that are more resilient to disease.

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Isolation of two novel *Fusobacterium necrophorum* variants from sheep in Australia

The detection of three novel *Fusobacterium necrophorum* leukotoxin (*lktA*) sequences (B, C and D) in sheep, goats, cattle (Zhou et al., 2009) and pigs (Zhou et al., 2010), suggests the existence of un-identified strains or subspecies of *F. necrophorum*. However, these strains or subspecies have not been isolated, and hence their identities await confirmation. We have recently isolated five strains of *F. necrophorum* from sheep hoof scrapings collected into Stuarts transport agar (0.6% agar) and cultured onto Wilkins-Chalgren agar (Oxoid, Australia) containing 5% horse blood. These were incubated over five days at 37 °C in an anaerobic atmosphere (BD Gaspak™ EZ anaerobe container system, Becton, Dickinson & Co. USA). Biochemical identification using the Vitek 2 Anaerobe and Corynebacterium (ANC) identification card on the Vitek 2 Compact (Biomerieux) gave a confidence interval (CI) for *F. necrophorum* ranging from 93% to 99%.

Bacterial DNA was isolated using the QIAamp DNA mini kit (Qiagen, Australia) with the bacterial pellet protocol and using the Qiacube DNA extraction robot. A *lktA* PCR amplicon was produced from all five *F. necrophorum* isolates, using the PCR primers described previously (Zhou et al., 2009). This suggests that the *lktA* gene is present in all *F. necrophorum* isolates, a finding in contrast to that reported by Ludlam et al. (2009) in which *lktA* was only detected in a minority (47%) of *F. necrophorum* isolates. SSCP analysis of the *lktA* amplicons revealed that three isolates (CI of species identification as *F. necrophorum* 93–97%) carried a *lktA* sequence that matched with variant B, and the other two (CI 99%) carried a *lktA* sequence that matched with variant C. The isolation of *F. necrophorum* variants B and C is consistent with our previously finding that these variants were common in sheep (Zhou et al., 2009).

References


The presence of *Dichelobacter nodosus* and *Fusobacterium necrophorum* on the claws of lame dairy cattle in New Zealand

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**ABSTRACT**

Dairy cows face many environmental and management factors that increase the likelihood of lameness, but little is known about the microbiology of lameness. To understand which bacteria may be involved in bovine lameness, samples of debris were scraped from lame claws of dairy cows and examined for the presence of *Fusobacterium necrophorum* (*F. necrophorum*) and *Dichelobacter nodosus* (*D. nodosus*) using the polymerase chain reaction (PCR). *F. necrophorum* was detected in 79/148 of the samples, while *D. nodosus* was detected in 7/148 of the samples. The frequent finding of *F. necrophorum* on lame dairy cattle is noteworthy and the occasional finding of *D. nodosus* on some lame claws, suggested a possible role in bovine lameness. The presence of *F. necrophorum* was further investigated on the claws of cattle, sheep and goats with symptomatic footrot. Of the nine samples from cattle, all were positive for *F. necrophorum* and eight of these carried a variant of *F. necrophorum* that matched the type strain of *F. necrophorum* subsp. *necrophorum*. Of the 20 samples from sheep and goats, 18 were positive for *F. necrophorum* and 14 of these carried a different variant of *F. necrophorum*. This suggests that cattle may be infected by a *F. necrophorum* strain different to those affect sheep and goats. In contrast to *D. nodosus*, there was no evidence of mixed-strain infection for *F. necrophorum* in hooves.

**Keywords:** *Fusobacterium necrophorum*; *Dichelobacter nodosus*; dairy cattle; lameness; footrot.

**INTRODUCTION**

Dairy cattle face many environmental and management factors that increase the likelihood of lameness. Despite an increased awareness of these factors, lameness persists in herds and is a cost to the industry (Esslemont & Kossiabati, 1997), which accrues from expenditure on treatment and management and the adverse impact on milk yield and reproduction. Lameness also represents a welfare issue, as lame cows suffer pain. In the grass-fed New Zealand (NZ) dairy system, incidence can range from 5 to 50% (Gibbs & Laporte, 2006) and this has historically been attributed to non-nutritional causes (Chesterton et al., 1989).

The causes of lameness are multi-factorial and interrelated: much is due to damage to feet that are worn and related to the softening of keratin in wet conditions which increases the risk of wear, thin soles and solar bruising. The quality of tracks and lanes, exposure to long standing periods, poor stockman-ship and animal handling on concrete yards also contributes to lameness (Chesterton et al., 1989). The increase in farm and herd size in NZ has resulted in cows spending more time walking to and from pasture and standing in yards waiting to be milked and these are likely to be factors that increase the incidence of lameness.

Little is known about the microbiology of lameness, whether infections are causative or symptomatic, or whether improved understanding of this microbiology may allow better management. Pasture-based systems offer the potential for animal hooves to be exposed to a variety of environmental and animal-borne pathogens, especially where pastures are grazed rotationally and/or animals are “grazed off” in winter months in mixed animal systems. Accordingly, a PCR approach was used to assess the presence of two organisms: *Fusobacterium necrophorum* (*F. necrophorum*) which has been reported to be associated with cattle lameness and other diseases (Berg & Loan, 1975; Clark et al., 1985; Nagaraja et al., 2005) and *Dichelobacter nodosus* (*D. nodosus*), which has been described as the most common cause of lameness in sheep (Moore et al., 2005) and has been associated with cattle lameness for many years (Egerton & Parsonan, 1966; Berg & Loan, 1975; Laing & Egerton, 1978), but has not to our knowledge been found on lame cattle in the NZ pasture-based dairy production system.

**MATERIALS AND METHODS**

**Samples of claw debris and bacterial DNA extraction**

Scrapings (n= 148) from the surface of one in ten of the affected claws of lame cows treated between September 2005 and May 2006 were collected from 15 commercial dairy farms in NZ by farm staff. Swab samples (n=29) were taken from the axial skin-horn junction from the affected claw of sheep diagnosed as infected with footrot (n=14), goats (n=6) and cattle (n=9), from farms in the...
North and South Island of NZ. The ends of these swabs were removed and placed into 1.5 mL tubes containing 0.7 mL of phosphate buffered saline (PBS) and 20 mM Na₂EDTA (pH 8.0). Both the claw debris and swab samples were stored at -20 °C until the DNA extraction procedure could be completed. Bacterial DNA was extracted using a phenol/chloroform method described in Zhou & Hickford (2000), and eluted in 50 to 150 µL of TE buffer, depending on an estimate of the original amount of lesion material.

**PCR detection of D. nodosus and F. necrophorum**

*D. nodosus* was detected by PCR amplification of the *D. nodosus* fimA gene (Zhou & Hickford, 2000). *F. necrophorum* was detected by PCR amplification of the leukotoxin (*lktA*) gene using primers aatcgagtagtaggtctgg and cttgtgtaactgcactgc. Although *lktA* is also present in *F. equinum* (Zhou et al., 2009b), the PCR primers used would only amplify *lktA* from *F. necrophorum* under the conditions described (Zhou et al., 2009a).

**Genotyping of F. necrophorum**

*F. necrophorum* was genotyped by PCR-single-strand conformational polymorphism (PCR-SSCP) analysis, as described in Zhou et al. (2009a). Briefly, the *lktA* gene of *F. necrophorum* was PCR amplified and amplicons were subject to SSCP analysis in 12% polyacrylamide gels at 300 V for 18 h at 5 °C in 0.5× TBE buffer. Amplicons of five known *F. necrophorum* *lktA* sequences (Zhou et al., 2009a) were also included in each gel and these banding patterns were used as standards for determining the *lktA* sequences present in each sample.

**RESULTS AND DISCUSSION**

Of the 148 claw debris examined, a total of 79 (53%) samples tested positive for the presence of the *lktA* gene of *F. necrophorum* and seven (5%) tested positive for the *fimA* gene of *D. nodosus*. The detection of *F. necrophorum* on 53% of the claws of lame dairy cattle and *D. nodosus* at a low proportion (5%) of cases, suggested that *D. nodosus* was not a major contributing agent, while *F. necrophorum* was present in a greater proportion of cases and may contribute to lameness in NZ dairy cattle. This was consistent with the finding that vaccination against *F. necrophorum* reduced the incidence of lameness in some production systems (Checkley et al., 2005), although this work was completed in Canadian feedlot beef cattle. It should be noted that this survey may not be representative, as samples were not collected from healthy cattle and the positive results for *D. nodosus* were low in number (7 positives out of 148 samples).

The presence of *F. necrophorum* in debris scraped from lame claws of dairy cattle has led to the hypothesis that it may be associated with lameness, and that it may be present on the claw for a transitory period, or alternately it may potentially be found on the claws of NZ dairy cattle in general, but has not been detected previously. This has possible implications when managing herd health, since cattle claws could act as a source of *F. necrophorum* and potentially contribute to the spread of this bacterium.

The detection of *D. nodosus* on the claws of lame dairy cattle may be significant since this bacterium has typically been described as a pathogen found in the footrot lesions of sheep (Moore et al., 2005), and transmission of *D. nodosus* between cattle and sheep has been demonstrated (Wilkinson et al., 1970; Laing & Egerton, 1978). While, detecting this bacterium on some lame cattle may not indicate that the lameness is caused by *D. nodosus*, it confirmed that *D. nodosus* was present and able to persist on the claws of dairy cattle, even where the farm was a specialised dairy farm that did not stock sheep.

*F. necrophorum* was found to be present in all nine swab samples taken from footrot infected claws of dairy cattle, thirteen of fourteen samples collected from sheep and five of six samples collected from goats. There was only one *lktA* variant detected for individual animals. Variant A (matched with the type strain of *F. necrophorum* subsp. *necrophorum*) was predominantly found on cattle claws, whereas on sheep and goat claws, sequence C was most commonly detected (Table 1).

The almost absolute detection of *F. necrophorum* in footrot lesions, suggests a role for this bacterium in footrot infection, supporting the contentions of Beveridge (1941) and Roberts & Egerton (1969). There was no *F. necrophorum* subsp. *funduliforme* detected from the animals in this study, which is consistent with it either being less pathogenic (Smith & Thornton, 1993), or tending to only be related to human disease (Riordan, 2007).

Although there were four different variants of *F. necrophorum* present in footrot lesions, there was no evidence of mixed-strain infection of *F. necrophorum* in cattle, sheep and goats. This was in contrast to the essential causative agent of footrot: *D. nodosus*, for which mixed-strain infections are common (Zhou & Hickford, 2000) and where there may be up to seven strains present on a single claw (Zhou et al., 2001). The detection of only a single variant from any one sample would seem to suggest that *F. necrophorum* variants are either very specialised; are under strong inter-specific competition; only colonise footrot samples in very small numbers (and therefore lack strain diversity);
TABLE 1: Detection of F. necrophorum in footrot samples from cattle, sheep and goats.

<table>
<thead>
<tr>
<th></th>
<th>Cattle</th>
<th>Sheep</th>
<th>Goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples tested</td>
<td>9</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>No. of samples containing F. necrophorum</td>
<td>9</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>F. necrophorum variant</td>
<td>A 8</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B 0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C 1</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>D 0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

or a combination of these factors. This can only be revealed upon further detailed investigation.

Different host species also appeared to be infected by different variants of F. necrophorum. This would seem to suggest that either a host-specific effect occurs, with one variant favouring one host, or that host populations are only exposed to specific strains in the environment, as a consequence of some farm or management-specific effect.

In conclusion, F. necrophorum was frequently found on the lame claws of dairy cattle in NZ and may be associated with lameness. This suggests that a leukotoxic activity may be present on the lame claws of dairy cattle and that the presence of lame cattle should be considered when managing other diseases associated with F. necrophorum. D. nodosus may be present and persistent in lame cattle in NZ, which may have ramifications for both the management of cattle lameness and for ovine footrot quarantine and/or eradication programs, especially if the strains of D. nodosus carried by cattle are virulent in sheep. The presence of F. necrophorum in lame dairy herds may be important in ovine footrot management as F. necrophorum is thought to be involved in this disease as well (Roberts & Egerton, 1969).

REFERENCES


*Fusobacterium necrophorum, footrot and lameness in cattle, sheep and goats in New Zealand*

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Abstract

**Aim:** To investigate what variants of *Fusobacterium necrophorum* are present on the hooves of lame dairy cattle, sheep with footrot and goats with footrot in New Zealand.

**Methods:** A combination of PCR, DNA sequencing and SSCP was used to characterise the leukotoxin (*lktA*) gene of *F. necrophorum* variants present in claw scrapings from 32 lame dairy cattle and foot swabs collected from 15 sheep and 9 goats with footrot.

**Results:** It was found that *lktA* variants A and C of *F. necrophorum* were detected most frequently, being found in 53/56 samples analysed. The majority (55/56) of *F. necrophorum* infections appeared to be monoclonal, with only one variant of *F. necrophorum* detectable in each sample. Furthermore, variants A and C were not uniformly distributed between host species, rather variant A of *F. necrophorum* tended to be found on lame dairy cattle (29/32 samples) while variant C tended to be found on sheep and goats with footrot (21/24 samples).

**Conclusions and Clinical relevance:** These results imply that variants A and C of *F. necrophorum* are found on specific hosts. Given that the *F. necrophorum* strain represented by variant C has not been described, if it is a cause of footrot on sheep and goats, this has implications for future footrot research.

Keywords: Footrot, *Fusobacterium necrophorum*, leukotoxin (*lktA*) gene, cattle, sheep, goats

Abbreviations: *Fnn* (*F. necrophorum* sub-species *necrophorum*), *Fnf* (*F. necrophorum* sub-species *funduliforme*), US (Unites States), NZ (New Zealand), PCR-SSCP (PCR-single strand conformational polymorphism), National Collection of Type Cultures (NCTC), leukotoxin (*lkt*).
Introduction

The use of the polymerase chain reaction (PCR) to detect the genome of different organisms has revolutionised biology, not least clinical microbiology. In a clinical context PCR is used as a tool to identify a unique genome sequence from a microorganism with great precision and sensitivity. As a result PCR can detect organisms that are dead, non-culturable, not free-living (e.g. viral genomes) or present at very low levels. However due to its great sensitivity care must also be taken, since false positives can arise a sample is contaminated and as a detection based technology, it can never definitively prove an organism is absent, merely that it could not be detected.

The application of PCR has been particularly useful in the field of ovine footrot since it has allowed the detection of the anaerobic microbes Dichelobacter nodosus (D. nodosus) and Fusobacterium necrophorum (F. necrophorum) that cause the disease (Roberts and Egerton 1969) without the need to culture. By circumventing the requirement of anaerobic isolation and culture, it has allowed historical pen trials (Roberts and Egerton 1969) to be extended to on farm investigations of D. nodosus, F. necrophorum and footrot "in the wild". Furthermore, since PCR amplifies a specific piece of the target's genome, it has provided genetic information on D. nodosus and F. necrophorum in their in-situ context without the bias of what strain can be isolated or grown on a particular media. This is important in the case of F. necrophorum since no reliable isolation protocol from ovine footrot lesions is published and the strains of F. necrophorum ovine footrot appear un-described (Zhou et al. 2009a).
**F. necrophorum**

*Fusobacterium necrophorum* (*F. necrophorum*) is a gram-negative anaerobic bacterium associated with among other things footrot in sheep (Roberts and Egerton 1969), bovine liver abscesses and lameness in cattle (Nagaraja *et al.* 2005). Two sub-species have been described, *F. necrophorum* sub-species *necrophorum* (*Fnn*, formally Biovar A) and *F. necrophorum* sub-species *funduliforme* (*Fnf*, formally Biovar B) (Shinjo *et al.* 1991). These 2 sub-species tend to be isolated from specific hosts, with *Fnn* being isolated from cattle and *Fnf* from humans (Nagaraja *et al.* 2005). Both sub-species express a leukotoxin encoded by a the *lktA* gene (Oelke *et al.* 2005). The only other microorganism described as carrying the *lktA* gene is *F. equinum* (Tadepalli *et al.* 2008, Zhou *et al.* 2009b), a bacterium isolated from horse that is phenotypically similar to, but genetically distinct from *F. necrophorum* (Dorsch *et al.* 2001).

*F. necrophorum* has been linked to lameness in beef cattle in Brazil (Cruz *et al.* 2005) and had been found in lame dairy cattle in New Zealand (NZ) (Bennett *et al.* 2009a). However, it should be noted that lameness in cattle in NZ is thought to have a wide variety of causes beyond bacterial infection including interactions between the physical environment, climate, genetics, nutrition and stock management practices (Chesterton *et al.* 1989).

In contrast to lameness in cattle, footrot in sheep and goats is a distinctive, contagious disease. Footrot in sheep is characterised by extensive damage to the hoof or horn through detachment of the hard horn layers in a process called ‘under-running’. Under-running footrot symptoms result from infection by a gram-negative anaerobic bacterium, *Dichelobacter nodosus* (*D. nodosus*) (Beverage 1941) although, in pen trials, *D. nodosus* is unable to induce footrot in sheep without *F. necrophorum* also being present (Roberts and Egerton, 1969).
Support for this relationship has been confirmed in the field with sheep on pasture, where both *D. nodosus* and *F. necrophorum* were highly associated with footrot (p<0.001) and each other (p< 0.025) (Bennett *et al.* 2009b). A similar result was also seen in caprine footrot with *D. nodosus* and *F. necrophorum* being highly associated with footrot (p<0.01) and each other (p< 0.039) (Bennett *et al.* 2009c).

**Fusobacterium lktA genetics**

In a recent NZ study, Zhou *et al.* (2009a) used the *lktA* gene of *F. necrophorum* to detect *F. necrophorum* variants from cattle, sheep and goats diagnosed with footrot (i.e. signs of under-running and a positive test for *D. nodosus*). It was observed that a particular variant of *F. necrophorum* lktA (designated variant A) tended to be detected on cattle with footrot and that this *lktA* sequence matched that from an overseas type strain of *Fnn* (NCTC 10575) isolated from a bovine liver abscess. In contrast, sheep and goats with footrot tended to carry a different and un-described variant of *lktA* from *F. necrophorum* (designated variant C), that did not match the *lktA* from either sub-species of *F. necrophorum* (Zhou *et al.* 2009a). Zhou *et al.* (2009) also observed that these *F. necrophorum* infections appeared monoclonal and it was possible that variant C could represent a strain of *F. necrophorum* specific to ovine footrot. Variant C of *F. necrophorum* represented 11/13 of the *lktA* detections made from ovine footrot samples (the other 2/13 were Variant B) and appears genetically distinct from previously described *lktA* sequences found in *F. equinum* (Zhou *et al.* 2009b), *Fnn* or *Fnf* (Figure 1).
Repeat testing of variant C of *F. necrophorum lktA* is specific to footrot

While Zhou *et al.*'s (2009a) results were intriguing, the experiment was relatively small consisting of samples from 9 cattle, 13 sheep and 5 goats. As a result a repeat study was undertaken on larger scale using similar techniques to Zhou *et al.* (2009a) to test if the variants of *F. necrophorum* found on footrot could be specific to footrot in sheep and goats. Samples were scraped from the hooves of 32 lame dairy cattle from 13 farms, 15 sheep with footrot from 10 farms and 9 goats with footrot from 4 farms. The scrapings were taken from cattle that were being treated for lameness, or sheep and goats who displayed obvious under-running of the hoof (a distinctive symptom of footrot) and were derived from flocks with a high incidence of disease. In sheep and goats with footrot, scrapings were taken from the skin-horn junction of the diseased hoof. DNA was extracted from hoof scrapings, as described by Bennett *et al.* (2009a). A *lktA* PCR was performed, as described by Bennett *et al.* (2009a). *LktA* PCR products were screened and typed using PCR-single strand conformational polymorphism (PCR-SSCP), as described by Zhou *et al.* (2009a).

Of the 56 samples typed (Table 1), three variants of *lktA* were observed and these matched the previously described variants of the A, B and C of the *lktA* gene (Zhou *et al.* 2009a). Lame dairy cattle tended to carry variant A (29/32 samples), while sheep and goats with under-running footrot tended to carry variant C (21/24 samples). Variant B was both uncommon (3/24 samples) and only found on sheep and goats. Mixed infections by *F. necrophorum* appeared to be uncommon, with only one out of the 56 samples tested positive for both variants A and C of the *lktA* gene.
Discussion

This study confirms the findings of Zhou et al. (2009a) that cattle tend to carry variant A of the lktA gene of *F. necrophorum*, while sheep and goats with under-running footrot tended to carry variant C. We also noted that the vast majority of the *F. necrophorum* infections in this study (55/56) appeared to be monoclonal, reinforcing this observation made by Zhou et al. (2009a).

The repeatable finding that a specific, genetically distinct variant of lktA tends to be detected on animals with footrot supports the contention that a specific strain of *F. necrophorum* carrying variant C of lktA is found on sheep and goats with footrot. Furthermore, given its genetic distance from either sub-species of *F. necrophorum* (Fnn and Fnf), it is possible that this strain could represent an un-described sub species of *F. necrophorum* specific to sheep and goats with footrot.

The observation that *F. necrophorum* tends to be observed in a monoclonal state has also been made overseas where only a single strain of *F. necrophorum* tended to be found in a variety of animal infections (Jang and Hirsch 1994). The finding that *F. necrophorum* tends to be monoclonal is interesting in the context of ovine footrot, since it contrasts with *D. nodosus*, which tends to be found as a multi-strain infection, with up to 7 strains being found on a
single hoof (Zhou and Hickford 2000). This suggests that within the context of footrot, *D. nodosus* and *F. necrophorum* may have quite different life histories, population biology, and evolutionary drivers of inter-strain variation, despite both being necessary for footrot to be initiated (Roberts and Egerton 1969).

There are three possible explanations of the apparent monoclonal state and host specificity of the *F. necrophorum* variants found in this and Zhou *et al*’s (2009a) study. First, hosts may only be exposed to a specific variant of *F. necrophorum* as a result of farm management practices or localisation in different environments on NZ farms. Second, specific variants could exhibit a strain-dominance mechanism allowing a single strain to exclude other strains from an environment or host, similar to what has been observed to occur between symbiotic *Vibrio fischeri* strains colonising the light producing organs of squid (Nishiguchi 1998). Third, particular variants of *F. necrophorum* may be adapted to particular hosts. It should be noted, none of these explanations are mutually exclusive and it is conceivable that all three could mechanisms could be at play at different times in the life cycle of *F. necrophorum*.

In lame dairy cattle in NZ, previous studies highlighted that *F. necrophorum* is widespread on the claws of lame dairy cattle, being detected on 79/148 claw scrapings while *D. nodosus* was less common, being detected in 7/148 claw scrapings (Bennett *et al*. 2009a). This is in contrast to NZ cattle with under-running footrot where *D. nodosus* was detected in 9/9 samples (Zhou *et al.* 2009a). Despite the differences between the lame dairy cattle studied here and cattle with footrot studied by Zhou *et al*. (2009a), it is notable that variant A of *F. necrophorum* is detected in both conditions and that this variant of *lktA* matches the *Fnn* type
strain (NCTC 10575) isolated from a bovine liver abscess. This suggests that variant A of *F. necrophorum* may be host-specific to cattle, rather than specific to a particular environment, production system or hoof disease. Such host-specificity is striking given the differences in production systems between NZ pastoral dairy farms and US feed lots, where *Fnn* is found (Tadepalli 2009).

**Future Work**

The finding that variants of *F. necrophorum* may be both host-specific and monoclonal, raises several intriguing possibilities for future research and treatments of lameness and footrot. Given that *F. necrophorum* is required for ovine footrot to be induced in pen trials (Roberts and Egerton 1969) and its high association with under-running footrot in the field (Bennett *et al.* 2009b), it could be also be a target for vaccine development for footrot control. Historically, difficulty has been experienced raising protective immune responses against *F. necrophorum* (Egerton and Roberts, 1971), but recent technological advances in producing leukotoxin vaccines have circumvented this problem in mice (Narayanan 2003) and cattle (Jones *et al.* 2004, Checkley *et al.* 2005). This suggests that it may now be technologically feasible to target *F. necrophorum* with a vaccine to control footrot. In New Zealand other applications of *F. necrophorum* bovine vaccines include distinguishing if *F. necrophorum* infection on lame NZ dairy cattle increases the prevalence or severity of lameness or if *F. necrophorum* is merely a "harmless" coloniser of lame dairy cattle.

Other avenues of future work include studying the relationship of *F. necrophorum, D. nodosus* and footrot. This would require development of an *in vitro* co-culturing systems for *D.*
nodosus and F. necrophorum to measure if and how these organisms “communicate” with each other and their environment. Examples such interactions include, measuring if D. nodosus supplies F. necrophorum with amino acids via its protease or if the leukotoxin of F. necrophorum protects D. nodosus from the host's immune system.

Conclusions

This work supports the postulate that variant C of lktA represents an un-described strain or sub-species of F. necrophorum specific to sheep and goats with footrot. Furthermore this study also found that lktA variant A of F. necrophorum was found on lame dairy cattle in NZ, and this variant appeared identical those found on NZ cattle diagnosed with footrot and the strains found overseas in bovine liver abscesses (Zhou et al. 2009a).

Acknowledgements

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Figure 1. Phylogenetic tree comparing \textit{lktA} found in type strains and clinical samples. A comparison was made of the \textit{lktA} from type strains \textit{Fnn}, \textit{Fnf} (Oelke et al. 2005), \textit{F. equinum} (Zhou et al. 2009b) and \textit{lktA} variants A, B, C and D found in clinical samples from cattle, sheep and goats (Zhou et al. 2009a). Since the \textit{lktA} sequences seen in \textit{F. necrophorum} sub sp. necrophorum (\textit{Fnn}) and Variant A appear identical, they are combined in this figure. The number at the forks show bootstrap confidence levels. This phylogenetic tree was created using DNAMAN (Version 4.0, Lynnon BioSoft), the Neighbour-Joining method (Saitou and Nei 1987) and 1000 bootstrap replications to estimate confidence levels.
Table 1. Distribution of the *lktA* gene variants of *F. necrophorum* detected on the hooves of lame cattle, sheep with footrot and goats with footrot

<table>
<thead>
<tr>
<th><em>lktA</em> variant</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
</tr>
<tr>
<td>A</td>
<td>28/32</td>
</tr>
<tr>
<td>B</td>
<td>0/32</td>
</tr>
<tr>
<td>C</td>
<td>3/32</td>
</tr>
<tr>
<td>A+C</td>
<td>1/32</td>
</tr>
</tbody>
</table>
SUMMARY

Footrot is a destructive hoof disease that has a variety of pathologies observed as the disease begins, progresses, and either persists, heals or forms a cryptic lesion. While footrot is known to be transmitted by *Dichelobacter nodosus (D. nodosus)*, to initiate the disease *D. nodosus* appears to require the presence of a second pathogen *Fusobacterium necrophorum (F. necrophorum)*. Beyond *F. necrophorum* being required as an initiator of disease and being found in under-running footrot, the role it plays as footrot pathologies develop and change over time, is still unclear. To study the role *F. necrophorum* could be playing in different footrot pathologies as the disease develops and changes, 16 control sheep and 15 experimental sheep challenged with footrot were observed over an extended period of time. Data on pathology were collected in combination with prevalence of *D. nodosus* and *F. necrophorum* (detected using species-specific PCR diagnostics). This allowed descriptions of when and where these bacteria were found as the disease progressed. Disease and prevalence data were analysed using a case study approach describing what was observed for individual sheep. At the flock level, stepwise regressions and path analyses were used to assign mathematical relationships between the presence of pathogens and disease.

Findings include: 1) support for the model that *F. necrophorum* acts as a initiator of disease; 2) that *F. necrophorum* persists in cryptic lesions with *D. nodosus*; 3) that it is possible that *F. necrophorum* determines whether footrot develops into a destructive disease or if it becomes a chronic, static disease; 4) and that variance in *D. nodosus* detection was strongly correlated (P<0.01) with disease, suggesting that as footrot develops, *D. nodosus* may undergo a “boom and bust” life cycle.

**Key Words:** Footrot, *D. nodosus*, *F. necrophorum*, sheep
Introduction

Footrot is a destructive hoof disease that has a variety of pathologies observed as the disease begins, progresses, and either persists, heals or forms a cryptic lesion. While footrot is known to be transmitted by *Dichelobacter nodosus* (*D. nodosus*), to initiate the disease *D. nodosus* appears to require the presence of a second pathogen *Fusobacterium necrophorum* (*F. necrophorum*) (Roberts and Egerton 1969). Over and above *F. necrophorum* being required as an initiator of disease and being found in cases of under-running footrot (Bennett et al. 2009), the role it plays as footrot pathologies develop and change over time, is still unclear.

To study what role *F. necrophorum* could be playing in different footrot pathologies as the disease develops and changes, 16 control sheep and 15 experimental sheep challenged with footrot were observed over an extended period of time. Data on hoof pathology was collected in combination with data recording the prevalence of *D. nodosus* and *F. necrophorum* (detected using species-specific PCR diagnostic tests).

Materials and Methods

Footrot challenges

During the springs of 2006 and 2007, two flocks of sheep were challenged with footrot. Each challenge consisted of two groups of sheep, a control group sourced from a footrot free flock and an experimental group sourced from a flock that had footrot and which were kept separated from the controls at all times, but were in adjacent paddocks.

The experimental group were a mixture of sheep that had no previous history of footrot and those that had under-running footrot at the outset. The sheep with under-running footrot were used so as to provide a footrot challenge to other members of the experimental group.

Each challenge was conducted in four, 0.4 hectare paddocks (Effective stocking rate of between 20 -25 sheep/ha). The two groups were moved to adjacent paddock as demand for feed dictated. Irrigation was utilised as considered appropriate by the farm managers to create “wet” conditions to promote footrot spread and the demand for this irrigation varied depending on the weather during the two challenges.
**Sampling procedure, Challenge 1**

Over the course of Challenge 1 (2006), measurements were taken three times a week (every Monday, Wednesday and Friday). Sheep were assessed for the presence of footrot and scored using the scoring system described below. Swabs were taken from the skin-horn junction of the hoof with the highest footrot score or, if no clinical signs were apparent, from the front right hoof. If a footrot lesion was present, it was sampled in preference to the skin-horn junction.

**Sampling procedure, Challenge 2**

Over the course of Challenge 2 (2007), samples were taken weekly and each sheep was assessed and scored for footrot, as described below. Swabs were taken from the skin-horn junction of the hoof with the highest footrot score or, if no clinical signs were apparent, from the front right hoof. If any lesions were present, these were sampled in addition to the skin-horn junction (a different regime from that used in Challenge 1).

**Footrot scoring system**

A footrot scoring system based on the system of Egerton and Roberts (1971) was used (see below). This system was modified and expanded to allow the description of sheep without footrot and those sheep which had under-running footrot in more than one hoof. Footrot pathology for all the sheep (control and experimental groups) was described using the foot-scoring system in both Challenge 1 and 2.

**Table 1. Footrot Scoring System.** The most important criteria in determining foot score are bolded progressively through the table

<table>
<thead>
<tr>
<th>Description of pathology</th>
<th>Foot Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inflammation, dry</td>
<td>1.0</td>
</tr>
<tr>
<td>Slight inflammation or dampness</td>
<td>1.5</td>
</tr>
<tr>
<td>Inflammation and/or dampness</td>
<td>2.0</td>
</tr>
<tr>
<td>Inflammation, very damp, clammy, no smell</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Smells ‘footrotty’, very inflamed, usually damp, no lesions</strong></td>
<td>3.0</td>
</tr>
<tr>
<td>‘Footrotty’ smell, initial lesions present on inside of inter-digital skin or hoof, and/or a small lesion on outside of hoof, no signs of under-running</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Under-running and/or extensive hoof damage and/or shedding of hoof may be beginning (note, disease only present on one foot)</strong></td>
<td>4.0</td>
</tr>
<tr>
<td>Two or three feet have under-running footrot</td>
<td>4.5</td>
</tr>
<tr>
<td>All four feet have under-running footrot</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Detection of *D. nodosus* and *F. necrophorum* from swabs taken from sheep

Once swabs were taken DNA was extracted and stored at 4°C. These DNA samples were tested as described previously for the presence of *D. nodosus* (Zhou et al. 2000) and *F. necrophorum* (Bennett et al. 2009).

**Analysis of bacterial detection and disease**

Statistical analyses were carried out to describe the relationships over time between the detection of *D. nodosus*, *F. necrophorum* and disease. This analysis comprised stepwise regression analyses using Pearson correlation coefficients and presented graphically using path analysis (Li 1975). After incomplete data sets were excluded from analysis, a total of 34 sheep were analysed. The variables examined were: detection site (skin-horn junction or lesion), detection means (positive detection was assigned a score of 1 and non-detection a score of 0), detection variance, foot score and foot score variance.

The analyses used remove variables based on the significance of the correlation, which left the variables of skin-horn junction *D. nodosus* variance, skin-horn junction *F. necrophorum* mean, and either lesion *D. nodosus* mean or lesion *F. necrophorum* variance. To maintain simplicity within the analyses, the variables lesion *D. nodosus* mean and lesion *F. necrophorum* variance were analysed separately, as the remaining variables were highly significant (p<0.01). Analysis was performed using Minitab (Version 15) and GenStat (Version 12).

**Results**

**Summary of the Raw Data**

The raw data from this study are summarised in Table 2.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>No. of cases</th>
<th>No. of samples taken</th>
<th><em>D. nodosus</em> detection frequency</th>
<th><em>F. necrophorum</em> detection frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic under-running footrot</td>
<td>5</td>
<td>154</td>
<td>59.1%</td>
<td>39.6%</td>
</tr>
<tr>
<td>Destructive under-running footrot</td>
<td>1</td>
<td>45</td>
<td>26.7%</td>
<td>57.8%</td>
</tr>
<tr>
<td>Self curing footrot</td>
<td>1</td>
<td>24</td>
<td>12.5%</td>
<td>16.7%</td>
</tr>
<tr>
<td>Cryptic lesions</td>
<td>3</td>
<td>80</td>
<td>17.5%</td>
<td>23.8%</td>
</tr>
<tr>
<td>Benign footrot and/or inter-digital dermatitis</td>
<td>3</td>
<td>64</td>
<td>7.8%</td>
<td>14.1%</td>
</tr>
<tr>
<td>Subclinical infections</td>
<td>11</td>
<td>243</td>
<td>4.5%</td>
<td>4.5%</td>
</tr>
<tr>
<td>Sheep with no infection or signs of disease detected</td>
<td>14</td>
<td>277</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Detailed case studies

Specific case studies have been selected that demonstrate examples variously of destructive under-running, chronic under-running and cryptic lesion pathologies. These examples are shown in detail.

**Destructive Under-running Footrot**

During the first month of the Challenge, a new lesion appeared and developed into under-running footrot. Initially, a small (~5 mm), damp, lesion was observed on the skin-horn junction and within two weeks signs of under-running were observed. This under-running lesion also developed an “abscess like” pathology in the under-run claw and this was sampled concurrently with the under-running lesion.
Figure 1. Detection of *D. nodosus* and *F. necrophorum* from destructive under-running footrot. During the first month of the Challenge, a new lesion was observed on the skin-horn junction that developed into under-running footrot before healing. As this disease developed a distinct abscess like pathology formed and was sampled separately. Foot score is indicated by a dark blue line, site and timing of where samples were taken from, is indicated by black dotted boxes. Detection of *D. nodosus* is indicated by blue columns and detection of *F. necrophorum* is indicated by red columns. It should be noted before the initial lesion formed; both wet weather and hoof weakness (hoof unusually pliant and soft) were observed.

**Chronic footrot**

These two cases were known chronic carriers of footrot added to the experimental group to promote spread of the disease. They had footrot in at least two feet at any given time.

Figure 2. Foot score and detection of *D. nodosus* and *F. necrophorum* from case NT during Challenge 2. Foot score is shown by a dark blue line, site and timing of where samples were taken from is indicated by black dotted boxes. Detection of *D. nodosus* is indicated by blue columns and detection of *F. necrophorum* is indicated by red columns.
Figure 3. Foot score and detection of *D. nodosus* and *F. necrophorum* from case Y296 during Challenge 2. Foot score is indicated by a dark blue line, site and timing of where samples were taken from is indicated by black dotted boxes. Detection of *D. nodosus* is indicated by blue columns and detection of *F. necrophorum* is indicated by red columns.

**Cryptic lesions**

This case was part of the experimental group and was found to be carrying a cryptic footrot lesion. This cryptic lesion burst out from the toe on the abaxial wall of the left front hoof on the 20/10/06. Both *D. nodosus* and *F. necrophorum* could be detected from this cryptic lesion as it opened, healed and closed. This site briefly reopened on the 6/11/06 as the hoof healed, but no signs of under-running were observed. Later in the challenge this sheep occasionally showed signs of slight inflammation or smelling “footrotty” prior to a non-under running inter-digital lesion being found.
Figure 4. Detection of *D. nodosus* and *F. necrophorum* from case G49 during Challenge 1. Case G49 appeared not to have footrot, but 2 weeks after a wet weather event, a cryptic lesion opened on the front of the hoof before closing within 10 days. After irrigation began, benign footrot symptoms were observed including formation of a new lesion on the skin-horn junction at the same time as lambs were removed from the challenge. Foot score is indicated by a dark blue line, site and timing of where samples were taken is indicated by black dotted boxes. Detection of *D. nodosus* is indicated by blue columns and detection of *F. necrophorum* is indicated by red columns. Two data points (16/10/06 and 16/11/06) had no foot score recorded. As a result the missing score was replaced by a mean of the preceding and following foot score.

**Path analysis**

Data from 34 cases were used to conduct Pearson correlations, and regressions were presented using path analysis. The two variables, skin-horn *D. nodosus* variance and skin-horn *F. necrophorum* mean were correlated with foot score to a high significance (P<0.01) (Figures 5 and 6). Other variables which correlated with foot score were lesion *F. necrophorum* variance (P=0.034) and
lesion *D. nodosus* mean (P= 0.027).

Figure 5. Path analysis of relationship between variables including “Lesion, *F. necrophorum* variance”.

Stepwise regression eliminated low significance variables relative to high foot scores. The path diagram has been rooted to foot score under the assumption that *D. nodosus* and *F. necrophorum* cause footrot. The “Residual” variable represents unknown factors that affect foot score. Correlations between variables are shown, highly significant correlations (P<0.01) are marked by ** and significant correlations (0.01<P<0.05) by *.
Discussion

This research suggests a number of things about the complex microbiology of the contagious hoof disease footrot.

Firstly *F. necrophorum* is frequently found associated with footrot confirming the findings of Bennett et al. (2009). The nature of this association is probably complex, but the case studies appear to suggest that the presence of *F. necrophorum* correlates with both the onset of severe disease and with increases in the frequency of under-running footrot lesions. These case studies are summarised in the pathway analysis, where once again the correlation between the occurrence of *F.*
necrophorum and footscore is high. This does not tell us what role *F. necrophorum* plays in disease aetiology, but given its production of a powerful leukotoxin (Narayanan et al. 2001), it could be speculated that it is involved in providing an immune-compromised setting on the hoof that is a better habitat for *D. nodosus* growth and activity. This is consistent with other reports of *F. necrophorum* occurrence, including a role in liver abscesses (Nagaraja et al. 2005), calf diphtheria (Nagaraja et al. 2005) and Lemierre’s syndrome (Lemierre 1936) in humans. Such a leukotoxic effect would be particularly important in footrot since *D. nodosus* is known to be susceptible to attack by the immune response (Roberts and Egerton 1969).

The importance of the relationship between *D. nodosus* and *F. necrophorum* was highlighted by comparison of chronic and destructive under-running footrot, where *F. necrophorum* was more frequently detected in the most destructive cases. In combination, our results, and those of Roberts and Egerton (1969), suggest that *F. necrophorum* infection is essential and predates *D. nodosus* infection during footrot. This would suggest the relationship between the two organisms is important and possibly that *D. nodosus* would not pose such a disease problem, if posing a problem at all, unless it is in a partnership with *F. necrophorum* on the hoof. Whether this means we could target *F. necrophorum* with a vaccine or an antibiotic approach as a means of footrot control needs to be ascertained.

A relationship between *D. nodosus* and *F. necrophorum* activity may also occur outside regions of active disease, since in cryptic lesions both bacteria are found persisting together. In such an environment a model of synergism could be envisaged, where both bacteria coexist together resisting environmental and immune stresses. In such a model, the leukotoxin secreted by *F. necrophorum* may play a protective role allowing *D. nodosus* to escape phagocytosis by the immune system (demonstrated to occur by Roberts and Egerton [1969]). In turn, *D. nodosus* may have a role in maintaining anaerobiosis as well as nutrition by supplying *F. necrophorum* with metabolites in the form of digested hoof proteins. Alternatively cryptic lesions may have other cyst-like attributes which allow them to avoid the immune response of the host.

The correlation between variance in *D. nodosus* detection on the skin-horn junction and high foot score suggests *D. nodosus* populations tend to wax and wane in numbers when the hoof is diseased. There are several possible mechanisms that might cause this. *D. nodosus* may exist in a ‘boom and bust’ life cycle where populations quickly expand when nutrients are briefly available on the skin-horn junction. Such a brief availability of nutrients would be particularly growth limiting to *D. nodosus* since it must import most of its amino acids, being unable to synthesize them
metabolically (Myers et al. 2007) from its small and simple genome. Once growth of *D. nodosus* was halted, the ‘bust’ in its numbers could be intensified since it is vulnerable to both phagocytosis (Roberts and Egerton 1969) and oxygen (Myers et al. 2007).

Beyond such changes in populations dynamics driving variance, it is possible that variance in detection of *D. nodosus* using PCR is also driven by the environment, affecting how many cells can be sampled and detected. Examples of this might include cells being washed on or off surfaces, the horn becoming softer or harder during disease or intermittent shedding of PCR inhibitors from putrefying tissue. Changes in the horn or lesions during disease development could also affect the migration of *D. nodosus* cells onto the skin-horn junction from foci of *D. nodosus* cells sequestered within the skin (Morgan et al. 1972) or “covert infections” within the horn (Egerton et al. 1989). It is conceivable that this could result in varying numbers of detectable cells of *D. nodosus* on the skin-horn junction and in our context false negatives being observed. In contrast it is difficult to see, laboratory-derived contamination aside, how PCR-based detection would give false positives given the specific nature of the detection methodology.

In summary, this investigation found that *F. necrophorum* may be involved in new, under-running footrot cases, although it may not necessarily be involved in chronic footrot cases to the same degree. *D. nodosus* and *F. necrophorum* also appear to persist together in cryptic lesions. *F. necrophorum* was found in benign footrot and inter-digital lesions on the skin-horn junction and these inter digital-lesions can act as a precursor to under-running footrot. Notably, in these cases, *D. nodosus* only became detectable once under-running began. Statistical analysis indicated that both detection of bacteria and variance in detection was correlated with disease, implying that the relationship between pathogens and footrot is complex.

**Acknowledgements**

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**References**


Improved method for isolating bacterial DNA from rumen fluid

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Abstract

DNA extraction from the rumen is difficult and impedes molecular studies of ruminant ecosystems and bacteriology. Therefore, we have sought to improve existing methodology for the isolation of ruminant bacterial DNA to facilitate ecological studies. We improved the quality of the extracted DNA from rumen fluid by washing bacterial cells with phosphate buffered saline and by adding soluble polyvinyl pyrollidine and 2-β-mercaptoethanol to the extraction buffers used in a silica column-based SV Wizard genomic DNA extraction kit.

A previously reported PCR method for detecting the methyl-coenzyme M reductase gene of methanogens found in the rumen, was used to test the quality of DNA isolated from these bacteria in rumen fluid. DNA purified in this way, was found to give PCR amplicons of the correct size, when the DNA eluted comprised up to 78% (v/v) of the PCR reaction. This suggests the effective removal of PCR inhibitors by this modified method.

Keywords: Methanogens; DNA extraction; PCR; Rumen fluid

Abbreviations:

- 2-BME (2-β-mercaptoethanol)
- PVP (soluble polyvinyl pyrollidine)
- PCR (polymerase chain reaction)
The rumen is a biologically active, diverse and dynamic biological system of great significance to agricultural production world-wide. Traditional microbiology is very difficult in such an environment due to the vast variety, complexity and numbers of organisms present, but the polymerase chain reaction (PCR) provides a useful tool for identifying particular organisms because of its specificity and sensitivity. The utility of PCR to detect specific bacterial populations in this environment is compromised by the co-isolation of plant-derived inhibitors of DNA polymerases and chemicals capable of reacting with DNA [1, 2].

The chemical constituents of rumen fluid can contain PCR inhibitors, affect bacterial DNA yields during extraction and reduce DNA purity. The most destructive compounds in rumen fluid are butyric acid(s), large polysaccharides [1], reactive phenolic compounds and plant-derived enzymes such as polyphenol oxidase [2-4], that can rapidly bind phenolic substances to DNA.

While protocols have been described for the purification of bacterial genomic DNA from rumen fluid, they tend to be time-consuming, involve the use of phenol [5] and may involve difficult procedures such as bead-beating lysis as part of the extraction regime [6-7]. Phenol-based extractions are particularly time-consuming, laborious and not always consistent, although protocols based on DNA-binding columns that avoid the use of phenol, have been described for both protozoan DNA extraction [8] and archaea DNA extraction [9] from rumen fluid.

In this work, rumen fluid was collected from a cannulated cow. Two different DNA extraction methods were performed from a single sample of rumen fluid and the quality of DNA isolated by each method was assessed using PCR. Each extraction method is described below. The first is a modified silica-column based method and the second uses a combination of cell washes, boiling lysis and phenol extraction. DNA quality was assessed using PCR
primers designed to amplify the methyl coenzyme M reductase gene from methanogenic bacteria [10], followed by gel electrophoresis, to view the amplicons (Figure 1).

In the first method, total DNA was isolated from rumen fluid using the Wizard® SV Genomic DNA Isolation kit (Promega, Madison WI, USA) according to the manufacturer’s instructions with the following modifications. Cells were washed in sterile phosphate-buffered saline (PBS, pH 7.3) (Oxoid, Hampshire, UK), before being centrifuged (13,000×g for 10 min) and re-dissolved in 500 µl of nuclei lysis buffer (Promega). To each aliquot of 500 µl of nuclei lysis buffer (Promega) was added, 4 µl RNAase solution (Promega), 2% (w/v) soluble polyvinyl pyrollidine (PVP) (Sigma Aldrich, Milwaukee WI, USA) and 5% (v/v) 2-β-mercaptoethanol (2-BME) (Sigma), before incubation at 80°C for 10 min.

From the same batch of rumen fluid, DNA was isolated using a phenol-chloroform extraction method. The rumen fluid was centrifuged (13,000 × g for 10 min) and the pellet washed in PBS and re-centrifuged (13,000 × g for 10 min) twice before being resuspended in 750 µl sterile water. This was boiled for 10 min and centrifuged (13,000 × g for 10 min). The supernatant was extracted with 750 µl of Tris-buffered phenol and then centrifuged (5,000 × g for 5 min). The supernatant was re-extracted with 750 µl of chloroform and isoamyl alcohol (24:1). After centrifugation (5,000 × g for 5 min), the supernatant was precipitated with an equal volume of ice-cold iso-propanol. This solution was centrifuged (14,500 × g for 10 min) and the pellet was air dried and re-dissolved in either 50 µl or 200 µl of sterile water.

The DNA extracted by both methods was amplified using 50 µl PCR reactions, each reaction contained 0.25 mM of each primer (ME1, 5’ GCMATGCARATHGGGWATGTC; ME2, 5’ TCATKGCRTAGTTDGGRTAGT) [10] (synthesised by Proligo LLC, Colorado, CA, USA), 200 µM of dNTPs (Eppendorf, Hamburg, Germany), 2.5 U of Taq polymerase (Qiagen, Hilden, Germany), 5 µl of 10 × buffer and a final Mg²⁺ concentration of 1.5 mM,
and either 39 µl of DNA or 4 µl of extracted DNA, with water to make up the volume. The thermal profile consisted of a 4 min denaturation at 94°C, followed by 35 cycles of 94°C for 40 s, 50°C for 90 s and 72°C for 180 s, with a final elongation step of 72°C for 7 min.

Electrophoresis was carried out using a 1.5% agarose gel and 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na₂EDTA) containing ethidium bromide at 0.2 µg ml⁻¹. Aliquots (10 µl) of each PCR product were combined with loading dye (50% sucrose, 0.25% bromophenol blue and 0.25% xylene cyanol) before being loaded into the wells.

The DNA extracted by the silica column method was found to be free of PCR inhibitors and could be amplified via PCR even when used at 78% of the PCR reaction mix (Fig. 1). The PCR products produced from the phenol-chloroform extraction suggested that the DNA was of poorer quality, producing a much wide-range of amplicon sizes and intensities when compared to the modified Wizard™ SV genomic DNA purification system.

If large amounts of genomic DNA had been extracted from rumen fluid, it might be expected that genomic DNA would be visible when the extracted DNA made up 78% of the aliquot loaded into the agarose gel. None was visible (lane 5 fig. 1). Spectrometry data has suggested that the Wizard™ SV columns may lose DNA binding capacity when other molecules such as RNA overload the column (José Laporte, Personal Communication). In most situations this would be prevented by the presence of RNAase in the lysis buffer, but this enzyme may have been deactivated by the rumen fluid used in this study.

Despite these issues, chemical modification of DNA extraction protocols based on columns may be useful when applied to ruminant bacteriology. During extraction of DNA from plant tissues, PVP is used to prevent phenolic compounds forming covalent bonds to
DNA while 2-BME prevents oxidative cross linking of DNA in solution after lysis [2-4]. The combination of these chemicals with multiple pre-lysis PBS washes has created an effective, quick, DNA extraction protocol that could be suitable for working with bacteria in rumen fluid.

References


Fig. 1. PCR amplification of the methanogen, methyl-coenzyme M reductase gene from bovine rumen fluid. Amplifications were performed using 39 µl (Lanes 1-3) or 4 µl (Lanes 4-6) of DNA. DNA extracted using a modified Wizard® SV Genomic DNA Isolation kit (Lanes 3 and 6: DNA eluted in 200 µl of dH2O) or by a phenol-chloroform method (Lanes 1 and 4: DNA suspended in 50 µl of dH2O; Lanes 2 and 5: DNA suspended in 200 µl of dH2O). Lane M was 1 kb plus DNA ladder and a negative control is shown in Lane 7. Each lane was loaded with 10 µl of PCR product and visualised using electrophoresis carried out on a 1.5% agarose gel and 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na2EDTA) containing ethidium bromide at 0.2 µg ml⁻¹.
Appendix C. Weather data from Trial 1

During the spring of 2006 when Trial 1 was run, maximum temperatures were usually above 10°C with minimum temperatures above 0°C (Figure C.1). Weather was generally warm with occasional intermittent storms followed by cooling (Figures C.1, C.2). A notable heavy rainfall event occurred on day -8 before the challenge began.

![Trial 1, min. and max. temperatures](image)

**Figure C1. Daily min. and max. temperatures during Trial 1.** Daily min. and max. temperatures were measured throughout Trial 1 by Station 17603, at Plant & Food Research in Lincoln. Data were accessed using the CliFlo system maintained by NIWA.
Figure C.2. Rainfall during Trial 1. Rainfall was measured throughout Trial 1 by Station 17603, at Plant & Food Research in Lincoln. Data were accessed using the CliFlo system maintained by NIWA. Pre-trial sampling was conducted on day -31, before the trial began on day 0 and irrigation was conducted from day 18.
Appendix D. Weather data from Trial 2

Throughout Trial 2, maximum daily temperatures were above 10°C, while minimum temperatures fluctuated, occasionally dipping below 0°C (Figure D.1). In the second half of the trial, minimum temperatures approached 10°C. Rainfall was observed throughout the trial with irrigation applied as required from day 55 (Figure D.2)

![Trial 2, max. and min. temperatures](image)

**Figure D.1. Daily min. and max. temperatures during Trial 2.** Daily min. and max. temperatures were measured throughout Trial 2 by Station 17603, at Plant & Food Research in Lincoln. Data were accessed using the CliFlo system maintained by NIWA. Pre-challenge sampling was undertaken on day 13 before the trial began on day 0.
Figure D.2. Rainfall during Trial 2. Rainfall was measured throughout Trial 2 by Station 17603, at Plant & Food Research in Lincoln. Data were accessed using the CliFlo system maintained by NIWA. Pre-challenge sampling was undertaken on day -13, before the trial began on day 0 and irrigation was conducted from day 55.
Appendix E. Output of model comparing bacterial detection frequencies between control and experimental groups

Generalized linear mixed model analysis

=======================================
Method: c.f. Schall (1991) Biometrika¹
Response variate: bugs
Distribution: poisson
Link function: logarithm
Random model: Expt
Fixed model: Constant + Treatment

Dispersion parameter estimated

Monitoring information

----------------------
Iteration Gammas Dispersion Max change
1 0.6946 0.8473 4.0878E-01
2 0.6828 0.9058 5.8497E-02
3 0.5950 1.047 1.4140E-01
4 0.5887 1.058 1.1302E-02
5 0.5887 1.058 2.2087E-05

Estimated variance components

-------------------------------
Random term component s.e.

Expt                          0.623       0.895

Residual variance model

-------------------------------------
Term   Factor  Model(order)  Parameter  Estimate  s.e.
Dispersn  Identity  Sigma2  1.058  0.121

Estimated variance matrix for variance components

---------------------------------------------------------------------------------
Expt  1  0.8002
Dispersn  2  -0.0001  0.0146
1  2
---------------------------------------------------------------------------------

Tests for fixed effects

-----------------------
Sequentially adding terms to fixed model

Fixed term  Wald statistic  n.d.f.  F statistic  d.d.f.  F pr
Treatment  110.45  1  110.45  153.0  <0.001

Dropping individual terms from full fixed model

Fixed term  Wald statistic  n.d.f.  F statistic  d.d.f.  F pr
Treatment  110.45  1  110.45  153.0  <0.001

Table of effects for Treatment

-------------------------------------
Treatment  Control  Treated
0.000  2.514

Standard error of differences: 0.2392
Tables of means with standard errors

Table of predicted means for Treatment

<table>
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<tr>
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<th>Treated</th>
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<td>1.073</td>
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Standard error of differences: 0.2392

Table of predicted means for Treatment

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<th>Control</th>
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<td>1.073</td>
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Standard errors

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<th>Maximum</th>
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Back-transformed Means (on the original scale)

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<tr>
<td>Treated</td>
<td>2.925</td>
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</table>
Appendix E shows the output of a statistical model compared bacterial prevalence between control and experimental groups. This model statistically describes the effect “treatment” had on the experimental group. The model was created and applied by Dr. Richard Sedcole using the Genstat (Version 12).
Appendix F. Matrix plot of pairs of variables used in path analysis.

Matrix Plot of Sco mean, Sco var, SDn mn, SDn va, SFn mn, SFn va, ...

LeDn mn
LeDn va
LeFn mn
LeFn va
Appendix F shows a matrix plot of pairs of variables used in path analysis. These variables were foot score mean (Sco mean), foot score variance (Sco va), skin-horn junction D. nodosus mean (SDn mn), skin-horn junction D. nodosus variance (SDn va), skin-horn junction F. necrophorum mean (SFn mn), skin-horn junction F. necrophorum variance (SFn va), lesion D. nodosus mean (LeDn mn), lesion D. nodosus variance (LeDn va), lesion F. necrophorum mean (LeFn mn) and lesion F. necrophorum variance (LeFn va). Detection means were calculated by assigning a positive detection a score of 1 and a lack of detection a score of 0.
Appendix G shows a multiple alignment between sequences of potential fimA amplimers generated from ovine gastro-intestinal tract samples and a library of known fimA sequences (Zhou and Hickford 2000a). The multiple alignment was performed using DNAMAN (Version 4.0, Lynnon BioSoft). Known fimA sequences are marked as B-nz3, A-nz1, B-nz1, B-nz2, D-nz1, B-nz4, B-nz5, B-nz6, C-nz1, C-nz2, M-nz1, E-nz1 and F-nz1. Sequences of potential fimA amplimers generated from the gastro-intestinal tract of sheep are marked as 30F, 8A, 8C, 39C, 34F and 38F.
Appendix H. Instructions to farmers taking hoof scrapings from lame dairy cattle

Sampling and posting procedure

1) after hosing/preparing the foot and identifying the cause of the lame foot, unscrew jar and use scraper in the wound to collect either pus, crumbling horn, or simply cut off a small piece of horn (3mm size, that is about 3 size) and put it in the jar. The scraper is a tough plastic design and can be used to vigorously scrape (‘sandpaper’) horn material off.

2) Screw the scraper back into the jar tightly

3) Write the cow ID (the same ID as in the lameness recording book) on the jar

4) Put the jar in the Freepost envelope provided and drop it in the mail – there are no addresses or postage charges required.
Appendix I. Multiple sequence alignment of the lktA variants A, B, C and D with lktA genes of F. necrophorum sub sp. necrophorum (Fnn) and F. necrophorum sub sp. funduliforme (Fnf).
Appendix I shows a multiple alignment of the \textit{lktA} variants A, B, C and D with \textit{lktA} genes of \textit{F. necrophorum} sub sp. \textit{necrophorum} (labelled \textit{Fnn}) and \textit{F. necrophorum} sub sp. \textit{funduliforme} (labelled \textit{Fnf}). When variation in nucleotides has resulted in a change of the predicted amino acid sequence, a red highlight is used. \textit{LktA} sequences described from \textit{Fnn} (GenBank accession number AF312861), \textit{Fnf} (GenBank accession number AY972049) and the sequences generated from \textit{lktA} variants A, B, C and D are shown. Since variant A and \textit{Fnn} have identical sequences they are shown as a single entry (A \textit{[Fnn]}). Amino acids are represented in one-letter code and shown in bold to the top of the corresponding codons. Nucleotides and amino acids identical to the A (\textit{Fnn}) sequence are presented by hyphens, and dots have been introduced to improve the alignment. This multiple alignment was performed using DNAMAN (Version 5.2.10 Lynnon BioSoft). The original version of this alignment and its description can be found in a peer reviewed publication in Appendix B.
Appendix J. Predicted leukotoxin sequence of *F. equinum*

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<th>Fnf</th>
<th>Fnn</th>
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<tr>
<td><strong>F. equinum</strong></td>
<td>TADSKQGISSFVGVGAGGAGIAGTVSVNQLSGKTEVDEKSH</td>
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<td>---------------</td>
</tr>
<tr>
<td><strong>Fnf</strong></td>
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<td>AA---------</td>
<td>FA---------</td>
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<tr>
<td><strong>Fnn</strong></td>
<td>---------------</td>
<td>AA---------</td>
<td>FA---------</td>
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<table>
<thead>
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<th><em>F. equinum</em></th>
<th>Fnf</th>
<th>Fnn</th>
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<tbody>
<tr>
<td><strong>F. equinum</strong></td>
<td>IAVKEADISSKHYGVSVGNGLGAAVKGAAGAASVTVKLTLNT</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Fnf</strong></td>
<td>-F--K-E-TV-R-SS-AI--AAV-V-A---------A----ES--</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Fnn</strong></td>
<td>-L--K-E-TA-R-SS-AI--AAV-V-A---------A----ES--</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th><em>F. equinum</em></th>
<th>Fnf</th>
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<tbody>
<tr>
<td><strong>F. equinum</strong></td>
<td>KLRIKDSNSTKTKLDAIAKNTKLNSGIGIAGAGAGVSVGV</td>
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<tr>
<td><strong>Fnf</strong></td>
<td>RA-V-N-K-M-RN----V--E-EI-SGT--GSA--GILA------</td>
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<tr>
<td><strong>Fnn</strong></td>
<td>RA-V-N-K-M-RN----V--E-EI-SGT--GSA--GILA------</td>
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</table>

Appendix J shows a multiple alignment of the predicted leukotoxin amino acid sequence of *F. equinum* aligned with homologous sequences from *F. necrophorum* sub sp. *funduliforme* (labelled Fnf) and *F. necrophorum* sub sp. *necrophorum* (labelled Fnn). Conserved regions between these species are shown by hyphens and the multiple alignment was performed on DNAMAN (Version 5.2.10, Lynnon BioSoft). This multiple alignment and its description originally appeared in a peer reviewed publication that can be found in Appendix B.
Appendix K. Multiple alignment of \( \text{lktA} \) sequences from \( F. \text{necrophorum} \) sub sp. \( \text{necrophorum} \) (\( \text{Fnn} \)), \( F. \text{necrophorum} \) sub sp. \( \text{funduliforme} \) (\( \text{Fnf} \)), \( \text{lktA} \) variants B, C, D and a portion of \( \text{lktA} \) from \( F. \text{equinum} \)

Appendix K shows a multiple alignment of \( \text{lktA} \) sequences from \( F. \text{necrophorum} \) sub sp. \( \text{necrophorum} \) (\( \text{Fnn} \)), \( F. \text{necrophorum} \) sub sp. \( \text{funduliforme} \) (\( \text{Fnf} \)), \( \text{lktA} \) variants B, C, D and a portion of \( \text{lktA} \) from \( F. \text{equinum} \). \( \text{lktA} \) sequences are described from \( \text{Fnn} \) (GenBank accession number AF312861), \( \text{Fnf} \) (GenBank accession number AY972049) and sequences generated from \( \text{lktA} \) variants B, C, D and \( F. \text{equinum} \). Since \( \text{Fnn} \) and \( \text{lktA} \) variant A have identical sequences, they were combined in this analysis (labelled as Variant A [\( \text{Fnn} \)]). Note, dots have been introduced to improve the alignment. This alignment was performed on DNAMAN (Version 4.0, Lynnon BioSoft) and was used to generate the phylogenetic tree shown in Figure 6.3 of Chapter 6.
Appendix L. List of bacterial strains used in this thesis

*Bacteroides fragilis* (ATCC 25285)
*Bacteroides vulgatus* (ATCC 8482)
*Clostridium perfringens* (ATCC 13124)
*Clostridium sordelli* (ATCC 9714)
*Dichelobacter nodosus* (strain A198 acquired from Professor Julian Rood Monash University, NSW, Australia)
*Fusobacterium equinum* (NCTC 13176)
*Fusobacterium necrophorum sub sp. funduliforme* (ATCC 51357)
*Fusobacterium necrophorum sub sp. necrophorum* (NCTC 10575)
*Fusobacterium nucleatum sub sp. nucleatum* (ATCC 25586)
*Fusobacterium pseudonecrophorum* (ATCC 51644)
*Fusobacterium varium* (ATCC 8501)
*Prevotella buccae* (ATCC 33574)
*Porphyromonas levii* (ATCC 29147)
*Peptostreptococcus anaerobius* (ATCC 27337)
Appendix M. Major suppliers of reagents, enzymes, equipment and software

Applied Bio Systems, Victoria, Australia  
Beckman Coulter, Fullerton, CA, USA  
Becton Dickinson Labware, Franklin Lakes, NJ, USA  
Biolab, Victoria, Australia  
Bio-Rad, Hercules, CA, USA  
Click-Clack, Palmerston North, New Zealand  
Eppendorf, Hamburg, Germany  
Integrate DNA Technologies, Coralville, IA, USA  
Invitrogen, Carlsbad, CA, USA  
Lynnon BioSoft, Vaudreuil, Canada  
Merck, Darmstadt, Germany  
New England Biolabs, Ipswich, MA, USA  
Oxoid, Hampshire, United Kingdom  
Proligo LLC, Colorado, CA, USA  
Promega, Madison, WI, USA  
Qiagen, Hilden, Germany  
Quantum Scientific, Milton, Australia  
Roche Applied Science, Mannheim, Germany  
Sigma, St Louis, MI, USA