Persistence of *Dichelobacter nodosus*, the causal agent of ovine footrot

By

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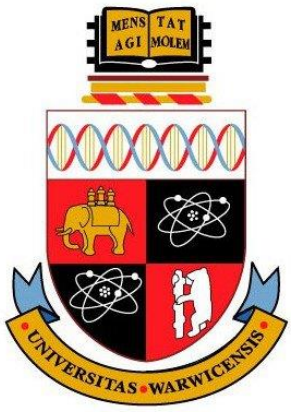

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Declaration

The results presented in this thesis are the work of the author. The work in chapter 5 was based on a research collaboration with the veterinary department of the University of Cordoba, Spain, who contributed to this work by sourcing suitable sheep flocks for scientific investigation and by facilitating sampling visits. This work has not been previously submitted for a degree application. All sources of information presented in this thesis have been acknowledged by a reference.

Summary

Ovine footrot (FR) is an economically important disease that causes lameness and affects sheep flocks worldwide. It is characterized by interdigital skin inflammation (interdigital dermatitis [ID]) with, or without, separation of the hoof horn from the underlying tissue (severe footrot [SFR]). The primary causative agent is the gram-negative anaerobic bacterium *Dichelobacter nodosus,* which is present in diseased feet and thought to be transmitted via contaminated surfaces. Periods of apparent zero prevalence of FR in a flock can be followed by disease occurrence when the climate becomes favourable for pathogen transmission. This suggests that there are sites were *D. nodosus* persists in the absence of disease. These sites might include healthy feet, the gingival cavity and faeces of sheep and also the environment. The aim of this thesis was to investigate persistence of *D. nodosus*, by investigating possible sites of survival of *D. nodosus* over time. Prospective longitudinal studies were used to investigate persistence. Samples were collected from sheep and from the pasture in three studies (Studies 1 and 2: England, study 3: Spain). Quantitative PCR was used to detect and quantify *D. nodosus* and to investigate associations between *D. nodosus* presence in feet, in the gingival cavity and on pasture and a range of predictor variables including climate. A multiple locus variable number tandem repeat analysis (MLVA) suitable for use on mixed DNA and environmental samples was optimized and validated to investigate *D. nodosus* strains within and between sites. A novel approach to characterize individual strains in a sample was designed. *D. nodosus* was detected in all sample types in all studies but not on all occasions.

The feet of sheep were the only site where *D. nodosus* was detected in loads exceeding 10^3 cells per swab. In study 1, *D. nodosus* was detected in amounts exceeding10^3 cells in samples collected from the pasture in week 1 only, when detection frequency of *D. nodosus* on feet was high and the weather was wet. A minimum of 14 strains of *D. nodosus* were detected on the feet of sheep by MLVA. A decline in detection of *D. nodosus* in the environment coincided with periods of dry weather, however, dry weather did not coincide with a decline in *D. nodosus* loads on feet or incidence of disease. *D. nodosus* was more likely to be detected in the gingival cavity when a sheep had FR. It was detected in 25 % of gingival cavity samples and strain types identified in the gingival cavity were the same as the dominant strain types on the feet of sheep. In study 2, disease prevalence and *D. nodosus* detection frequencies were lower than in study 1. When sheep from the study group were separated from the main flock in week 1 and moved onto pasture that had been unoccupied for 10 days, *D. nodosus* was transferred to the study group on healthy feet. One dominant strain of *D. nodosus* persisted throughout an episode of disease and this strain was present on the healthy feet of sheep until up to 5 weeks before the development of lesions in high bacterial loads. There was a reduction in lesion severity and reduced detection of *D. nodosus* in soil in a period of dry weather. Only 1 sample from the gingival cavity was positive for *D. nodosus.* Two faecal sample were positive for *D. nodosus*, indicating for the first time that faecal shedding is possible. In study 3, there were high loads of *D. nodosus* on healthy feet of a sheep that was classed as susceptible when there had been no cases of FR for at least 2 month. *D. nodosus* was still present in the flock during the long non-transmission period in the summer.

We conclude that *D. nodosus* is more likely to persist on the feet of sheep, whereas long-term environmental reservoirs of *D. nodosus* are unlikely. Future research should focus on the feet of sheep and possibly faeces as possible sites of persistence of *D. nodosus* in the absence of disease.

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Abbreviations

AHDB	Agriculture and Horticulture Development Board
AEMET	Agencia Estatal de Meteorología (climatological agency, Spain)
AWERB	Animal Welfare and Ethical Review Body
BLAST	Basic local alignment search tool
DNA	Deoxyribose nucleic acid
DL	Detection limit
ELB	Elution buffer
EB	Extraction blank
SEB	Soil extraction blank
SHP	Soil hoof print
FD	Fluorescent dye
FEB	Faecal extraction blank
ETOH	Ethanol
HA	Hoof agar
HSD	Honestly significant difference
HTA	High traffic area
IUPAC	International Union of Pure and Applied Chemistry
LB	Lysogeny broth
LNA	Locked nucleotides
LTA	Low traffic area
MLVA	Multiple locus variable number tandem repeat (VNTR) analysis
MO	Microorganism
NOAA	National Oceanic and Atmospheric Administration
NTC	Non-template control
OR	Odds ratio
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVPP	Polyvinylpolypyrrolidone
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
RR	Recovery rate
Rpm	Rounds per minute
qPCR	Quantitative PCR
RFU	Relative fluorescence units
SNP	Single nucleotide polymorphism
rpoD	RNA polymerase sigma 70 factor alpha
µg	Microgram
µl	Microliter
UV	Ultraviolet
VNTR	Variable number tandem repeat
(w/v)	Weight/Volume percent

CHAPTER 1

General Introduction

1.1 Ovine footrot and its impact on economy and health and welfare of sheep

Ovine footrot (FR) is an economically important disease with negative effects on animal productivity and welfare (Stewart *et al*., 1984; Marshall *et al.*, 1991; Ley *et al*., 1995; Wassink *et al*., 2010a; Wassink *et al*., 2010b). It causes lameness (Beveridge *et al*., 1941), pain and discomfort, weight loss, poor ewe body condition, increased mortality and reduced wool growth (Stewart *et al*., 1984; Marshall *et al*., 1991; Wassink *et al*., 2010b). Financial losses occur because of reduced rates of lambs born and reared and slower growth rates of lame lambs (Wassink *et al*., 2010). The annual cost of FR in Great Britain in 2006 was estimated to be £24 - £80 million (Nieuwhof and Bishop, 2005, Wassink *et al*., 2010a).

1.2 Global prevalence of footrot and prevalence of lameness in the United Kingdom

Ovine FR is a health concern in sheep flocks across the globe and occurs on all continents where sheep are farmed. It occurs in countries with cold, temperate, tropical, sub-tropical, arid and semi-arid climates (Depiazzi, *et al.*, 1998; Gradin, *et al.*, 1993; Ghimire, *et al.*, 1996; Zakaria, *et al.*, 1998; Cagatay and Hickford, 2005; Moore, *et al.*, 2005; Gurung, *et al.*, 2006b; Aguiar, *et al.*, 2011; Friedrich, 2011; Taku, *et al.*, 2011; Frosth, *et al.*, 2012; Gilhuus, *et al.*, 2014; Sreenivasulu, *et al.*, 2013). Scientific evidence of FR has been reported in all countries highlighted in Figure 1.1, but this does not encompass all countries where sheep are kept and it is likely that the disease is more widespread than reported. Prevalence of FR has been researched in number of countries using a range of methods and a summary (Table 1.1) shows a disease prevalence ranging from 3.1-78%.

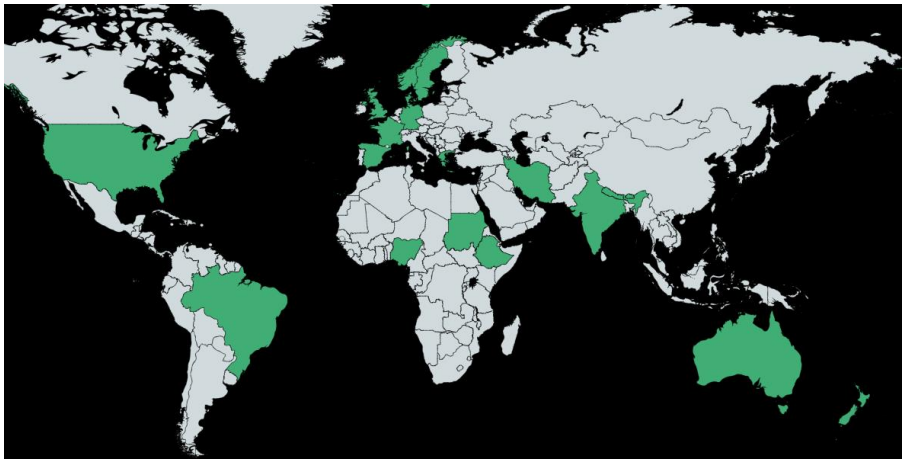


Figure 1.1: Countries with published scientific evidence of footrot occurrence.

Table 1.1: Published disease prevalence estimates in some countries where footrot occurs.

Country	Data collection	Prevalence (%)	Period studied	Reference
Sweden	Inspection at abattoir	5.8	2009	Koenig *et al*., 2011
Bhutan	National sampling survey	3.1	1998	Gurung *et al.*, 2006b
Germany/Austria/ Switzerland	Questionnaire	66/66/78	2010	Friedrich *et al.*, 2012
Northern India	Sampling survey	12.54	2008-2009	Rather *et al*., 2011
Southern India	Sampling	15	2009-2011	Sreenivasulu *et al.*, 2013
Southern Brazil	Inspection of feet	13.69	2009	Aguiar *et al*., 2011

In the UK farmers attribute > 90% of lameness in their sheep to FR (Kaler and Green, 2008). In 2013, 68% of foot lesions observed in sheep were FR (Winter *et al*., 2015). Winter *et al*. (2015) analyzed postal questionnaire responses and reported that the UK global mean prevalence of lameness fell from 10.6% to 4.9% between 2004 and 2013. They suggest that this decrease in prevalence may be attributable to the increased uptake of beneficial management techniques, as reported by farmers between 2004 (Kaler and Green, 2009) and 2013 (Winter *et al*., 2015).

1.3 Disease expression and epidemiology

The two clinical presentations of FR (Figure 1.2) that cause lameness and loss of condition are interdigital dermatitis (ID), characterized by inflammation of the interdigital skin and severe FR (SFR), characterized by various degrees of separation of the hoof horn from underlying tissues (Egerton and Parsonson, 1969). Clinical signs are usually accompanied by a characteristic foul smell and grey/white exudate (Beveridge *et al*., 1941). Although previously viewed as separate diseases by farmers, veterinarians and researchers, these presentations are different stages of the same disease (Wassink *et al*., 2003; Moore *et al*., 2005). In Australia ID and SFR are termed benign FR (BFR) and virulent FR (VFR) respectively. In this thesis, the terms ID and SFR are used, because the Australian definitions refer to clinical presentations, which do not necessarily correlate with the virulence of the infecting pathogen (Stewart *et al*., 1986).

A **B**

Figure 1.2: Interdigital dermatitis (ID) (**A**) and severe footrot lesions (SFR) (**B**). Source: Research group FR archives, University of Warwick.

The main causal agent of ovine FR is the gram-negative anaerobic bacterium *Dichelobacter nodosus*. Although the presence of *D. nodosus* is necessary for FR to occur (Beveridge, 1941; Roberts and Egerton, 1969; Kennan *et al*., 2010), FR is a polymicrobial disease, with a complex pathogenesis and large bacterial community whose structure changes in different disease states (Calvo-Bado *et al*., 2011). Another key player in the disease process is the gram-negative anaerobic bacterium is *Fusobacterium necrophorum*. *F. necrophorum* is associated with both ID and SFR (Bennet *et al*., 2009; Witcomb *et al*., 2014).

D. nodosus has been detected in sheep with ID and in sheep with no clinical signs of disease (Moore *et al*., 2005; Kaler *et al*., 2010; Calvo-Bado *et al*., 2011). Calvo-Bado *et al*. (2011) sampled three flocks and identified *D. nodosus* by quantitative polymerase chain reaction (qPCR) in all feet irrespective of disease state. This shows that presence of *D. nodosus* does not necessarily coincide with disease expression and that molecular analyses based on detection may not provide meaningful information about the disease process. Witcomb *et al*. (2014) found that *D. nodosus* load increased before and during an episode of ID and before the occurrence of SFR. Maboni *et al*. (2016) collected biopsies from ovine feet and found that more *D. nodosus* were present on feet with ID than on feet with SFR. Stäuble *et al*. (2014) reported that healthy sheep had a much smaller *D. nodosus* load than affected animals. These papers indicate that *D. nodosus* is a key player in disease initiation and progression. Recently, *F. necrophorum* load has been shown to increase once SFR is established on a foot, whereas no association of bacterial load with ID was found (Witcomb *et al*., 2014). Whereas previously *F. necrophorum* was believed to be essential for the initiation and development of FR (Roberts and Egerton, 1969), this new evidence supports that *F. necrophorum* may play a secondary role in the disease process, possibly increasing severity.

1.4 Treatment of footrot

The most common procedures for control of FR are administration of topical and parenteral antibiotics, footbathing, vaccination and culling. The latter two will be discussed in later sections. Parenteral antibiotics have been commonly used for many years to treat FR (For example Grogono-Thomas *et al*., 1994; Jordan *et al*., 1996; Kaler *et al*., 2010; Duncan *et al*., 2012). Kaler *et al*. (2010) showed that sheep that had been treated with parenteral antibiotics recovered from lameness (65%) and lesions (78%) within 5 days of administration. The likelihood of recovering from lesions and lameness was also increased in sheep that had received parenteral antibiotic treatment, compared to sheep that were not treated with antibiotics. Duncan *et al*. (2012) showed that a single injection of long-acting amoxicillin lead to recovery rate of 81.54%. Topical antibiotic spray is also widely used in the treatment of FR, although the efficiency seems to vary according to the severity of the lesions (Kaler *et al*., 2010).

Evidence suggests that prompt antibiotic treatment of individual sheep is key to controlling FR (Kaler *et al*., 2010, Wassink *et al*., 2010). Early treatment with parenteral antibiotics resulted in a decreased risk of lameness (Winter *et al*., 2015) and sheep that

were lame for fewer days and less severely lame (Wassink *et al*., 2010; Green *et al*., 2012).

Footbathing sheep in solutions of formalin, zinc sulphate or copper salts are also used to limit the spread of FR (Raadsma and Egerton, 2013; Raadsma and Dhungyel, 2013). UK evidence shows that footbathing was associated with a decreased prevalence of ID, but an increased prevalence of SFR (Wassink *et al*., 2003; Wassink *et al*., 2004; Kaler and Green, 2009; Winter *et al*., 2015), possibly due to the superficial nature of the treatment.

Foot trimming is considered as standard practice (Abbot and Lewis, 2005) and is traditionally used by many farmers to treat footrot. Recent evidence, however, suggests that foot trimming may be associated with an increased FR prevalence, an increased risk of lameness and delayed recovery time of FR lesions (Kaler *et al*., 2010, Wassink *et al*., 2010; Winter *et al*., 2015). Mounting evidence suggests that foot trimming should not be considered as a routine treatment for FR.

1.5 Characterization of *Dichelobacter nodosus*

D. nodosus (Figure 1.3) is a rod-shaped, non-sporing, fimbriated, aerotolerant anaerobic bacterium that is highly adapted to the ovine foot (Ellemann, 1988; Depiazzi *et al*., 1990; Kennan *et al*., 2001b; Rood *et al*., 2005) Formerly known as *Fusiformis nodosus* and *Bacteroides nodosus*, it was later reclassified as belonging to the class *Proteobacteria* and re-named *D. nodosus* (Dewhirst *et al*., 1990; La Fontaine & Rood, 1990). It exists as a single species in its genus in the Cardiobacteriaceae family (Rood *et al*., 2005).

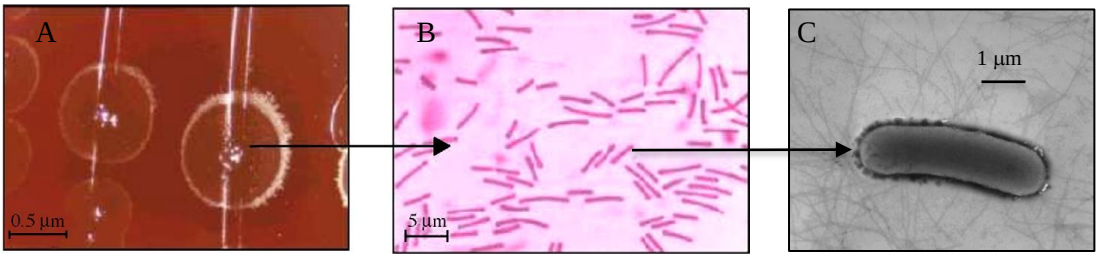


Figure 1.3: *D. nodosus* culture, gram stain and *D. nodosus* cell **A**: *D. nodosus* culture grown on Eugon agar, showing characteristic circular shape growing outwards from the streak. **B**: Gram stain of *D. nodosus* showing characteristic gram negative rods with bulbous ends (Catagay and Hickford 2005). **C**: *D. nodosus* cell showing fimbriae that facilitate motility (adapted from Kennan *et al*., 2001a).

D.nodosus has a compact genome and consists of a single small circular chromosome of 1,389,350 base pairs (bp) (Myers *et al*., 2007) (Figure 1.4). The *D. nodosus* genome is highly conserved, displaying a 95% sequence similarity between isolates with 8 major regions of sequence variability (Kennan *et al*., 2014).

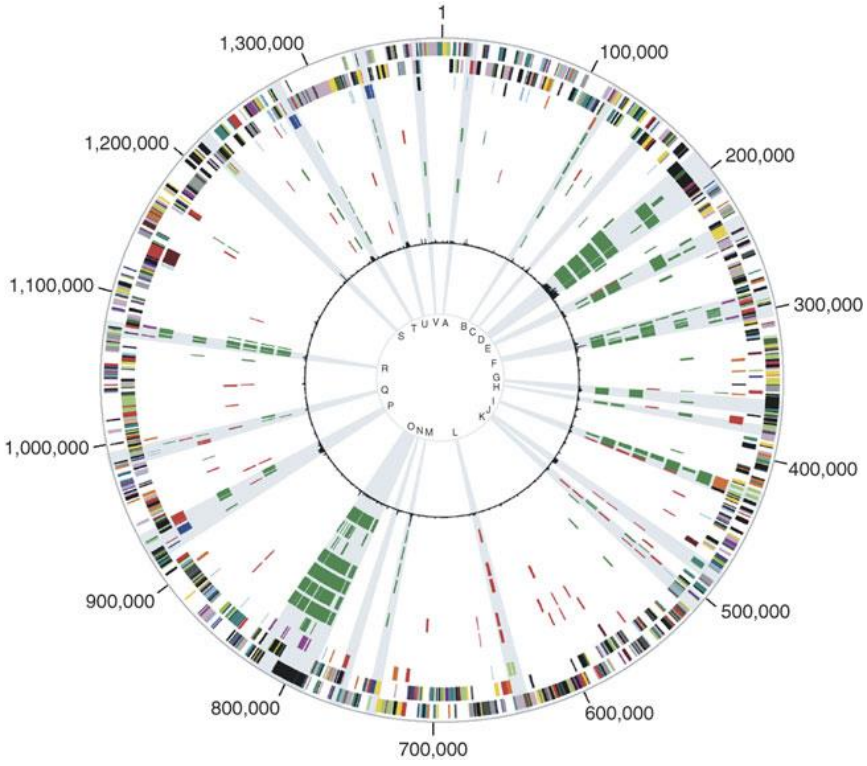


Figure 1.4: Circular presentation of the *D. nodosus* genome of strain VCS 1703A (Myers *et al.*, 2007). Different colours indicate cellular roles, location of genetic elements and genes.

1.5.1 Main virulence factors of Dichelobacter nodosus

The pathogenicity of an organism is determined by its virulence factors (Russell & Herwald, 2005) and expression and progression of FR is partially determined by the virulence of the strain involved. The major virulence factors of *D. nodosus* implicated in FR are type IV fimbriae and extracellular proteases (Han *et al*., 2008).

Type IV fimbriae are the major surface antigens of *D. nodosus* (Every &Skerman, 1982*)*. Fimbriae play an important role in host colonization in a wide range of pathogens (Skerker & Berk, 2001) as they mediate attachment and adherence to the epithelium as well as twitching motility, facilitated by a process of fimbrial extension and retraction (Merz &Forest, 2002). Avirulent strains of *D. nodosus* show low levels of motility compared with virulent strains (Depiazzi & Rood, 1984). Fimbrial biogenesis, presence

of the fimA gene encoding the fimbrial subunit protein, as well as presence of the pilT and pilU genes required for fimbrial retraction are essential for twitching motility, cell adherence, natural transformation as well as protease production of *D. nodosus* (Kennan *et al*., 2001a; Kennan *et al*., 2001b; Han *et al*., 2007; Han *et al*., 2008).

Proteases are produced by many bacterial pathogens and are responsible for the degradation of the host's extracellular matrix and so facilitate colonization (Siezen & Leunissen, 1997). They are thought to be responsible for tissue invasion in FR, and *in vitro* research has shown that they efficiently degrade keratin (Kennan *et al*., 2010; Wong *et al*., 2011).

D. nodosus secretes three acidic and basic extracellular proteases encoded by three separate genes via a type II secretion-like pathway utilizing the type IV fimbrial machinery (Parker *et al*., 2006; Han *et al*., 2007). Virulent strains produce the acidic isoenzyme proteases 2 and 5 (AprV2, AprV5) and the basic protease BprV. Benign strains produce three corresponding proteases, genetically similar to their counterparts, that are termed AprB2, AprB5 and BprB (Kortt *et al.*, 1993; Riffkin *et al.*, 1993; Kortt *et al.*, 1994; Kortt & Stewart, 1994; Riffkin *et al.*, 1995). These three proteases are thought to act synergistically. AprV2 is essential for virulence, as replacement with AprB2 resulted in a benign strain unable to cause disease (Kennan *et al*., 2010) and AprV5 is required for optimal processing of all three proteases (Han *et al*., 2012).

Strains considered as benign have less protease activity in vitro than virulent strains (Depiazzi & Rood, 1984) and produce thermolabile proteases, whereas virulent strains produce thermo-stable proteases (Kortt & Stewart, 1994; Depiazzi & Richards, 1979). Differences in properties of proteases have been used in diagnostic tests to distinguish virulent and benign isolates. These tests do not always correlate with each other or diseases state and so yield inconsistent results (Liu and Yong, 1993; Dhungyel *et al*., 2013b), indicating that virulence is complex and linked to more than one process. Recent genome sequencing of *D. nodosus* isolates suggested that single nucleotide polymorphisms (SNP) divide the *D. nodosus* population into 2 clades that coincide with the single amino acid difference between AprV2 and AprB2 and with the definitions of an isolate being benign or virulent, highlighting a possible central role of these proteases in defining virulent and benign isolates (Kennan *et al*., 2014). However, it was also stated that there was insufficient information to determine the virulence status of many isolates. Moreover, definitions of benign and virulent isolates are not uniform among laboratories; therefore, one cannot relate the SNP's with certainty to virulence.

1.5.2 Benign and virulent strains of Dichelobacter nodosus in sheep populations

The exact nature of co-existence and interactions of virulent and benign strains that may lead to initiation of disease is unknown. In addition, what exactly determines whether a strain is virulent or benign is still to be elucidated. Information on the characteristics of *D. nodosus* strains circulating in the European sheep population is limited. Inoculation of feet with a benign strain as defined by its proteolytic activity was less likely to cause separation of the hoof-horn than inoculation with a virulent strain (Egerton and Parsonson, 1969). Benign strains have therefore been associated with the clinical expression of ID (Egerton & Parsonson, 1969). However, the severity of clinical disease with FR is not completely consistent with the virulence of the infecting *D. nodosus* strain (Stewart *et al.*, 1986). Virulent *D. nodosus* is detected on clinically healthy feet and on feet presenting with ID only (Moore *et al*., 2005; Stäuble *et al.*, 2014), whilst under-running of the horn was observed in sheep infected with only benign strains (Gilhuus *et al.*, 2013). Virulent and benign strains co-exist in sheep flocks and on feet (Younan *et al*., 1999; Rather *et al*., 2011). In the UK, where FR is endemic, many virulent strains are present and benign strains have been difficult to detect (Moore *et al*., 2005). Recently Maboni *et al.* (2017a) detected benign strains and mixed populations of benign and virulent *D. nodosus* strains in 7% of post-slaughter interdigital biopsies using qPCR. In Norway, a country where a virulent strain has only recently been introduced, only benign strains of *D. nodosus* were present except for one region, which appears to act as the source of spreading SFR (Gilhuus *et al*., 2013; Gilhuus *et al*., 2014).

1.5.3 Antigenic diversity of Dichelobacter nodosus

The fimbriae of *D. nodosus* form the basis of classification of *D. nodosus* into 10 major serogroups (A-I + M) and at least 21 serotypes, based on the variation in the fimbrial subunit proteins (Claxton, 1989; Mattick *et al*., 1991; Ghimire *et al*., 1998; Zhou *et al*., 2010; Bhat *et al*., 2012; Bhat *et al*., 2013) with several amino acid differences between serogroups and up to 15 differences between serotypes (Mattick *et al.*, 1991). Categorization of serogroups is traditionally based on agglutination reactions from antisera raised against *D. nodosus* (Claxton *et al*., 1989). Serogroup dominance varies between countries and regions within countries and in the UK serogroup H is the main serogroup (Thorley & Day, 1986; Moore *et al*., 2005). Mixed serogroups co-exist on farms and on individual feet (Claxton *et al*., 1983; Hindmarsh and Fraser, 1985; Gradin *et*

al., 1993; Ghimire and Egerton, 1996; Jelinek *et al*., 2000; Hussain *et al*., 2009; Catagay and Hickford 2011).

Sequence analysis of the fimA gene has shown that recombinational exchanges of the FimA gene and gene region have occurred between strains (Hobbs *et al*., 1991) and it has been suggested that such recombination events also occur in the field (Ghimire *et al*., 1998; Zhou & Hickford, 2000). *D. nodosus* is naturally competent for transformation (Kennan *et al*., 2001a, b) and Kennan *et al*. (2003) demonstrated that *D. nodosus* is able to undergo serogroup conversion by natural transformation and homologous recombination events (Kennan *et al*., 2003) and occurrence of serogroup conversion on farms has been suggested (Gilhuus *et al*., 2014). This has important implications for disease control, as virulent strains of *D. nodosus* may be able to seroconvert and so evade the immune response of targeted vaccines. In addition, the possibility exists that benign strains may act as reservoirs of alternative fimbrial antigens.

More than one serogroup of *D. nodosus* can often be found in a diseased flock (Claxton, *et al.*, 1983; Hindmarsh and Fraser, 1985; Gradin *et al.*, 1993; Ghimire *et al.*, 1996; Cagatay and Hickford, 2011; Jelinek *et al.*, 2000; Hussain *et al.*, 2009). Immunity to FR is serogroup specific and the success of vaccination is largely restricted to targeting the serogroup involved, hence where co-infections are rare, chances of disease eradication increase. Successful vaccines to control FR should contain the major known serogroups of *D. nodosus* present in the flock during an outbreak. (Lambell, 1986, Hindmarsh *et al*., 1989, Egerton *et al*., 2002).

FR has been successfully eradicated with mono and bivalent vaccines when only one or two serogroups were present in Australia, Bhutan and Nepal (Egerton *et al.*, 2002; Gurung *et al.*, 2006a; Dhungyel *et al.*, 2013a; Dhungyel *et al.*, 2015). Vaccination with multivalent vaccines is less successful due to antigenic competition, which occurs when pilus antigens of multiple different serogroups are combined in a single vaccine (Hunt *et al*., 1994). In the UK, the currently available multivalent footrot vaccine contains the antigens of 9 serotypes and has shown to improve recovery and reduce new infection rates. Recovery rates were further improved by combining vaccination with antibiotic treatment (Duncan *et al*., 2012). Evidence regarding vaccine efficacy is however variable and depending on the time of administration, pathogen load in the flock and environmental conditions (Duncan *et al*., 2012). In addition, protection may only last for a short period and manufacturers recommend vaccination of a flock at 4-5 month intervals.

1.6 The role of climate and environment in footrot initiation, severity and elimination

A number of factors, including breed, age, lambing status, endemicity, host immunity, as well as management factors such as housing conditions and stocking densities can influence incidence, prevalence and severity of FR (Emery *et al.*, 1984; Skerman *et al*., 1988; Woolaston, 1993; Raadsma *et al.*, 1999; Wassink *et al.*, 2003).

Climatic patterns and seasonal conditions may affect disease behaviour in terms of spread, diffusion range, amplification and persistence (De La Roque *et al*., 2008). Climate can affect the physiology, metabolism, development rate, distribution and adaptation of hosts, and pathogens and their vectors. It is generally accepted that climate influences the range of an infectious disease, whereas weather affects the timing and the intensity of disease outbreaks (Hughes, 2000).

The environment is the most important factor to consider with regards to transmission, severity, as well as eradication of FR (Graham & Egerton, 1968; Cross, 1978; Depiazzi *et al.*, 1998; Green & George, 2008). Factors directly or indirectly associated with climate have been associated with the spread of FR including improved pastures, rich soils and irrigation (Beveridge *et al.*, 1941; Graham and Egerton, 1968, Stewart *et al*., 1984; Woolaston 1993; Whittington *et al*., 1995; Depiazzi *et al.*, 1998).

1.6.1 The environment in disease initiation and transmission of footrot

FR is transmitted horizontally, by contaminated surfaces. Muzafar *et al*. (2015) detected high loads of *D. nodosus* on the feet of lambs 5-13 hours after they touched the ground. The rate of infection is likely to be determined by environmental challenge from *D. nodosus*, which in turn is dependent upon environmental contamination from infectious sheep (Green & George, 2008), partly determined by stocking density (Wassink *et al*., 2003).

Transmission patterns of FR vary according to climatic characteristics of a region or country. In many countries spread of FR is highly seasonal, peaking in the wet climate of spring and autumn. In countries and regions characterized by semi-arid climates such as Southern Spain, South Brazil, Ethiopia, Western Australia and New South Wales FR prevalence peaks during and after the short period of annual rainfall preceded by a prolonged hot and dry non-transmission period (Parsonson *et al*., 1967, Graham, 1968; Fernandez *et al*., 1996; Aguiar *et al*., 2011; Chanyalew and Alemu, 2014). In tropical Southern India FR peaks in the rainy season between June and September (Sreenivasulu

et al., 2013). In the Himalayan regions of Norther India and Nepal FR transmission occurs during migration of sheep to alpine pastures (Ghimire and Egerton, 1996; Kaler *et al*., 2012).

Seasonal fluctuations of infectious diseases imply an association with climatic factors (Hughes, 2000). In Australia spread of FR was associated with increased rainfall and temperatures exceeding 10 °C (Graham and Egerton, 1968). In the UK, FR is transmitted at temperatures below 10 °C and can occur at any time of the year (Ridler *et al*., 2012; Smith *et al*. 2014). Therefore, the effects climate on FR may not be consistent in all regions or countries. In the UK, there is no defined hot and dry non-transmission period, due to all year-round high rainfall. One UK study reported an increased incidence of disease when rainfall increased 2 and 4 weeks before examination of lesions (Smith *et al*., 2014), indicating the importance of rainfall and possibly moisture level in disease transmission. Whether ambient temperature, rainfall or level of moisture in the ground has the highest impact on disease initiation and progression is unknown, but it is likely that a combination of these factors is important.

1.6.2 The role of the environment in control and elimination of footrot

Disease control is the reduction of disease incidence, prevalence, morbidity or mortality to a locally acceptable level as a result of deliberate control efforts (Dowdle, 1998). In the case of FR, the aim is to reduce the force of infection, minimize pathogen spread and reduce the impact of the disease on the animal (Raadsma and Egerton, 2013). Elimination of the disease is the reduction to zero of the incidence of a specific disease in a geographical area due to deliberate efforts (Dowdle, 1998).

Control and eradication programs commonly rely on the long periods of zero transmission during dry summers. In Australia culling sheep is considered more effective than treating sheep for FR (Raadsma and Egerton, 2013). In New South Wales and Western Australia total destocking and culling of affected animals has successfully contributed to the elimination of FR in these regions. The objective of eradication was to eliminate sources of reinfection before climate conditions became favourable for *D. nodosus* transmission, hence elimination efforts were concentrated on the dry and hot summer period (Mills *et al*., 2012; Raadsma and Egerton 2013). Culling is also used in the UK as a FR control measure (Grogono-Thomas and Johnston 1987; Wassink *et al*., 2003; Winter 2009; Winter *et al*., 2015). The UK however lacks a predictable non-transmission period due to year-round high rainfall and there is little evidence regarding the success of culling as control measure in the UK. Winter *et al*. (2015) found that the

recovery rate (RR) of sheep was not improved when farmers culled sheep that had been repeatedly lame. Clements and Stoye (2014) suggest a 5-point plan that recommends combining culling of repeatedly affected sheep with other measures to avoid of spread of infection, including quarantining incoming stock and biannual vaccination.

Programs that aim to control FR are based on the expectation that *D. nodosus* does not survive off the ovine foot for more than 7-10 days and that all pastures are uninfected during non-transmission periods (Abbott & Lewis, 2005). Again, these assumptions originate from research conducted in Australia. Elimination programs should be based on the seasonal behavior of *D. nodosus* in the country where FR is present. The lack of seasonality of FR in the UK presents a challenge for disease control and renders the Australian eradication program unfeasible. One reason why eradication attempts relying on low transmission periods during the winter have failed may be that *D. nodosus* is able to survive at lower temperatures than previously expected as suggested by Smith *et al*. (2014). This highlights the necessity to investigate the seasonal behaviour of *D. nodosus* and the climatic conditions that influence *D. nodosus* survival in the UK.

1.7 Persistence of *Dichelobacter nodosus* on sheep and in the farm environment

The ability of microorganisms to survive is defined as their persistence to withstand prevailing conditions long enough to become established on susceptible hosts. For prevention and control of infectious disease agents it is important to quantify the organisms' capacity to invade naive host populations, as well as its ability to persist in such populations (Jesse *et al*., 2011). As mentioned previously, climatic factors may influence the epidemiology of a disease via direct effects on pathogens and their hosts and these effects may affect the ability of a pathogen to persist at certain sites. A number of bacteria are capable of surviving in the environment in a viable but non culturable state (Rollins and Cotwell, 1986; Jin *et al*., 2017).

Throughout this project presence is defined as the detection of an organism once, at one location. Persistence is defined as the detection of an organism (strain) at one location at least twice on consecutive samplings (see also section 1.9).

The fact that FR occurs after a disease-free period in flocks when the climatic conditions become conducive for pathogen survival and transmission (Beveridge, 1941; Depiazzi *et al.*, 1998), suggests that *D. nodosus* may be able to persist in the host, in the environment or both. It is possible that survival of *D. nodosus* outside the host is dependent on a moist

environment and ambient temperature (Graham & Egerton, 1968), as these are the conditions that favour its transmission as described in Section 1.6.

Most scientific investigations into the impact of climate on FR originate from Australia. Apart from the existing climatic differences between Australia and the UK, these studies investigated disease transmission by assessing FR lesions or establishing presence of *D. nodosus* via culturing for characterization purposes (Graham and Egerton, 1968; Depiazzi *et al*., 1998). Therefore, these studies provide limited information on how climatic conditions affect *D. nodosus* persistence.

1.7.1 Detection of Dichelobacter nodosus in feet, in the oral cavity and in faecal samples

Previous studies have suggested that diseased sheep are a reservoir of infection (Whittington *et al.*, 1995; Green *et al*., 2007; Kaler *et al*., 2010), but it is unknown whether sheep can carry *D. nodosus* and for how long. *D. nodosus* has been isolated from healthy feet and it has been suggested that it might be harbored for several months, surviving in a latent stage, with no clinical signs of FR, presenting a possible reservoir of infection (Beveridge, 1941; Depiazzi *et al.*, 1998; Moore *et al.*, 2005; Kaler *et al.*, 2010; Witcomb, 2012). It has been suggested from a genome study that the ability of *D. nodosus* to tolerate exposure to oxygen, leads to prolonged viability on the ovine foot (Myers *et al*., 2007). Maboni *et al*. (2017) found *D. nodosus* in a large proportion (58%) of ID post-slaughter biopsies samples from healthy feet and suggest that it may be present on the foot without necessarily causing disease.

Bennett *et al*. (2009) reported presence of *D. nodosus* in the gingival cavity, but did not comment on sampling procedures, detection frequencies or bacterial load. Witcomb (2012) isolated *D. nodosus* from 71.4% (25/35) of oral cavity swabs. The oral cavity might constitute a reservoir of infection, as it has been described as an ideal environment for anaerobic bacteria and is heavily colonized (Cheng & Costerston, 1986). Whether *D. nodosus* is able to replicate in the mouth is unknown.

There is no evidence that *D. nodosus* is shed in ovine faeces. *D. nodosus* has only been detected in faecal samples that have been in contact with the ground (Witcomb, 2012; Muzafar *et al*., 2015), therefore, environmental contamination with *D. nodosus* is likely.

1.7.2 Distribution of Dichelobacter nodosus in the farm environment

Limited information is available on the distribution and persistence of *D. nodosus* in the farm environment. Early research estimated survival times on paddocks according to the time span between vacating a paddock where infection was present and introduction of a naive flock. On this basis, survival times on pasture have been estimated at 7-10 days (Gregory, 1939; Beveridge, 1941; Woolaston, 1993; Whittington, 1995), which led to the assumption that *D. nodosus* uses the environment merely as a transitional medium.

More recently, *D. nodosus* was detected in a number of environmental sources, using PCR-based detection methods: Witcomb (2012) detected *D. nodosus* in 24.2% of all faecal samples, but failed to detect it in samples collected before faeces contacted the ground, suggesting that faeces may become contaminated through contact with contaminated soil or bedding. *D. nodosus* presence in faecal material has also been reported more recently (Muzafar *et al*., 2015), but samples had also come in contact with the environment.

Recent evidence suggests that *D. nodosus* may be able to survive in soil for longer and at lower temperatures than previously assumed. Cederlof *et al*. (2013) evaluated *D. nodosus* survival time in laboratory microcosms and found viable cells until up to 24 days in samples stored at 5 °C, whereas storage at 15 °C decreased survival time to 4 days. Muzafar *et al*. (2016) investigated the impact of soil type and temperature on viability of *D. nodosus* in soil microcosms and found that although *D. nodosus* survived for 40 days in all soil types (clay, sandy, sandy loam) at 5 °C and 25 °C, survival was higher at 5 °C compared to 25 °C. This could explain why Smith *et al.* (2014) detected transmission of FR at temperatures below 10 °C in the UK. *D. nodosus* also survived significantly longer in clay soils than in all other soil types. Increased incidence of FR in areas with clayey soils has been reported previously (Depiazzi *et al.*, 1998; Aguiar *et al*., 2011), highlighting a possible role of soil type in the outbreaks of FR and transmission of *D. nodosus*. Clay soils retain water and so may maintain moisture content suitable for longer pathogen survival than other soil types. In Muzafar *et al.* (2016) soil moisture was significantly associated with increased survival time with *D. nodosus* surviving longer constantly moist soils.

D. nodosus was detected in one (1/20) sample of farm soil (Witcomb, 2012) but significant levels of PCR inhibition were reported. DNA extracted from soil may contain inhibitors that interfere with the use of molecular techniques to detect *D. nodosus* (Purdy, 2005). Humic acid in soil is known as an inhibitor of Taq polymerase (Tsai and Olson, 1992), highlighting the need to develop suitable DNA extraction methods.

The above evidence suggests that *D. nodosus* may be able to persist on the feet of sheep, in the gingival cavity or on the pasture. The cross-sectional nature of some of the above studies (Witcomb, 2012), indicate presence, but not necessarily persistence of *D. nodosus*. Longitudinal studies may provide correct temporal sequences of association and allow the investigation of patterns of association of a variety of variables over time (Webb & Bain, 2011).

1.8 Methods for the detection, quantification and characterization of *Dichelobacter nodosus*

1.8.1 Molecular epidemiology

Molecular epidemiology has a number of definitions, but is generally defined as a discipline that resolves epidemiological problems using molecular approaches (Foxman and Riley, 2001; Nelson *et al*., 2007). Molecular and genetic markers are used to trace disease development. In addition, techniques that investigate the relatedness between strains from different sources, geographic locations and different time periods aid in the establishment of epidemiological linkage between cases (Wang *et al*., 2015). A number of molecular approaches used in this study to improve the understanding of *D. nodosus* persistence are described in the next section.

1.8.2 Culture dependent methods

Traditionally the identification of *D. nodosus* has relied on culture of *D. nodosus* from lesions followed by observations of phenotypic characteristics and microbiological and biochemical tests (Skerman, 1989). These tests include microscopic examination and a range of assays based on the differences in the properties of the *D. nodosus* extracellular proteases (e.g. Kortt *et al*., 1983; Depiazzi and Richards, 1985; Stewart *et al*., 1986; Depiazzi *et al*., 1991; Palmer, 1993). Although established culturing protocols exist (Thomas, 1955; Thorley, 1976), *D. nodosus* isolation is challenging and time consuming, because of its fastidious and anaerobic nature. In addition, effective isolation is challenged by the large number of different bacteria present on the ovine foot (Catagay and Hickford, 2005).

1.8.3 Culture independent methods

Recently molecular techniques have been applied in FR diagnosis. Comparison of the 16S rRNA gene sequence has emerged as a preferred genetic technique, as it can identify poorly described, rarely isolated and phenotypically difficult to describe strains. Polymerase Chain Reaction (PCR) is an *in situ* DNA replication process that allows for the exponential quantification of target DNA in the presence of synthetic oligonucleotide primers and a thermostable DNA polymerase (Adzitey *et al.*, 2013) and although PCR cannot prove the absence of genetic material it is a more sensitive and rapid test compared to more time-consuming culturing methods (Moore *et al*., 2005; Hussain *et al*., 2009; Adzitey *et al*., 2013). Bacterial PCR assays targeting the 16S rRNA gene are a common tool in molecular biology for the detection of microorganisms (Rowlinson *et al*., 2007) and their application for the detection of *D. nodosus* is discussed below.

1.8.4 Dichelobacter nodosus 16s rRNA gene sequence analysis

A *D. nodosus* specific PCR targeting the 16S rRNA gene (La Fontaine *et al.*, 1993) is the primary test for detecting *D. nodosus* in field samples (Moore *et al*., 2005; Belloy *et al*., 2007; Wani *et al*., 2007; Hussain *et al*., 2009; Farooq *et al*., 2010; Rather *et al*., 2011; Taku *et al*., 2011; Baht *et al*., 2012) increasing sensitivity of detection by 12-17% compared to culture (Moore *et al*., 2005). The 16S rRNA gene is present in three copies in *D. nodosus*, which is an advantage when specific and sensitive detection is required (La Fontaine *et al*., 1993).

Low copy numbers of *D. nodosus*, crude DNA extraction methods and presence of PCR inhibitors have been named as possible reasons for limited detection capacity (Hussain *et al.*, 2009; Calvo-Bado *et al*., 2011; Taku *et al*., 2011). Non-specific amplicons, primer mismatches and faint bands have also been reported as an issue when using this PCR (Frosth *et al*., 2012). Development of a real-time PCR targeting the 16S rRNA gene increased detection to 81.7-97% (König *et al*., 2011; Frosth *et al*., 2012), however this approach is more costly than conventional PCR (Adzitey *et al*., 2013) and has not been designed for quantification (Froths *et al.*, 2012), ruling out simultaneous processing of samples for identification and quantification in one PCR.

Calvo-Bado *et al*. (2011) used nested PCR (Described in chapter 2, section 2.10.5) for detection of *D. nodosus* and detected the pathogen in 100% (n = 420) of samples. Nested PCR is more sensitive than conventional PCR (Belloy *et al.*, 2007; Adzitey *et al*., 2013). However, the presence of *D. nodosus* on hooves irrespective of disease state (Calvo-Bado

et al., 2011) highlights the need for a suitable quantification method. Muzafar *et al*. (2015) detected *D. nodosus* in both soil and faecal samples using this assay.

1.8.5 Real-time quantitative PCR (qPCR)

Real-time quantitative PCR (qPCR) is a culture independent method for the detection and quantification of nucleic acids to determine the absolute or relative number of bacteria in a sample. Although it may overestimate bacterial load due to detection of DNA from lysed cells, it eliminates the need for post-PCR processing, leads to high throughput and reduces amplicon contamination risk (Adzitey *et al.,* 2013). Investigation of bacterial load using PCR-based methods is a common tool in microbiology and avoids bias that can be introduced with culture-based methods (Amann *et al*., 1995). In addition, evidence suggests that load can be correlated with disease development in bacterial infections (Hill *et al.,* 2000; Hackett *et al.,* 2002; Witcomb *et al.,* 2014), making it a useful approach in epidemiological studies.

A number of qPCR assays have been developed for *D. nodosus* (Frosth *et al*., 2012; Stäuble *et al*., 2014), which where sensitive for detection, however not designed for quantification. Calvo-Bado *et al.* (2011) designed specific and sensitive TaqMan qPCR targeting the RNA polymerase sigma 70-factor gene (*rpoD*) that detected and quantified *D. nodosus* on 68.4% - 86% of all feet. The use of probes and primer sets can increase the sensitivity, specificity and reproducibility of real-time PCR's (Kuhar *et al*., 2013; Yan *et al.*, 2014) and is more sensitive than measuring bacterial load by culture (Nadkarni *et al*., 2002).

1.8.6 Multiple Loci Variable Number Tandem Repeat (VNTR) Analysis (MLVA)

Bacterial typing methods contribute useful information to epidemiological surveillance of infectious diseases due to their potential to detect strains responsible for disease outbreaks (Van Belkum *et al*., 2007).

Bacterial genomes contain a high percentage of DNA consisting of tandem repeats that vary in size, location and complexity (Lindstedt, 2005, Nadon *et al*., 2013). Repetitive DNA is often incorrectly replicated through slipped strand mispairing, which leads to deletion and insertion of repeat units and hence creating strain diversity (Van Belkum *et al*., 2007). These repeat sequences are termed variable-number tandem repeat (VNTR) loci. VNTR's contribute to the phenotypic variation in bacteria and to the adaptation of a pathogen to the physiological environment imposed by its host (Van Belkum *et al*., 1997;

Le Fleche *et al*., 2001). The Multiple loci variable number tandem repeat (VNTR) Analysis (MLVA) assay takes advantage of these natural occurring tandem repeats to assess the molecular fingerprint of bacteria (Nadon *et al*., 2014). Flanking primers, that can be fluorescently labeled, are used to target the VNTR sequence in order to assess variation in repeats between strains, by determining the length of the PCR product (Keim *et al*., 2002, Hyytia-Trees *et al*., 2007). Bacterial strains can subsequently be grouped into strains or allelic profiles by comparing the variation of VNTR's at each locus (Figure 1.5). MLVA profiles of bacterial isolates obtained from disease outbreaks can be compared to other profiles from isolates originating from affected or non-affected populations which enables control and surveillance of the disease (Van Belkum *et al*., 2007). MLVA has been adopted as part of laboratory based disease surveillance in many countries and has been described as performing well in meeting the criteria needed for an effective typing assay, such as robustness, reliability, discriminatory power, portability, throughput and objectability of data (Lindstedt, 2005; Van Belkum *et al*., 2007; Hyytia-Trees *et al*., 2007; Nadon *et al*., 2013).

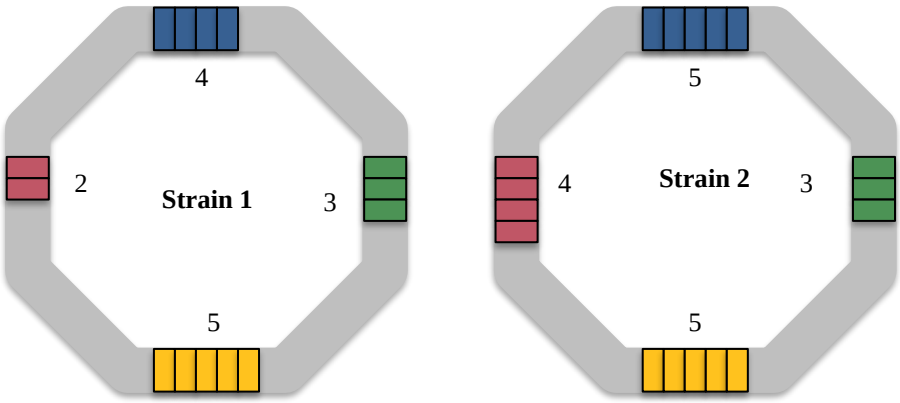


Figure 1.5: Example of variation in the number of tandem repeats (TR) in four loci of *D. nodosus* (each colour represents a locus where tandem repeats arise (**DNTR02**, **DNTR09**, **DNTR10**, **DNTR19**) in 2 strains**.** The colours of the loci correspond to the dye used in the fluorescent forward primers. The numbers indicate the number of repeats at a locus. The two strains differ in number of repeats in 2 of the 4 VNTR loci which enables differentiation.

1.11.6.1 Assay technology

A set of suitable loci containing tandem repeats are identified. PCR is used to amplify the fragments of interest using as set of primers often containing a fluorescently labeled

primer to help differentiate between loci visually. The identification of loci and development of primers are specific for each pathogen. The number of repeats in a locus can be quantified by submitting the sample for sequencing and comparing the length of the sequence to a reference sequence with known numbers of repeats. This technique is often applied when working with bacterial isolates (e.g. Le Fleche *et al*., 2001; Russell *et al*., 2014). When analyzing fragment size of amplified PCR products originating from mixed DNA samples, a method based on capillary electrophoresis is required (e.g. Vranckx *et al*., 2011; Muzafar *et al*., 2015). This method provides the high resolution required for sizing fragments that differ by only a few basepairs in size and is able to detect several strains of a pathogen in the same sample.

Fragment analysis is based on capillary electrophoresis and provides a size estimate for DNA fragments relative to a size standard containing DNA fragments of known length. During capillary electrophoresis, the PCR products are injected electrokinetically into capillaries filled with polymer. During the application of high voltage, the fluorescent DNA fragments are separated by size (Whatley, 2001). Results can be visualized by using adequate software such as GeneMapper® or PeakScanner™).

1.11.6.2 Previous uses of MLVA for the typing of bacteria and in farm animal disease research

In recent years, MLVA has emerged as a valuable method for subtyping bacterial pathogens and has been implemented for investigation of a variety of foodborne disease outbreaks and diseases with zoonotic impact (Table 1.2). MLVA has also become popular in epidemiological studies of bacterial disease in farm animals (Table 1.3). MLVA assays are traditionally applied to bacterial isolates obtained from culture (Table 1.2). More recently a range of assays have been developed that enable strain typing directly from mixed DNA samples (Table 1.3).

Table 1.2: Summary of research investigating the use of MLVA to type a range of bacterial species using varying numbers of loci and a range of fragment sizing methods.

Pathogen	Associated disease	Origin of Isolates	Loci*	Sizing method	Reference
Brucella spp.	Brucellosis	Various origin (not specified)	15	Gel electrophoresis	Le Fleche *et al*. 2006
Brucella melitensis	Brucellosis	Camel, goat, gazelle	16	Gel electrophoresis	Gyuranecz et al., 2016
Salmonella enterica	Salmonellosis	Human	8	Gel electrophoresis	Ranjbar *et al.*, 2016
Streptobacillus monoliformes	Rat bite fever	Various origin (human/animal)	3	Gel electrophoresis Sequencing	Eisenberg *et al*.,2016
Leptospira interrogans Leptospira borgpetersenii	Leptospirosis	Kidneys from feral and wild mammals	11	Gel electrophoresis Sequencing	Koizumi *et al*., 2015
Bacillus antracis	Anthrax	Various countries	5	Fluorescent gel electrophoresis	Keim *et al.*, 2000
Yersinia pestis	Bubonic plague	India, human origin	25	Gel electrophoresis	Kingston *et al.*, 2009)
Escerichia coli (O157:H7)	Bloody diarrhea, Hemolytic uremic syndrome	Range of Isolates (not specified	6	Sequencing and sequence alignment	Noller *et al*., 2003
Haemophilus influenza	Various including meningitis, pneumonia	Isolates, various origin	4	Gel electrophoresis	Van-Belkum *et al*., 1997

*Number of VNTR's targeted.

Table 1.3: Summary of research investigating the use of MLVA in farm animal diseases

Pathogen	Disease	Species investigated	Loci*	Sizing method	Isolates/ clinical sample	Reference
Mycobacterium avium	*M. avium* infection	Pigs	8	Capillary electrophoresis	Isolates	Agdestein *et al*., 2014
Mycoplasma hyopneumoniae	Porcine enzootic pneumonia	Pigs	4	Fragment analysis	Mixed DNA	Vranckx et al. (2011)
Staphylococcus aureus	Mastitis	Cattle, sheep, goat	16	Capillary sequencer	Isolates	Bergonier *et al*., 2014
Mycobacterium bovis	Bovine tuberculosis	Cattle	28	unknown	Isolates	Biffa *et al.*, 2014
Coxiella burnetii	Q-fever	Cows, goats	5	Capillary electrophoresis	Mixed DNA	Ceglie *et al*., 2015
Anaplasma phagocytophilum	Tick borne fever (ruminants), anaplasmosis (humans)	Cattle, sheep, deer, reindeer, horses, dog	5	Gel electrophoresis and sequencing	Mixed DNA	Dugat *et al*., 2014, 2016

*Number of VNTRS's targeted.

1.11.6.3 The Dichelobacter nodosus MLVA assay

The *D. nodosus* genome was sequenced for the first time in 2007 (*D. nodosus* VCS1703A, Meyers *et al*., 2007). Using this *D. nodosus* genome sequence, Russell *et al*. (2014) developed an MLVA assay for *D. nodosus,* identifying four polymorphic loci across the genome for analyses (DNTR 02, 09, 10, 19) (Table 1.4). Subsequently, population analyses divided *D. nodosus* into clonal complexes containing single and double locus variants (Figure 1.6), highlighting the global geographical clustering and distribution of *D. nodosus* isolates. The assay was also used on mixed community foot swabs by Muzafar *et al*. (2015) without modifications to the original assay. 2 loci could not be included in the analysis due to insufficient amplification (DNTR09) and non-specific binding (DNTR02). More recently Smith *et al*., (2017) characterized 268 isolates of *D. nodosus* to investigate population dynamics and persistence and reported presence of 87 MLVA profiles and 2 major MLVA complexes that persisted over time although in the majority of cases *D. nodosus* was isolated only twice from the same sheep.

Table 1.4: VNTR loci identified by Russell *et al*. (2014), including size of tandem repeats, number of repeats in *D. nodosus* strain VCS 1703A and corresponding primers

VNTR locus	Repeat size	Number of repeats	Primer sequence (5'– 3')
DNTR02	5	6	F*:GATCCATCGTTTCATCGTCA R*: CGCACTTTAGCCGTTATGTTT
DNTR09	108	5	F:GGCGTAAACGAAATGCCTAA R: ATCGGCGGAAGATTGTCTC
DNTR10	48	7	F:CCGTCTATCCACCCGATTTA R: TTGAACCGCGTCACTATCAG
DNTR19	84	3	F:CCCGTCGAATCACTCCAG R: GGTAGCGCCGAAGAAAGA.

* F=forward primer, R= reverse primer.

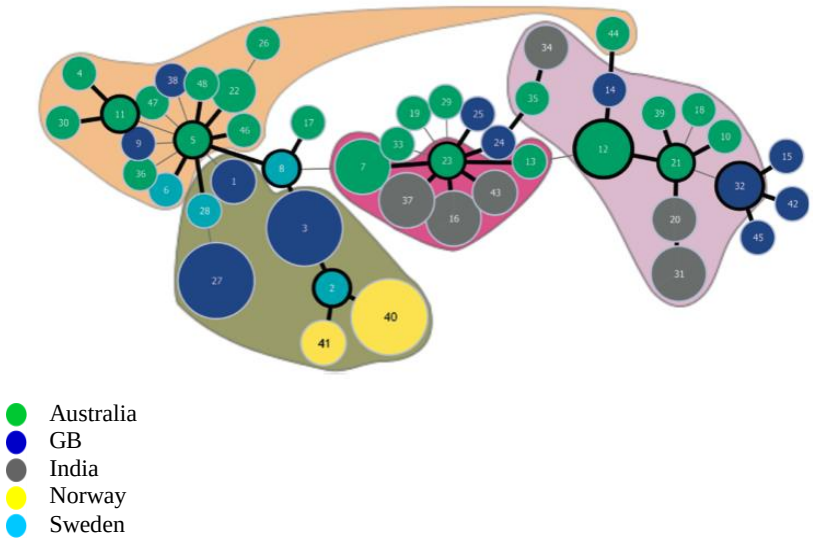


Figure 1.6: Spanning tree created with geographic cluster analysis of isolates characterized by MLVA. Isolates are grouped by country of origin. Thin lines represent double locus variants, indicating different numbers of tandem repeats at two loci between two strains; thick lines represent single locus variant, indicating different numbers of tandem repeats at a single locus indicating a closer relatedness of isolates (Russell *et al.*, 2014).

With regards to this project, information on bacterial load in combination with an effective typing system to establish whether there is genetic variation between populations ***over time*** could be used to investigate pathogenic trends and to identify possible reservoirs of infection.

1.9 Aims, objectives and hypotheses

The aim of this project is to investigate the persistence of *D. nodosus* in the environment (sheep, pasture), by investigating possible sites of survival that may facilitate the occurrence of infection and re-infection. The associations between *D. nodosus* presence/persistence and levels of disease in a flock over time will be investigated with particular emphasis on *D. nodosus* load, using a range of molecular tools, including PCR, qPCR and MLVA.

Presence is defined as the detection of an organism once, at one location. Persistence is defined as the detection of an organism (strain) at one location at least twice on consecutive samplings.

Primary hypothesis

There is at least one site where *D. nodosus* persists when there are no diseased sheep in a flock during a period of non-transmission

Sub-hypotheses

1. The number of sites where persistence occurs decreases as the environmental conditions become less conducive to *D. nodosus* survival.
2. The number of sites where persistence occurs decreases as the time from the last diseased sheep increases.
3. There is a positive association between *D. nodosus* load on the foot, *D. nodosus* load in the environment and level of disease in the flock.
4. Environmental loads of *D. nodosus* correlate with disease outbreaks.

1.10 Thesis structure

Chapter 1 is a general introduction to the thesis

Chapter 2 describes in detail the laboratory processes, including all protocols, tool development, and validation of the methodologies where applicable.

Chapter 3 is based on the results of 2 field studies (Study 1 and 2) and investigates the persistence of *D. nodosus*, focusing mainly on the changing patterns of bacterial load over time, in range of sample types.

Chapter 4 concentrates on the development and optimization of the MLVA analysis and investigates its potential uses with clinical samples and its potential to provide information on persistence of *D. nodosus*. The results are based on samples collected in studies 1 and 2.

Chapter 5 presents the result of a third field study (Study 3) conducted in Spain, investigating whether *D. nodosus* can be detected during periods of non-transmission.

Chapter 6 is a general discussion, discussing key results from the 3 field studies

CHAPTER 2

Materials, methods and laboratory tool development

The approximate workflow and procedures are outlined below (Figure 2.1) for clarity.

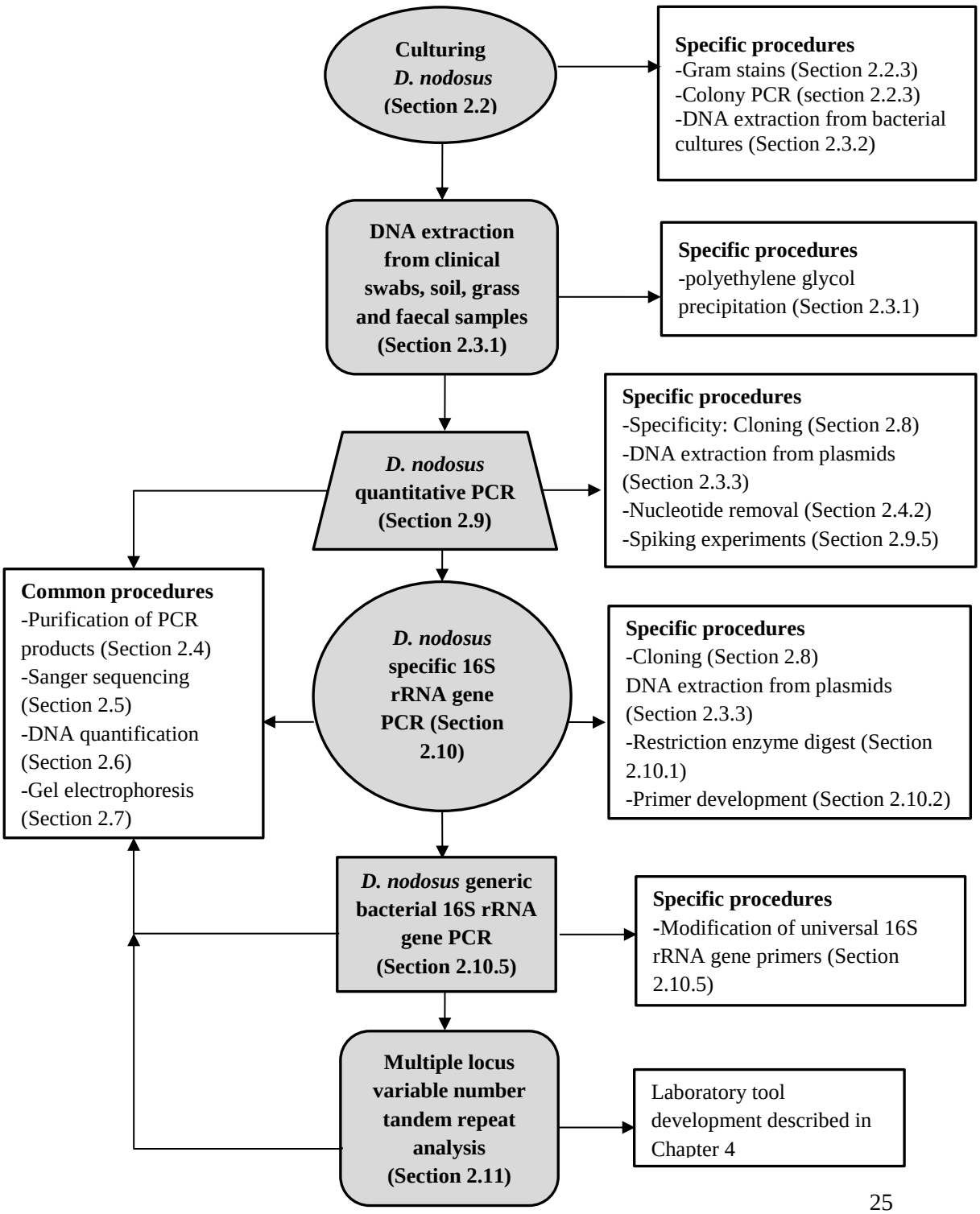


Figure 2.1: Laboratory tools development flowchart.

2.1 Bacterial strains and control DNA samples used throughout the project

The bacterial strains used in this study are shown in Table 2.1 and 2.2. DNA from *D. nodosus* strain 4303 LBV was used as positive control in individual PCR assays. Other *D. nodosus* strains (Table 2.1) were used as target controls to ensure amplification of all strains following assay developments and optimizations conducted in this project. Table 2.2 shows all organisms used as non-target controls for testing the specificity of the *D. nodosus* 16S rRNA gene PCR assay (Section 2.10.3) and multiple locus variable number tandem repeat analysis (MLVA) (described in detail in chapter 4).

Table 2.1: *D. nodosus* strains used as target control organisms.

Organism/Strain id.	Country of isolation	Source	Virulence	Serogroup
VCS 1703A	Australia	J.R. Egerton, University of Sydney	Virulent	G
4303 LBV	UK	University of Warwick	Virulent	unknown
C305	Australia	J.R. Egerton, University of Sydney	Benign	H
UNE135	Australia	B.F. Cheetham, University of new England	Benign	unknown
UNE149	Australia	B.F. Cheetham University of new England	Virulent	unknown
CS101	Australia	D.J. Steward, CSIRO*, Parkville	Benign	unknown
VCS1690	Australia	J.R. Egerton, University of Sydney	Unknown	H
13295C	Australia	Wagga Wagga University	Unknown	F
VCS1001 (A198)	Australia	J.R. Egerton, University of Sydney	Virulent	A
SP-02-418-C	Spain	Unknown	Virulent	E
SP-02-428-C	Spain	Unknown	Virulent	E
SP-02-473-C	Spain	Unknown	Virulent	E
SP-02-475-C	Spain	Unknown	Virulent	E
SP-02-508-C	Spain	Unknown	Virulent	E
SP-02-520-C	Spain	Unknown	Virulent	E
BS8	UK	L.J. Moore, University of Bristol	Virulent	H

All organisms are ovine isolates taken from the feet of sheep

Table 2.2: List of non-target organisms.

Non-target organisms	**Source of isolation**	**Location of isolation**
Staphylococcus uberis	Unknown	AHVLA*, UK
Staphylococcus epidermis	Unknown	AHVLA, UK
Staphylococcus intermedius	Unknown	AHVLA, UK
Staphylococcus aureus Newbould 305, isolated 1958	Cow with mastitis	Ontario, Canada
Staphylococcus hyicus	Unknown	AHVLA, UK
Staphylococcus chromogenis	Unknown	AHVLA, UK
Streptococcus dysgalactidae	Unknown	AHVLA, UK
Streptococcus agalactidae	Unknown	AHVLA, UK
Mannheimia spp.	Sheeps milk	UK, University of Warwick
Fusobacterium necrophorum, isolated 2014	Sheep with FR	UK, University of Warwick
Pseudomonas aeruginosa	Unknown	UK, University of Warwick
Escherichia coli	Unknown	UK, University of Warwick
Mycobacterium tuberculosis	Unknown	UK, University of Warwick

*Animal Health and Veterinary Laboratory Agency. All isolates were purchased as reference strains.

2.2 Culture media and bacterial growth conditions

D. nodosus strains were isolated and cultured using the following media and procedures, which were optimized and validated by initially growing a pure culture of *D. nodosus* (strain 4303 LBV**).**

2.2.1 Isolation of Dichelobacter nodosus from field samples (swabs) on Hoof-Horn Agar (HA)

Table 2.3: Two percent and 4% solid and liquid hoof agar (HA) media recipes (Thomas, 1955; Thorley,1976).

4% HA solid medium ingredients	**Quantity (g/l^{-1})**	**2% HA solid medium ingredients**	**Quantity (g/l^{-1})**
Protease peptone	10	Tripticase peptone	15
Beef extract	4	Beef extract	5
Yeast extract	1	Yeast extract	2
Sodium chloride	5	Proteose peptone	5
Ground hoof powder (4%)	15	L-arginine	5
Bacto agar	40	Serine	1.5
		Magnesium sulphate	25
		Ground hoof powder (2%)	10
		Bacto agar	20

Swab samples were cultured on a 4% hoof agar (HA) solid medium (Table 2.3) to selectively isolate *D. nodosus* (Moore, *et al.*, 2005). Short-term maintenance of pure isolates was on a 2% hoof agar (HA) (Table 2.3). Cultures were incubated at 30 °C in an anaerobic cabinet (MACS-MG-1000 anaerobic workstation, Don Whitley Scientific, Shipley, UK) and subcultured after 5 days of incubation. Pure colonies were harvested by scraping from agar plates into 1.5 ml Phosphate Buffer Saline pH 7.4 (PBS). Cells were collected by centrifuging at 13,000 rpm for 10 minutes. Pellets were stored for DNA extraction at -20 °C. 100 µl of colonies + PBS were re-streaked onto 2% HA, incubated for a further 4 days, harvested in 3ml PBS pH 7.4 + 50 % Glycerol and stored at -80 °C.

2.2.2 Culturing of Dichelobacter nodosus isolates on Eugon Agar

Glycerol stocks and pure isolates were grown and subcultured on Eugon agar (Table 2.4) and incubated for 5 days. Colonies were harvested as described above (section 2.2.1).

Table 2.4: Eugon agar recipe (Catagay and Hickford, 2005).

Eugon Agar ingredients	**Quantity (g l^{-1})**
Yeast Extract (0.2%)	2
Proteose Peptone	7.5
Peptone from Casein	7.5
Soy Peptone	5
Glucose	5.5
L-cystine	0.7
Sodium Chloride	4
Sodium Sulphite	0.2
Defibrinated sheep blood	5%
Bacto agar	40

2.2.3 Identification of pure isolates by examination of colony morphology and colony lysis PCR

Initial identification of pure isolates was made by examination of typical colony morphology in gram-stained smears described in detail by Bartholomew and Mittwer (1952) and performed according the protocol outlined in Coico (2005). Spore stains, using the Schaeffer-Fulton method (Prescott *et al*., 2002) were occasionally performed to check for purity in the cultures due to previous contamination issues with *Clostridia* spp. Presence of *D. nodosus* was confirmed by a colony lysis PCR following the protocol described in Cheetham *et al*. (2006).

2.3 DNA extractions

2.3.1 DNA extractions from clinical swabs, soil, grass, and faecal samples

All DNA extractions from clinical samples (swabs and environmental samples) were performed using the method described by Purdy (2005) with the following modifications: Only 0.5 ml of 120 mM sodium phosphate, pH 8.0 with 1% acid washed Polyvinylpolypyrrolidone (PVPP) was added the sample before the first bead-beating step and only 0.5 ml of 120 mM sodium phosphate, pH 8.0 was used for rewashing the pellet.

Spinning time during the ethanol precipitation step was increased to 30 minutes for each step. Precipitated DNA was suspended in 50 µl of 10mM Tris pH 7.5.

Following ethanol precipitation as described by Purdy (2005) an additional polyethylene glycol (PEG) precipitation step was applied to all environmental samples to obtain DNA of sufficient purity for analysis. Addition of this step increases intensity of bands on PCR gels, indicating improved cleanliness of DNA (Figure 2.2). PEG precipitation is described in Ogram *et al*. (1988) and the following modifications were applied:

0.2 volumes of 6M NaCl and 1 volume of 30% PEG 6000 was added to the suspended DNA. Centrifugation time after overnight incubation was modified to 20 min. at 13,000 rpm. Supernatant was removed from the pellet and washed twice with 70% ethanol, vortexing and centrifuging at 13,000 rpm for 20 minutes after each step. The resulting supernatant was removed and the pellet was air-dried before re-suspension in 50 µl of 10 mM Tris pH 7.5.

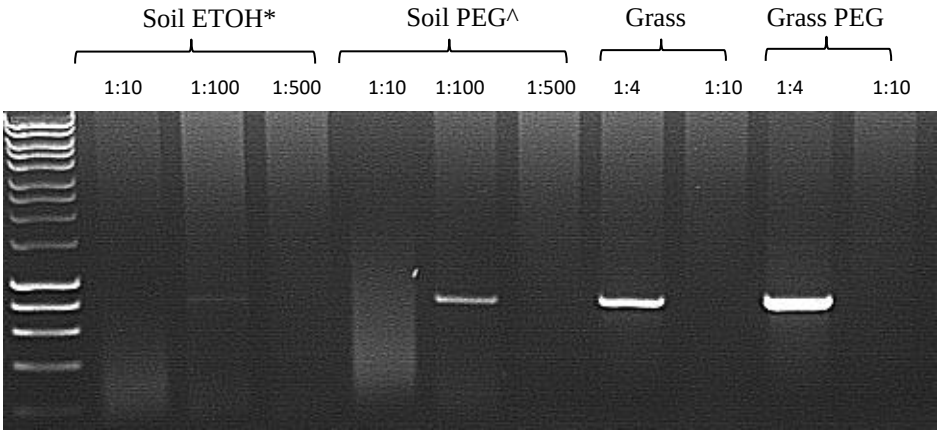


Figure 2.2: Gel shows amplification of the 783bp of *D. nodosus* using nested PCR (section 2.10.5). Samples were assayed with different concentrations of DNA. *ETOH= Ethanol precipitation, ^PEG= Polyethylene glycol precipitation. Soil and Grass samples that had previously tested positive for the presence of *D. nodosus* were used.

2.3.2 DNA extractions from pure cultured isolates (DNeasy®Blood & Tissue Kit)

This was applied to all pure *D. nodosus* and other bacterial cultures. Cultured cell pellets were re-suspended or cells from plates were harvested in 1.5 ml of PBS pH 7.4 and centrifuged for 10 minutes at 7500 rpm. DNA was then extracted using a commercial kit (Qiagen DNeasy®Blood & Tissue Kit, Hilden, Germany) according to the manufacturer's instructions and maximum incubation times. DNA was eluted in 50µl elution buffer to increase final DNA concentration.

2.3.3 DNA extractions from plasmid DNA (QIAprep®Miniprep plasmid extraction kit)

For the extraction of plasmid DNA following all cloning procedures, *Escherichia coli* cells in liquid medium were centrifuged for 10 minutes at 7500 rpm and the supernatant was removed. DNA was then extracted from cell pellets with a QIAprep®Miniprep plasmid extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and maximum incubation times advised.

2.4 Purification of PCR products

2.4.1 QIAquick® PCR purification kit

If required PCR products were purified using a QIAquick® PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturers instruction. Samples were eluted in either 30µl (stronger bands) or 20µl (weak bands) elution buffer (ELB) to increase the final DNA concentration.

2.4.2 QIAquick® Nucleotide removal kit

PCR products containing *D. nodosus rpoD* cloned sequences were purified using a QIAquick® nucleotide removal kit (Qiagen, Hilden, Germany) due to the small size of the inserts. Samples were eluted in either 30 µl (stronger bands) or 20 µl (weak bands) ELB to increase the final DNA concentration.

2.5 Sanger sequencing of PCR amplicons

Sanger sequencing was performed to confirm correct amplification of the desired inserts in all PCR assays used in this study. 5µl of purified PCR product (section 2.4) was mixed with 5µl of the corresponding sequencing primer at a concentration of 5 µM. DNA was sequenced by the molecular biology service of GATC Biotech AG (Cologne, Germany). Sequences were analyzed using the Basic local alignment search tool (BLAST) algorithm (Altschul *et al*., 1990) and traces were assessed for quality using CodonCode Aligner version 6.0.2.

2.6 Quantification of DNA

All extracted DNA samples obtained were tested for DNA purity and yield using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA).

2.7 Gel electrophoresis for visualization of PCR amplicons

If visualization was required, PCR products were electrophoresed on 1% agarose gels in 1X TAE pH 7.5, stained with 0.5 μg ml^{-1} ethidium bromide and visualized under ultraviolet illumination (UV) (GeneFlash, Geneflow, Lichfield, UK) using a 1kb DNA ladder (Hyperladder™ 1kb, Bioline, London, UK). All images were saved on a compact flash SD card (SanDisk® Ultra II).

2.8 Cloning

Cloning of PCR amplicons was conducted on three occasions: 1. In order to sequence *D. nodosus rpoD* PCR amplicons (quantitative PCR), cloning was necessary due to the small size (61bp) of the insert. 2. The specificity of the *D. nodosus* specific 16S rRNA gene primers (La Fontaine *et al*., 1993) was tested by sequencing clones and by restriction fragment length polymorphism analysis to obtain a DNA fingerprint (Section 2.10.1). 3. Quantitative PCR plasmid standards were obtained by cloning (Section 2.9.4).

Fresh PCR products were purified using a QIAquick® Nucleotide Removal Kit (Qiagen, Hilden, Germany) [qPCR] or a QIAquick® PCR purification kit (Qiagen, Hilden, Germany) [16S rRNA gene PCR] according to the manufacturer's instructions.

Purified PCR products were cloned using a TOPO® TA Cloning® Kit for sequencing (Invitrogen, Carlsbad, CA, USA) with a PCR™4-TOPO® plasmid vector supplied with single 3′ thymidine (T) overhangs for TA Cloning®. All reactions were set according to the manufacturer's instructions with the exception of using 0.5 μl of PCR™4-TOPO® plasmid vector instead of 1 μl. Maximum incubation times were applied to all processes and all recommended control TOPO® cloning reactions were performed.

The transformation step was performed using One Shot® TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA) cells according to the manual. However, only 25 μl of competent cells were used per transformation reaction. Maximum incubation times were applied.

Kanamycin was used as the selective agent for transformation and the transformants were

streaked on LB agar plates with 50 µg ml^{-1} Kanamycin and incubated for 24 hours at 37 °C. Harvested colonies were suspended in Liquid LB medium with 50 µg ml^{-1} Kanamycin and incubated in a shaking incubator at 37 °C for 24 hours. Cells were pelleted and plasmid DNA was recovered using a Plasmid Miniprep Kit (QIAprep® Miniprep, Qiagen, Hilden, Germany) following the manufacturer's instructions. Plasmid DNA was standardized to 25ng μl^{-1} screened for inserts using the M13f (5'-GTAAAACGACGGCCAG-3') and M13r (5'-CAGGAAACAGCTATGAC-3') vector-based sequencing primers.

2.9 A PCR for quantifying *Dichelobacter nodosus*: Amplification of the *Dichelobacter nodosus rpoD* gene

2.9.1 TaqMan® Probe chemistry

A TaqMan® probe consists of a fluorescent reporter dye attached to the 5'end of the probe and a non-fluorescent quencher at the 3'end to enable the detection of a specific PCR product as it accumulates during amplification. 5' - 3' nuclease activity of DNA Taq polymerase cleaves the dual-labeled probe while it binds to the complementary target sequence, displacing fragments of the reporter dye from the target, spatially separating the reporter dye from the quencher dye. Degradation of the probe releases fluorophore, resulting in an increase in fluorescence, which is proportional to fluorophore released and the amount of DNA present in the template. This permits quantitative measurement of the accumulation of the product during the exponential phases of the qPCR.

2.9.2 Dichelobacter nodosus quantitative PCR primer and probes

The qPCR assay targets a 61bp sequence within the RNA polymerase sigma 70 factor alpha (*rpoD*) gene of *D. nodosus* and was developed by Witcomb (2012). The primer and probe sets (Table 2.5) were synthesized commercially (TIB MOLBIOL GmbH, Berlin, Germany). TaqMan® probes were labeled with a FAM (6-carboxy-fluorescein) reporter dye and a Black Berry Quencher (BBQ).

Table 2.5: Primer and probe set targeting the 61bp *rpoD* sequence of *D. nodosus.*

Primer	Sequence (5'– 3')	Position	Reference
***rpoD* forward**	GCTCCCATTTCGCGCATAT	1547-1565	Calvo-Bado *et al*., 2011
***rpoD* reverse**	CTGATGCAGAAGTCGGTAGAACA	1607-1585	Calvo-Bado *et al*., 2011
TaqMan *rpoD*	6FAM-CATTCTTACCGGA+T+C +CG-BBQ	1567-1583	Witcomb, 2012

The plus symbol (+) indicates positions of LNA bases or 'locked' nucleotide bases.

2.9.3 Dichelobacter nodosus quantitative PCR cycling parameters

The qPCR assays were performed as previously described by Calvo-Bado *et al*., 2011 using the 7500-fast real-time PCR system (Applied Biosystems®, ThermoFisher, Loughborough, UK). DNA standards and samples were run in triplicate on each quantitative plate and included a non-template control in the form of DNase and RNase-free sterile water (Ambion®, ThermoFisher, Loughborough, UK). The *rpoD* copy number was estimated based on the standard curve obtained from analysis of the 10-fold serial dilutions of DNA extracted from *D. nodosus strain* VCS 1703A (see below).

2.9.4 Dichelobacter nodosus quantitative PCR plasmid standard curves and detection limit

A PCR product of the 61bp *rpoD* insert of *D. nodosus* VCS 1703A was purified and cloned (See section 2.8). Extracted plasmid DNA was then used to create a series of plasmids standards. Plasmid copy numbers where determined as described by the Applied Biosystems reference guide (2013). A set of serial dilutions was then prepared ranging from approximately 10^6 to 10^0 *rpoD* copies µl $^{-1}$. The lowest detected standard for this assay is 10^0 *rpoD* copies µl $^{-1}$. Only samples that amplified in technical triplicates were considered as positive for the *D. nodosus rpoD* insert. Samples that amplified in triplicate, but below the lowest detectable standard were classed as negative in order to minimize the possibility of detecting false positive. Only assays with an R^2 value of >0.90 were accepted as successful and were repeated if lower. Figure 2.3 shows the *D. nodosus* standards in a typical assay.

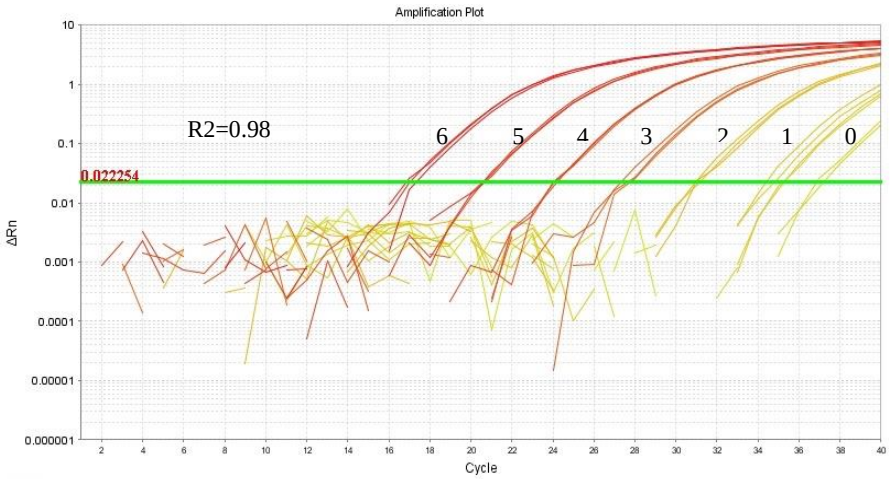


Figure 2.3: *D. nodosus* standards in a qPCR assay ranging from 10^0-10^6 (0-6) *rpoD* genome copies µl $^{-1}$. R^2 value: 0.98.

2.9.5 Spiking of swabs, soil and faeces

The reliability of the qPCR assay depends on efficient extraction of DNA from the environmental sample, which can be tested with spiking experiments. Witcomb (2012) reported significant inhibition of DNA samples extracted from soil when quantifying *D. nodosus* using this assay, but used a different DNA extraction method (Calvo-Bado *et al*., 2011). Therefore, a set of spiking experiments was set up to assess recovery of DNA from all sample types.

Soil from an urban garden and an ovine faecal sample that had previously tested negative for *D. nodosus* were selected for the experiment. *D. nodosus* strain 4303 LBV was cultured on Eugon agar as previously described and harvested in 1 ml of PBS pH 7.4. Suspended cells were quantified using a Petroff-Hausser counting chamber (Hausser Scientific, PA, USA) and serially diluted resulting in dilutions ranging from 10^{-6} to 10^{6} resulting in approximately 1.07 to 1.07 x 10^{6} *rpoD* copies per sample/swab. Tubes containing either 0.5 g of soil, 0.1 g of faeces or an inoculated swab were then spiked with 50 µl of undiluted and serially diluted culture. DNA was then extracted as previously described (Purdy, 2005). An additional PEG precipitation step was applied to all environmental samples (Section 2.3.1). Non-inoculated grass and soil samples, blank swabs were included in all extractions to act as negative controls. DNA concentration was measured (NanoDrop™ 2000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA USA) and A260/280 and 260/230 ratios were recorded. Samples were the screened for *D. nodosus* using the *rpoD*-based qPCR assay as described above.

The lowest detected dilution for swabs was 10^{2} cells μl^{-1} (Figure 2.4, A-B) and the lowest detected dilution for soil and faecal DNA was 10^{1} cells (Figure 2.4, A-F). The standard curves shown in Figure 2.4 however indicate that although the *rpoD* gene was detected quantification became less reliable. However, the information the test provided for the study is valuable, as it indicates that DNA recovery is positively correlated with DNA input.

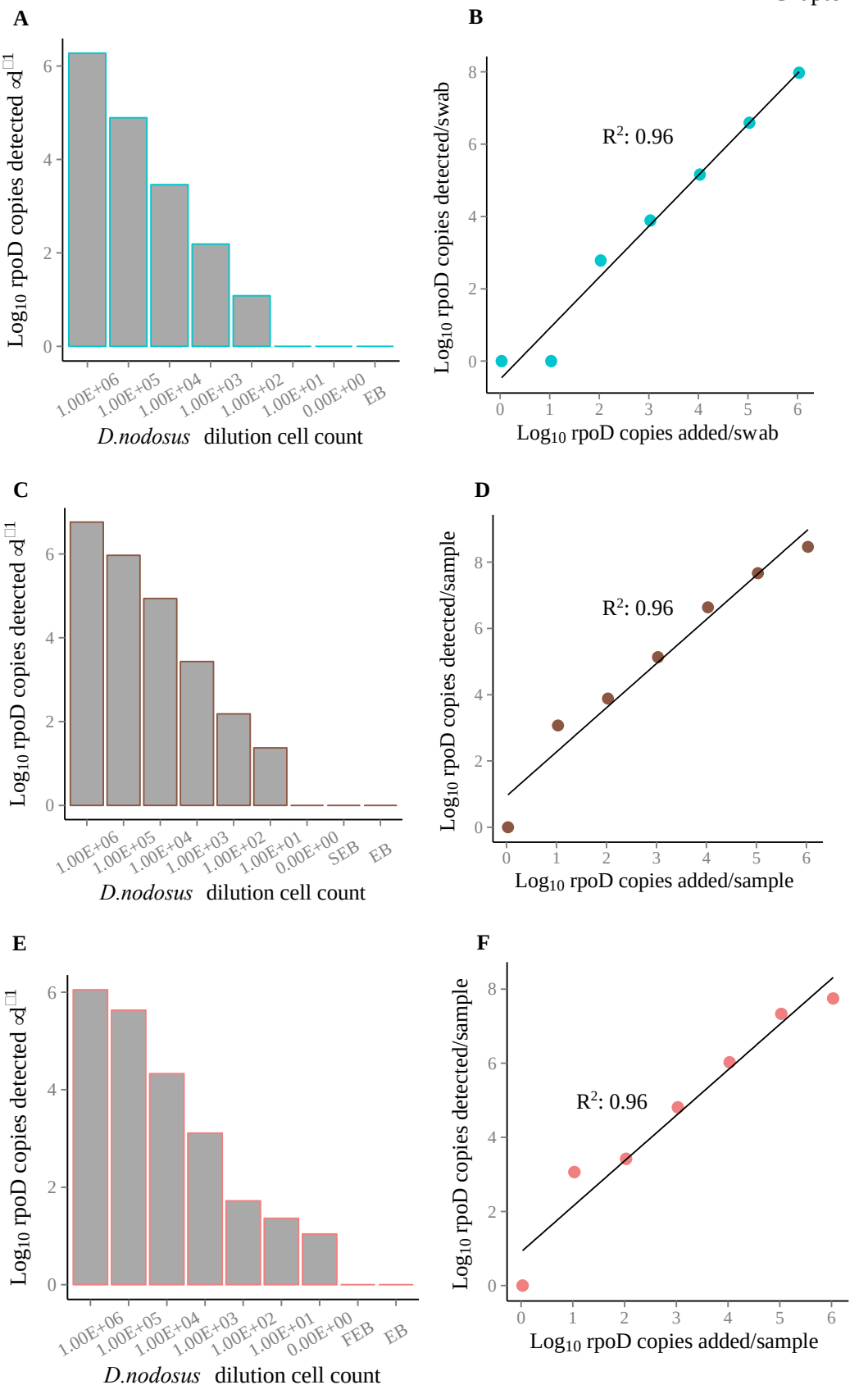


Figure 2.4: *D. nodosus* spiking experiments: *D. nodosus* log10 *rpoD* gene copies detected on swabs **(A)**, soil **(C)** and faeces **(E)** spiked with diluted and serially diluted cultures and log10 *rpoD* copies detected against Log10 *rpoD* copies added to swabs **(B)**, soil **(D)** and faeces **(F)**. EB/SEB/FEB= Extraction blank/Soil extraction blank/Faeces extraction blank (swab/sample spiked with 50 µl PBS). R^2-value shown on graph.

2.9.6 Cloning of the Dichelobacter nodosus rpoD amplicon for sequencing

The analytical specificity of the assay has previously been described using screening of non-target organisms (Calvo-Bado *et al*., 2011; Witcomb 2012) and cloning of the *rpoD* amplicon (Witcomb, 2012). Witcomb (2012) used this PCR for quantification of *D. nodosus* in samples collected from pasture, but reported low detection frequencies, particularly in soil where it was detected in only 1/20 samples. Subsequent cloning of the *rpoD* amplicon confirmed specificity of the assay, but only results from testing samples from the feet of sheep were reported. *D. nodosus* detection on grass had never been investigated. To confirm specificity of the assay 4 *rpoD* amplicons of each sample type were randomly selected (www.randomizer.org), purified and cloned (due to the small size of the insert) and subsequently submitted for sequencing as described in sections 2.4, 2.8 and 2.5.

The *rpoD* insert was observed in all tested soil, grass and faecal samples. No insert was observed for 2 gingival cavity samples, however presence of *D. nodosus* in the sample had already been confirmed using 16S rRNA gene sequencing. Clones with the 61 bp *rpoD* insert sequenced as *D. nodosus* with 99-100% sequence similarity to the published *D. nodosus rpoD* nucleotide sequence with no other matches.

2.10 Generic bacterial 16S rRNA gene PCR for the detection of *Dichelobacter nodosus*

2.10.1 Non-specificity of Dichelobacter nodosus 16S rRNA detection primers

Table 2.6: 16S primers for the detection of *D. nodosus* developed by La Fontaine *et al*. (1993).

Primer	Sequence (5'– 3')	Position*
16S rRNA (forward)	CGGGGTTATGTAGCTTGC	67-84
16S rRNA (reverse)	TCGGTACCGAGTATTTCTACCCAACACCT	820-849

*Nucleotide positions refer to the *D. nodosus* 16S rRNA gene sequence (Dewhirst *et al*., 1990).

A range of environmental samples was tested for the presence of *D. nodosus* using the published *D. nodosus* specific 16S rRNA gene primers (La Fontaine *et al*., 1993) (Table 2.6). Bands were observed for all sample types (Figure 2.5) and products of the correct size were sequenced. DNA was sequenced as described in section 2.5. Sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990).

The faecal DNA amplicon tested did not match published *D. nodosus* sequence when aligned and electropherograms showed overlapping traces, suggesting that DNA from more than 1 species was amplified.

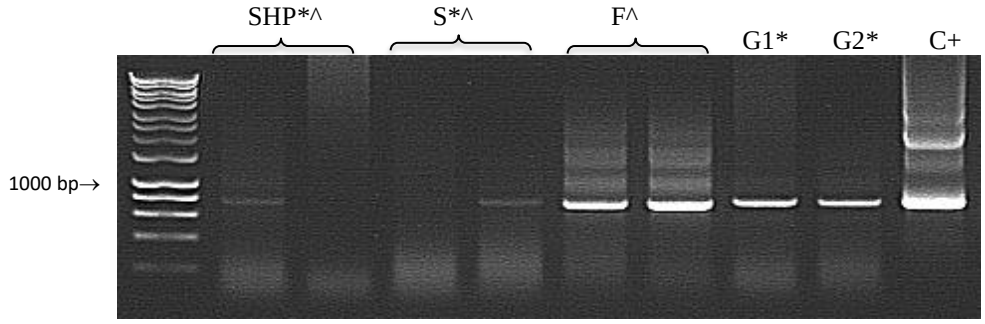


Figure 2.5: 1% (w/v) agarose gel showing the amplification of the 783 bp band characteristic for *D. nodosus*. **SHP**= Soil taken from hoof print, **S**= soil, **F**= faeces, **G1**= Grass where sheep had stood, **G2**: Grass, **C+**= positive control. *= Confirmed as *D. nodosus* by Sanger sequencing, ^= PCR reaction for each sample was performed at sample dilutions of 1:100 (left) and 1:500 (right).

The poor-quality sequence may have been caused by a lack of specificity in the primers or by multiple copies of the 16S gene with different sequences within the *D. nodosus* genome. To analyze which of these possibilities was correct the PCR amplicon was cloned and plasmid DNA extracted (Procedures are described in section 2.8 and 2.3.3). Amplicons containing the expected insert of 943 bp (Figure 2.6) were purified and sequenced (sections 2.4 and 2.5 above). All 14 clones had both *D. nodosus* 16S rRNA gene forward and reverse primer. There was an 83-88% sequence similarity to a range of bacterial 16S rRNA gene sequences, but none matched published *D. nodosus* sequences.

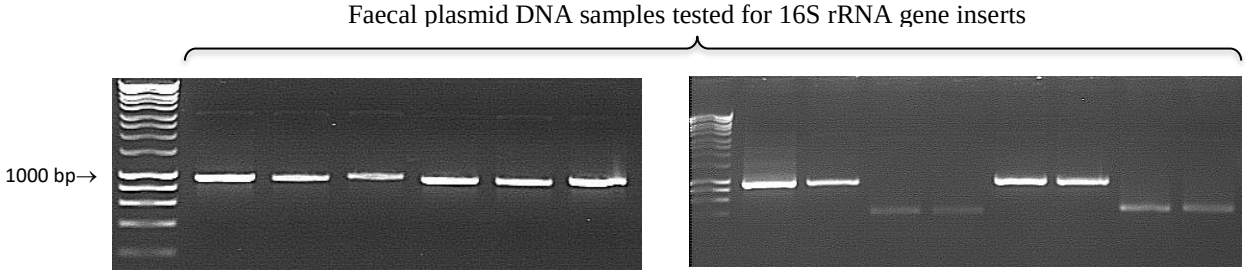


Figure 2.6: Cloning insert screen using M13f and M13R vector primers for 16S rRNA gene inserts on a 1% (w/v) agarose gel. Colonies with the insert have the expected 943bp product. (vector 166 bp + insert 785 bp). Negative control (sterile H_2O) was included, but not shown on this row. Bioline 1 kb DNA ladder with 1000 bp marker indicated.

Restriction digest was then applied to 11 cloned amplicons in order to obtain a DNA fingerprint. DNA fingerprint patterns of cloned 16S rRNA gene fragments can indicate whether more than one bacterial species is present in a PCR product. Webcutter 2.0 software was used to obtain a restriction digestion map for the 785 bp *D. nodosus* 16S rRNA gene sequence. Settings were adjusted to use linear sequence analysis and the number of cut-sites was specified (2-4). A suitable enzyme was chosen depending on the position of the cut-sites. Cloned faecal amplicons were digested with HaeIII (Promega, Madison, USA) according to the manufacturers guidelines.

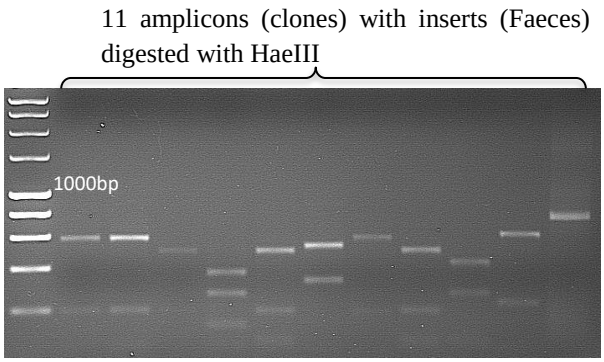


Figure 2.7: Restriction digest results from 11 cloned faecal amplicons. Amplicons were digested with HaeIII. Restriction sites in the amplified *D. nodosus* 16S rRNA gene were at 260 bp and 245 bp, resulting in approximate expected fragment sizes of 240 bp, 85 bp and 580 bp. Bioline 1 kb DNA ladder was used with the 1000 bp marker indicated. Negative control (sterile H_2O) was included, but not shown.

The results show that clones were diverse and Figure 2.7 shows the presence of 5 different fingerprint patterns. Bands did not correspond with the fragment sizes expected to be obtained from restriction digest of the *D. nodosus* 16S rRNA gene, confirming that these clones are not *D. nodosus*. Genome sequences from *D. nodosus* show it has 3 essentially identical copies of the 16S rRNA gene (Myers *et al*., 2007) that would produce identical fingerprinting in this analysis.

Therefore, the PCR assay is non-specific, especially when challenged with a highly diverse faecal sample. As the feet of sheep are often contaminated with soil and faeces, this lack of specificity could produce false positives. Hence there can be only limited confidence in the accuracy when using this PCR assay. In addition, given the lack of specificity is particularly poor with faecal samples to meet one of the aims of this project, to investigate faecal shedding of *D. nodosus*, highlights the need to develop more specific *D. nodosus* detection primers.

2.10.2 Development of Dichelobacter nodosus specific 16S rRNA gene primers

A list of 200 candidate assays based on the 16S rRNA gene sequence of *D. nodosus* was designed using BatchPrimer3 (v1.0). A second list of 8 *D. nodous* target 16S rRNA gene sequences were obtained from the National Centre for Biotechnology Information (NCBI) website. A third list of approximately 900 16S rRNA gene sequences from organisms that are most closely related to *D. nodosus* were obtained using ARB software (Ludwig *et al*., 2004). All lists were incorporated into the Thermophyl software (Oakley *et al*., 2011). Thermophyl software assesses the phylogenetic sensitivity and specificity of the candidate assays and running the software resulted in 156 possible primer pairs.

Only assays that matched all 8 target sequences and no non-target sequences were selected and tested, together with their resulting product, for similarity to *D. nodosus* using the BLAST algorithm (Altschul *et al*, 1990). A number of assays were chosen for sensitivity and specificity testing in the laboratory. The most promising assay was a combination between a new forward primer (436f) and the previously used *D. nodosus* reverse primer (La Fontaine *et al*., 1993) (Table 2.9, Section 2.10.5) amplifying a 413 bp product of the *D. nodosus* 16S rRNA gene. The *D. nodosus* reverse primer developed by La Fontaine *et al*. (1993) was not a candidate primer returned by the Thermophyl software, but was tested in combination with a new forward primer that was returned by the software and had shown some promise during initial testing in the laboratory. This assay resulted in clean products from soil and grass DNA and did not amplify the faecal DNA sample from section 2.10.1. Sequencing results showed a 100% sequence similarity and query cover to all 8 published *D. nodosus* sequences. Electropherograms showed one single clean trace (Figure 2.8).

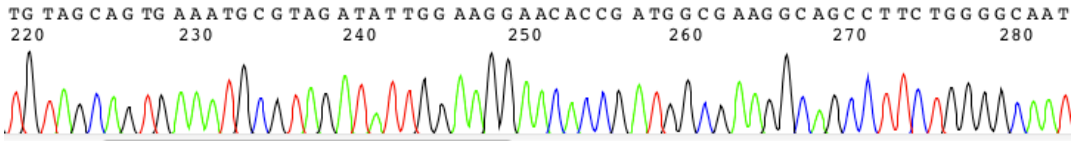


Figure 2.8: Partial electropherograms from sequence 18BC20 (soil sample). The electropherogram features a clean trace and well-formed peaks with no evidence of multiple sequences within the PCR product.

2.10.3 Specificity of the developed 16S rRNA gene primers

The assay was then tested against a number of non-target organisms using the final reaction set-up and cycling conditions described below. This resulted in no visible amplification of the selected non-target organisms. The assay produced single bands of the expected size, indicating that is specific for the selected target. (Table 2.7, Figure 2.9). Primers were tested on clinical swab samples from study 1 (Chapter 3).

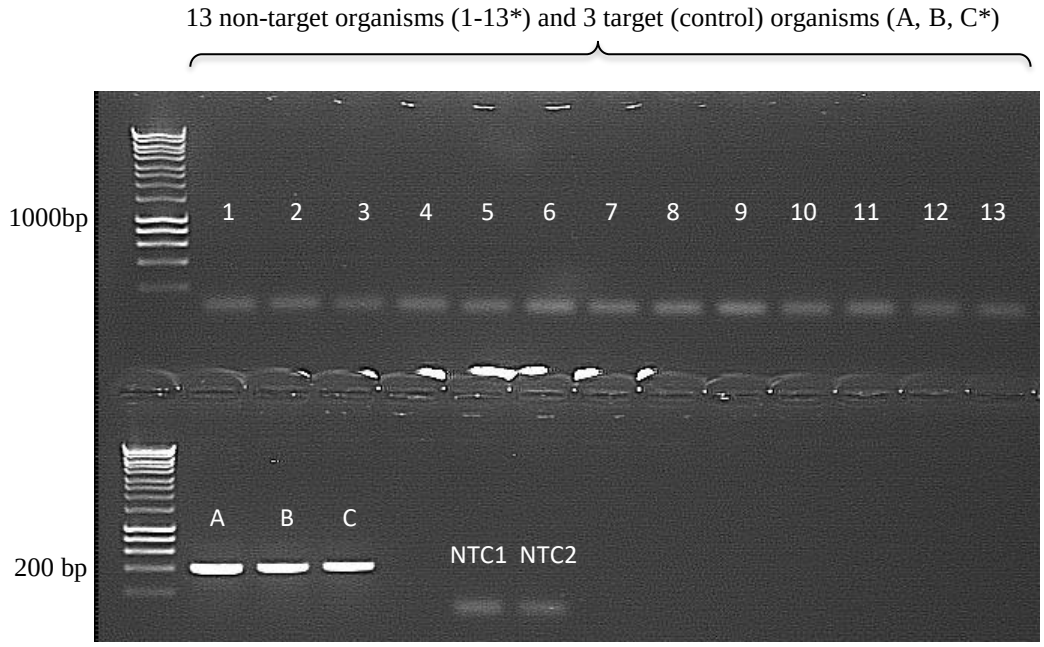


Figure 2.9*:* Non-target organism screen for *D. nodosus* 16S rRNA gene primers shown on 1% (w/v) agarose gel. * Numbers and letters correspond to organisms shown in Table 2.7. Amplicons from control organisms have the expected band size of 413 bp. **NTC**= Non-template-control. **NTC1**= Non-template control from the first round of PCR using universal 16S gene primers. NTC2= Non-template control from the second round of PCR (*D. nodosus* 16S rRNA gene specific PCR). Bioline 1 kb DNA ladder with 1000 bp marker indicated.

Table 2.7: List of target and non-target organisms tested.

Non-target organisms		
1	*Staphylococcus uberis*	-
2	*Staphylococcus epidermis*	-
3	*Staphylococcus intermedius*	-
4	*Staphylococcus aureus*	-
5	*Staphylococcus hyicus*	-
6	*Staphylococcus chromogenis*	-
7	*Streptococcus dysgalactidae*	-
8	*Streptococcus agalactidae*	-
9	*Mannheimia spp.*	-
10	*Fusobacterium necrophorum*	-
11	*Pseudomonas aeruginosa*	-
12	*Escherichia coli*	-
13	*BCG (Bacillus Calmette-Guerin)*	-
Target Organisms		
A	*D. nodosus Strain C305 (benign)*	+
B	*D. nodosus Strain VCS 1703A)*	+
C	*D. nodosus Strain 4303 LBV*	+

Minus (-) indicates that no amplification was observed. Plus (+) indicates that a band of the correct size was observed. For additional information on the strains above see chapter 2.

2.10.4 Dichelobacter nodosus specific final 16S rRNA gene PCR protocol

All primers were commercially synthesized (Sigma Aldrich, Dorset, UK). All PCR reactions had a final volume of 25 µl, containing 12.5 µl Mastermix (MyTaq™ Red Mix, Bioline, London, UK), 1 µl of each primer at a final concentration of 10 mM, 1 µl bovine serum albumin (10 mg ml^{-1}) (Sigma, Aldrich. Dorset, UK) and 8.5 µl of nuclease-free water (Fisher Scientific Loughborough, UK). 1 µl DNA template was added to each PCR reaction. The following cycling conditions were applied: 1 cycle of 95 °C for 2 minutes, 40 cycles of 95 °C for 1 minute, 62 °C for 1 minute and 72 °C for 2 minutes with a final extension of 72 °C for 10 minutes.

2.10.5 Nested PCR: Modification of the Universal 16S rRNA gene primers

If samples did not amplify directly with the new *D. nodosus* primer set, a nested approach was used. A first round using a modified 16S gene 27F primer (Table 2.9) and 1525R primer (Lane, 1991, Baker *et al*., 2003) was applied to the samples. The 27F primer was modified to correct two continuous mismatches in the sequence at the 5'end of the *D. nodosus* 16S rRNA gene and the primer (Table 2.8). Cycling conditions and reaction composition are identical to the *D. nodosus* specific 16S rRNA gene assay with the exception that the annealing temperature was reduced to 55 °C. DNA extracted from type strain of *D. nodosus* 4303 LBV was used as a positive control and each reaction included a non-template control in the form of DNase and RNase-free sterile water (Fisher Scientific Loughborough, UK).

Table 2.8: Modifications of the universal 16S rRNA gene primer.

D. nodosus 16S rRNA gene sequence	AGA GTTTGA **TTC** TGGCTCAG
Original 27f primer	AGA GTTTGA **TCM** TGGCTCAG
Modified 27f primer	AGA GTTTGA **TTC** TGGCTCAG

Degenerate basepairs are listed using IUPAC code.

Table 2.9: Primers used for the *D. nodosus* 16S rRNA gene assay.

Primer	Sequence (5'– 3')	Position	Reference
***D. nodosus* 16S rRNA**	f: TAGTGAAGAACGGTGCATGG	436 - 455	This study
	r: TCGGTACCGAGTATTTCTACCCA ACACCT	820 - 849	La Fontaine *et al*., 1993
Universal 16S rRNA	f: AGAGTTTGATTCTGGCTCAG*	27 - 46	Lane *et al*., 1991,
	r: AAGGAGGTGWTCCARCC	1525 - 1541	Baker *et al*., 2003

* Forward primer was modified in this study. Degenerate basepairs are listed using IUPAC code.

2.10.6 Testing of the developed Dichelobacter nodosus specific 16S rRNA gene primers

A selection of samples with the expected 413 bp PCR product obtained from study 1 (Chapter 3) were used to test the developed primer set for correct amplification using Sanger sequencing as described in section 2.5.

10/10 PCR amplicons from foot swabs sequenced *as D. nodosus* with a 100% sequence similarity (100% query cover) to all 8 published *D. nodosus* 16S rRNA gene nucleotide sequences. Electropherograms displayed good quality traces. Sequencing was unsuccessful for the single submitted grass samples, but both faecal (Figure 2.10) and soil DNA samples sequenced as *D. nodosus* with a 99-100% sequence similarity.

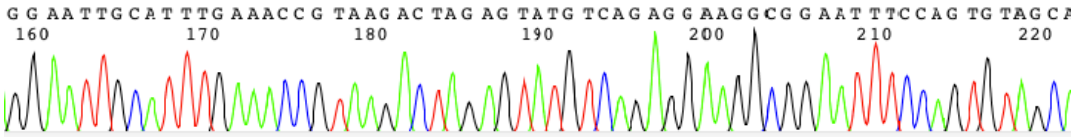


Figure 2.10: Partial electropherogram of sequence 51FA39 from a faecal sample. For the first time sequencing of the *D. nodosus* 16S rRNA gene DNA amplicon was successful for a faecal DNA sample. Electropherogram shows a clean trace with strong peaks and no evidence of non-specificity of the assay.

Sequences were obtained for 8 samples from the oral cavity. Seven samples sequenced successfully *as D. nodosus* with a 100% sequence similarity to published *D. nodosus* sequences (99-100% query cover). One sample showed 100% sequence similarity to the *D. nodosus* genome with query cover of 95%. Two other matches were returned for this sample displaying 100% sequence similarity and 96-99 % query cover to the two other members of Cardiobacteriaceae, indicating 2 cases of non-specificity of the developed primers.

Both samples displayed a higher sequence similarity to *Sutonella* spp. and *Cardiobacterium* spp., than to the published *D. nodosus* sequences. These two species are the only other two members of the family Cardiobacteriaceae that *D. nodosus* belongs to. They have not been previously associated with sheep, but are both assumed to be present in the respiratory tract (Garrity, 2005). This could explain why the cases of non-specificity occurred only in samples taken from the oral cavity. This also suggests that the developed PCR assay is a Cardiobacteriaceae-family specific assay and was therefore not used in subsequent studies.

2.11 Multiple Loci Variable Number Tandem Repeat (VNTR) Analysis (MLVA)

2.11.1 Assay optimizations and cycling conditions

The MLVA assay was for *D. nodosus* was developed by Russell *et al*. (2014) for use on bacterial isolates and later by Muzafar *et al*. (2015) on clinical swabs without modifications of the original assay. However, Muzafar *et al*. (2015) reported that multiple peaks could be detected in fragment analysis using DNTR09 and there was non-specific binding when using DNTR02. The MLVA assay was optimized in this study to increase both sensitivity and specificity (Chapter 3). These included changes in assay composition and thermal cycling conditions and resulted in the following final set-up for four *D. nodosus* VNTR loci (DNTR02, 09,10 and 19): All PCR reaction had a final volume of 25 µl containing 12.5 µl Mastermix (MyTaq™ Red Mix, Bioline, London, UK), 1 µl of each primer (10 mM) (Table 2.10) and 1 µl bovine serum albumin (20 mg ml^{-1}) (Sigma, Aldrich. Dorset, UK)). 1 µl DNA template was added to each PCR reaction. Reactions

were carried out using the following conditions: 1 cycle of 95 °C for 2 minutes, 40 cycles of 95 °C for 1 minute, 59 °C for 30 seconds and 72 °C for 1 minute with a final extension of 72 °C for 2 minutes. The full development process for the modified *D. nodosus* MLVA assay can be found in chapter 4.

Table 2.10: *D. nodosus* MLVA loci, primers and associated fluorescent dyes.

VNTR/ primer name	Primer sequence (5'– 3')	Dye name	Dye colour
DNTR02	Forward: GAT CCA TCG TTT CAT CGT CA (FL)* Reverse: CGC ACT TTA GCC GTT ATG TTT	6-Fam	Blue
DNTR09	Forward: GGC GTA AAC GAA ATG CCT AA (FL) Reverse: ATC GGC GGA AGA TTG TCT C	Vic	Green
DNTR10	Forward: CCG TCT ATC CAC CCG ATT TA (FL) Reverse: TTG AAC CGC GTC ACT ATC AG	Net	Yellow
DNTR19	Forward: CCC GTC GAA TCA CTC CAG (FL) Reverse: GGT AGC GCC GAA GAA AGA	Pet	Red

* FL = 5'fluorescent labeled.

2.11.2 Fragments analysis and data analysis

Amplified VNTR's were submitted for fragment analysis (DNA Sequencing and Services, University of Dundee, Scotland). The GeneScan™1200 Liz® size standard (Applied Biosystems, Warrington, UK) was used for sizing products and data obtained were analyzed with Peak Scanner Software (Applied Biosystems, Warrington, UK).

2.12 Presence of *Dichelobacter nodosus* in areas where sheep are historically absent

No data about the presence of *D. nodosus* outside a farm environment was available prior to the start of the study. Therefore, soil samples collected from urban gardens across Coventry and Warwickshire where sheep and other ruminants are historically absent were tested for the presence of *D. nodosus* using the 16S rRNA gene direct and nested PCR assays described in sections 2.10.4 and 2.10.5, as well as the quantitative PCR assay described in section 2.9.3. No amplification was observed in the samples (Figure 2.11).

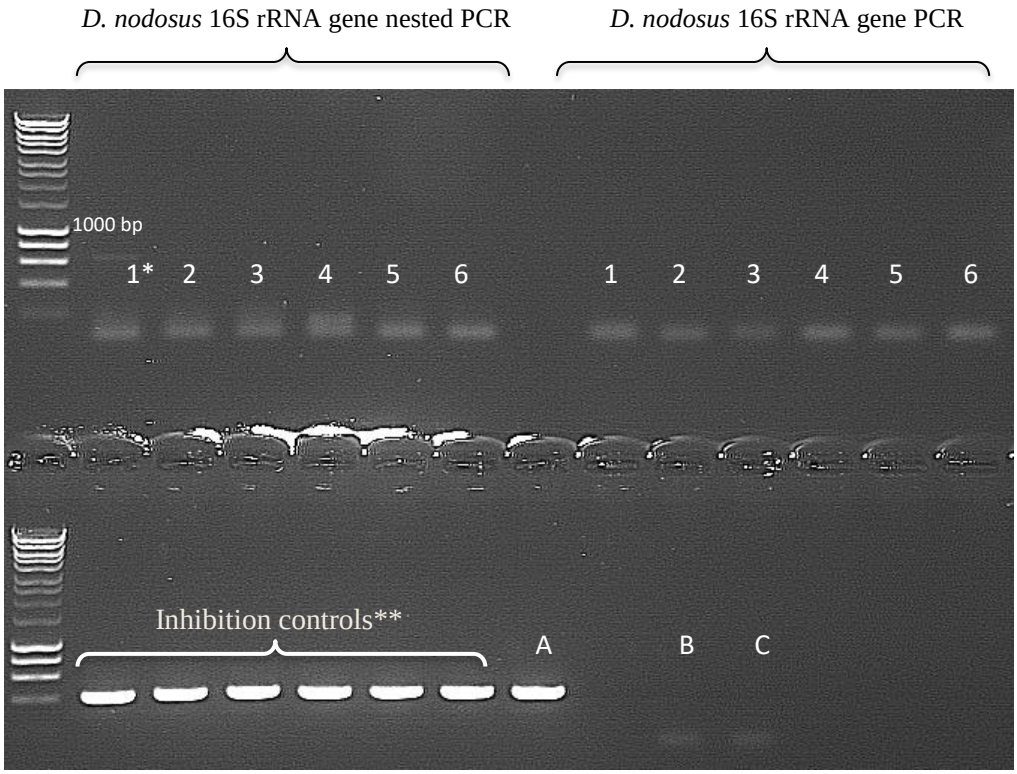


Figure 2.11: Amplification of *D. nodosus* in areas where sheep are historically absent shown on a 1% (w/v) agarose gel. *1-6 = 6 soil samples collected from urban gardens. One non-specific band was observed when using nested PCR (1*) but the band was the wrong size and therefore judged to be *D. nodosus* negative. Sterile water and a PBS extraction blank were included as negative controls (**B, C**) and DNA extracted from *D. nodosus* strain 4303 LBV (**A**) was included in the reaction as positive control. **1 µl of each soil sample (1-6) was added to DNA extracted from *D. nodosus* strain 4303 LBV to investigate whether amplification was inhibited. Bioline 1 kb DNA ladder with 1000 bp marker indicated.

These results indicate that *D. nodosus* was not detectable in environments that are free from sheep and other ruminants. This supports the idea that *D. nodosus* is closely associated with ruminants and, in the case of sheep, to FR.

CHAPTER 3

Detection and quantification of *Dichelobacter nodosus* on sheep and in environmental samples: Evidence from two field studies.

3.1 Introduction

Investigating the persistence of pathogenic bacteria and potential reservoirs of infection increases knowledge on disease pathogenesis and is essential for the design of effective disease control measures (Haydon, 2002).

It is not known where and whether *D. nodosus* persists. The seasonal behaviour of *D. nodosus* in the UK has not been investigated previously and there is little information on how its presence and survival might be affected by climate.

FR occurs in all continents were sheep are farmed and from reviewing the literature it is clear that disease initiation and progression differs depending on the climatic characteristics of a country or region. Whereas in many countries FR is highly seasonal with long periods of non-transmission preceding outbreaks (Cagatay and Hickford 2006; Sreenivasulu, *et al*., 2013; Aguiar, *et al*., 2013), in the UK FR can occur throughout the year in form of a series of mini-epidemics (Ridler *et al*., 2009).

Both ambient temperature and moisture have been described as conditions that favour transmission (Graham and Egerton, 1968; Smith *et al*., 2014). Research originating from Australia reports that FR does not spread at temperatures below 10 °C. However, the results of a number of studies from the UK suggest that FR can spread at lower temperatures (Ridler *et al*. 2009; Smith *et al*., 2014). These studies provide information on climate factors that influence disease transmission, but did not provide information on *D. nodosus* persistence.

Australian research suggests that *D. nodosus* does not survive on the pasture for more than 10-14 days (Graham and Egerton, 1968), but recent evidence from *in-vitro* studies suggest that *D. nodosus* may be able to survive for at least 40 days (Cederlof *et al*., 2013; Muzafar *et al*., 2016).

D. nodosus has been detected in soil, faecal samples collected from the field and in the gingival cavity, but these studies were cross-sectional in nature and there was no linkage to climate (Witcomb, 2012; Muzafar *et al*., 2015). The ability of *D. nodosus* to persist on the feet of sheep has been suggested by a number of authors (Beveridge, 1941; Depiazzi

et al., 1998; Kaler *et al.*, 2010; Moore *et al.*, 2005; Witcomb, 2012), but this is mostly based on findings that *D. nodosus* can be detected on healthy feet.

The aim of the 2 studies reported in this chapter was to investigate persistence of *D. nodosus* by conducting longitudinal studies in two sheep flocks with different levels of disease, to investigate FR disease patterns, *D. nodosus* detection and load variation on sheep and their pasture overtime. We further aimed to investigate patterns of associations between *D. nodosus* presence on the foot, in the gingival cavity and in the farm environment using a range of predictor variables, including climate, to elucidate were *D. nodosus* is most likely to persist. The results of both studies are compared and contrasted in this chapter.

3.2 Ethical approval

Ethical approval for studies 1 and 2 was obtained from the Animal Welfare and Ethical Review Body (AWERB) at the University of Warwick (Document reference number AWERB 33/13-14).

3.3 Materials and methods

3.3.1 Farms and animals

Study 1

The first aim of study 1 was to validate all optimized and developed laboratory tools. The second aim was to test *D. nodosus* detection frequencies and bacterial loads (including variation of loads) in a range of sample types. The third aim was to gain information on the persistence of *D. nodosus* by investigating patterns of associations between *D. nodosus* presence on the foot, in the gingival cavity and in the farm environment using a range of predictor variables, including climate. One goal was to use the resulting findings to design a second more extensive longitudinal study (Study 2), so that adequate decisions about the number of sheep to be sampled, the duration of the study and potential bacterial reservoirs to be investigated could be made.

The study was conducted between May and August 2014 on a commercial farm located near Kenilworth, Warwickshire. The flock was chosen based on its known history of FR and the presence of an outbreak at the time of the study. From the flock of approximately 150 animals, 5 North Country Mules and 5 lambs (North-country mule x Texel) were selected for the study. Sheep that were asymptomatic and symptomatic for ID and SFR lesions were chosen. All lambs were unrelated to the ewes. A total of four fortnightly farm-visits were arranged. All 10 animals remained with the rest of the flock on the same field throughout the trial and were sampled fortnightly according to the procedures outlined below (Section 3.3.2). The sample size and sample frequencies were chosen in order to gain adequate amounts of epidemiological information and to capture variations in climate, taking into account the aims of the study.

Study 2

Suitable sample sizes were calculated using Altmans Nomogram for sample size determination (Petrie and Watson, 2013). All procedures were based on specification of power (80%), significance levels (5%), the effect of interest in the population being observed and the variability of those observations. Data from Witcomb *et al*. (2014) was used to compute the number of samples needed in order to detect a difference in load of *D. nodosus* over time and associations between presence of *D. nodosus* on feet and in the gingival cavity and level of disease of an individual. Practical, ethical and economical parameters were also considered, as well as the results obtained from study 1.

The study was conducted from February to July 2015. A flock of 120 Suffolk x Wiltshire-horn ewe lambs, from a commercial sheep farm near Warwick, Warwickshire, was selected as the study population. Prior to commencement of the study, the farmer confirmed history of FR and presence of lame sheep in the flock.

During the first visit in February all 120 sheep were observed for lameness and divided in 3 groups: non-lame, mildly lame/uncertain and obviously lame. All non-lame sheep were turned and their feet examined for signs of footrot, interdigital dermatitis and other causes of lameness. Forty ewe lambs that showed no signs of lameness, had no FR lesions and scored ≤ 1 for ID were sampled as outlined in the sampling procedure described below (section 3.3.2), marked and moved to the study pasture as the study group.
The study group and pasture were examined and sampled weekly for five months from February to July 2015. Baseline pasture samples were taken 10 days prior to commencement of the trial and again at the end of the 10-days, before the selected study group was moved onto the pasture. The pasture was left unglazed for this period. All animals remained on the same pasture throughout the study. No cattle grazed the study field throughout the duration of the study.

3.3.2 Animal and environmental sampling procedures (Studies 1 and 2)

The total number of samples taken in study 1 was 342 and included 190 swabs taken from sheep and 152 samples collected from the pasture (Table 3.1). The total number of samples collected in study 2 was 5428, including 4788 swabs from sheep and 640 samples from the pasture (Table 3.2).

Table 3.1: Number of samples collected in study 1 each fortnight from different sampling sites.

Sample type/origin	Number per week	Total taken
Foot swab	40 (4 per sheep), 32 in week 7	152
Gingival cavity swab	10 (1 per sheep), 8 in week 7	38
Faeces	10	40
Soil		
Low traffic area 0-1cm	5	20
Low traffic area 4-5cm	5	20
High traffic area 0-1cm	6	24
High traffic area 4-5cm	6	24
Grass		
Low traffic area	5	20
High traffic area	1*	4
		342

* Grass was present at one site only.

Table 3.2: Number of samples collected in study 2 each week from different sampling sites.

Sample type/origin	Number per week	Total taken
Foot swab	160 (4 per sheep)	3192
Gingival cavity swab	40 (1 per sheep)	798
Faeces	40 (1 per sheep)	798
Soil		
Low traffic area 0-1cm	5	105
Low traffic area 4-5cm	5	105
High traffic area 0-1cm	6	126
High traffic area 4-5cm	6	126
Grass*		
Low traffic area	4-5**	78
High traffic area	5-6	100
		5428

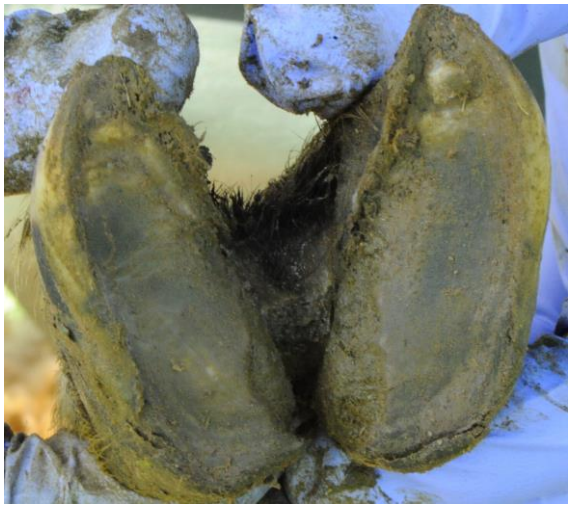
*Includes baseline pasture samples, ** On some occasions grass was not present.

3.3.2.1 Procedures for collection of samples from sheep

Sheep were individually identified, and body condition scored on a scale from 1-5 (Agriculture and Horticulture Development Board [AHDB], 2013). Sheep were then turned; feet were photographed when lesions were present and scored for ID and SFR lesions using two 5-point scales (Moore, *et al*., 2005) (Table 3.3). Sheep were defined as having SFR if at least one foot had a SFR lesion score > 0 (Kaler *et al.*, 2011). Sheep were defined as having ID if at least one foot scored lesions of > 1. If a foot scored ≥ 1 for SFR or >1 for ID, the farmer was informed and recommended to treat the sheep. All treatments given (including any given by the farmer in the interval between visits) were recorded and taken into account during data analysis. Other causes of lameness or foot abnormalities were also recorded. Figures 3.1 and 3.2 show ID and SFR lesions categorized according to the scoring system.

Table 3.3: Scoring system for interdigital dermatitis (ID) (A) and severe footrot (SFR) (B) lesions (adapted from Moore *et al*., 2005).

A: Classification of ID lesions	
0	Clean interdigital space with no dermatitis lesion or fetid smell
1	Slight interdigital dermatitis, irritation of the skin but dry
2	Slight interdigital dermatitis with a fetid smell < 5% skin affected
3	Moderate interdigital dermatitis with a fetid smell, 5-25% skin affected
4	Severe interdigital dermatitis with a fetid smell, >25% skin affected
B: Classification of SFR lesions	
0	A clean digit with no lesions
1	An active or healing footrot lesion with a degree of separation of the sole of the digit
2	An active footrot lesion with a marked degree of separation of the sole of the digit
3	An active footrot lesion with extensive under-running of the wall hoof horn
4	An active footrot lesion with complete under-running of the wall hoof horn



Healthy foot (No ID/SFR)

ID 1

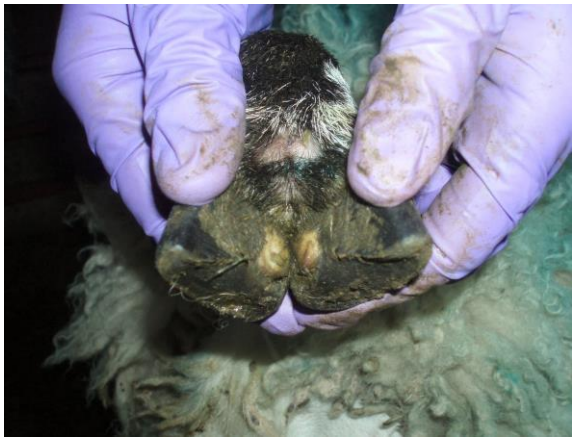
ID 2

ID 3

ID 4

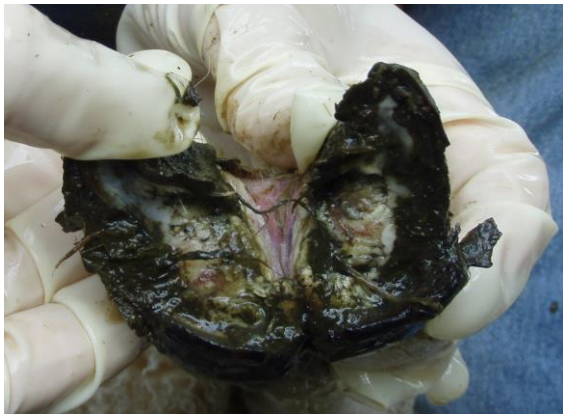
Figure 3.1: Interdigital dermatitis (ID) lesion chart. Images correspond to the description given in Table 3.3 (Source: Research group footrot archives, University of Warwick).

SFR 1



SFR 2

SFR 3



SFR 4

Figure 3.2: Severe footrot (SFR) lesion chart. Images correspond to the description given in Table 3.3 (Source: Research group footrot archives, University of Warwick).

One observer carried out all disease assessments to avoid inter-observer bias (Foddai *et al.*, 2012). All data were recorded using an Electronic Identification (EID) Reader. Swabs (Eurotubo® Collection swab with transport media, Deltalab, Rubi, Spain) were taken by trained researchers from the interdigital skin of the foot and from the gingival crevice of the mouth by passing the sterile wooden end of cotton swab 5 successive times over the skin while rotating it (Figure 3.3). Swabs were transferred into cryogenic vials with 300 µl PBS pH 7.4 and stored at -20 °C.

Figure 3.3: Sheep sampling procedure. Lesions were assessed (A); swabs were taken from the interdigital space of the foot (B) and the gingival cavity (C).

3.3.2.2 Sampling procedures unique to study 1

For study 1 only, an additional swab for culture was taken from each foot (Eurotubo® Collection swab with transport media, Deltalab, Rubi, Spain). Swabs were taken as above with the sterile wooden end of the cotton swab. Swabs for culture were transferred into transport media (BBL™ CultureSwab™ Plus, BD, New Jersey) and immediately streaked on HA plates and incubated (Chapter 2, section 2.2) to avoid sample deterioration (Catagay and Hickford, 2005).

Faecal samples were randomly collected from the pasture with sterile spatulas and transferred into sterile universal containers. Five samples were obtained from faecal parts that had become in contact with the environment and 5 samples were taken from the faecal sample core. All environmental samples were stored at -20 °C. On the day or processing samples were defrosted and weighed out (0.5 g of soil, 0.2g of grass and 0.1g of faeces).

3.3.2.3 Sampling procedures unique to study 2

For study 2 only (faecal samples were randomly collected from the field in study 1) a faecal sample weighing no more than 5g was collected by manual extraction with a gloved hand from the rectum of each animal and placed into a sterile universal container (procedure carried out under Home Office license – PPL 70/8392). If no faeces were

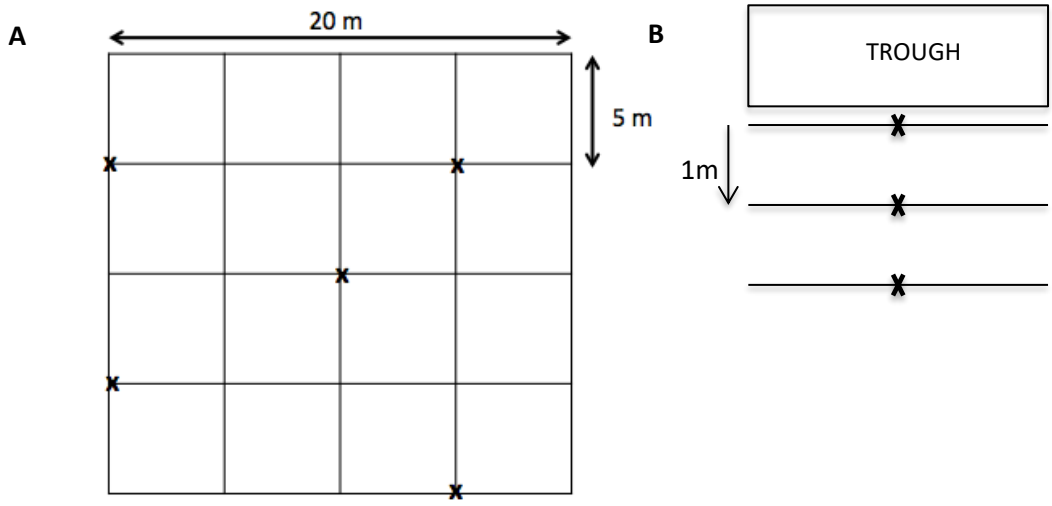
present in the rectum a swab sample was taken and placed into a cryotube containing 0.3 ml of PBS pH 7.4. If a sheep defecated prior to this procedure, efforts were made to collect the faecal sample before contact with the ground, removing the requirement for a rectal sample. Faecal samples from the pasture were not collected.

3.3.2.4 Sampling procedures for collection of soil and grass samples (Studies 1 and 2)

Soil and grass samples were collected from pre-designated sampling sites from the field were sheep were kept. Samples were taken from 2 high and 1 low-traffic area (HTA, LTA). Areas were identified by observation of sheep movement and farmer knowledge. LTA's were sampled using a quadrant approach with points on the quadrant for sampling chosen using a random number generator (http://www.randomizer.org/form.htm) each week One sample was taken from a chosen HTA and an additional 2 samples were collected 1m and 2m from the HTA respectively (Figures 3.4 and 3.5).

Soil samples at 0-1 and 4-5 cm depths were taken using a soil corer and transferred into sterile universal containers using sterile spatulas. When grass was present at a location, a sample was collected, and transferred to sterile universal containers. Fresh gloves were used between sample collections. Both soil corer and single use gloves were cleaned with DNA remover wipes between samples (PCR Clean™Wipes, Minerva Biolabs, Berlin, Germany). Two additional soil samples (0-1cm, 4-5 cm) were taken from the 3 traffic areas for the determination of soil moisture content.

Figure 3.4: High-traffic sampling areas for study 1 (**A**) and 2 (**B**). A gate and a tree were animals congregate were chosen as high traffic areas for study 1. For study 2, the area in front of a feeding trough and a water trough were chosen (image from www.google.co.uk/maps).



F**igure 3.5:** Sampling strategy for low traffic areas (LTA's) (**A**) and high traffic areas (HTA's) (**B**). **A**: A 20m^2 quadrant was chosen and soil and grass samples were taken from 5 random locations. Nodes on the quadrant were numbered 1-25 and sampling points (**x**) were chosen using a random number generator. **B**: Low traffic areas where sampled **(x)** in the centre of the low traffic areas and at 1m and 2m distance.

3.3.2.5 Collection of climate data (Studies 1 and 2)

In order to relate climate features to FR lesions and *D. nodosus* bacterial loads, local climate data (mean, minimum and maximum ambient temperatures [°C] and total rainfall [mm]) was collected by consulting climate data from the Warwick weather station (http://warwick-weather.com).

During every visit soil temperature was recorded in each area using a general-purpose thermometer (Fisher®Brand, Loughborough, UK).

3.3.3 Analysis of samples in the laboratory (Studies 1 and 2)

All samples from study 1 were analyzed in the laboratory. For study 2, all 640 soil and grass samples were analyzed. A selection of samples collected from sheep (feet, gingival cavity and faeces) was analyzed based on ID and SFR scores of sheep which were assessed after completion of the trial. The selection of the samples is therefore described in section 3.5.1 (Results).

3.3.3.1 DNA extractions

Samples were selected in random order and thawed before processing. Swabs were removed from cryotubes along with any liquid accumulated and placed in Eppendorf tubes containing 0.5 g micro-beads in preparation for DNA extraction. Samples from the pasture as well as faecal samples were weighted and 0.5g of soil, 0.2g of grass and 0.1g of faecal matter were also placed in Eppendorf tubes with micro-beads. DNA was extracted from all samples according to Purdy (2005) with the addition of a polyethylene glycol (PEG) precipitation step (Ogram *et al*., 1988) for soil grass and faecal samples, according the optimized protocols described in chapter 2, section 2.3. Every batch of DNA extraction included one blank as experimental control, using PBS pH 7.4 as a sample substitute.

3.3.3.2 Quantification of Dichelobacter nodosus using real-time PCR (Studies 1 and 2)

In order to assess detection and quantification of *D. nodosus* in all sample types, samples were submitted through the *D. nodosus rpoD* assay using the Applied Biosystems 7500 Fast Real-time PCR System (Calvo-Bado *et al*., 2011, Chapter 2, Section 2.9), for quantification. All samples were processed without technical replicates initially to determine detection of *D. nodosus.* Quantification of positive samples was subsequently carried out in triplicate. All protocols including cycling conditions and the preparation of standard curves are described in chapter 2 (Chapter 2, Section 2.9). Every PCR run included a non-template control (sterile water) and all DNA EB's were also tested.

3.3.3.3 Determination of soil moisture content (Study 2)

Two additional soil samples (0-1 cm and 4-5 cm) were collected per area for the analysis of soil moisture during each sampling visit. Soil moisture samples were collected from the pasture as described in section 3.4.2.4. Samples were weighed a maximum of 1 hour after collection and dried in the oven at 110 °C for 24 hours. Samples were re-weighed and soil moisture (%) was calculated using the following formula:

Equation 3.1: Calculation of soil moisture

$$MC\% = \frac{W_2 - W_3}{W_3 - W_1} \times 100$$

Where:

MC% = Moisture content (%)

W_1 = Weight of soil container

W_2 = Weight of moist soil + container (g)

W_3 = Weight of dried soil + container (g)

3.3.4 Data analysis

All statistical analysis was conducted using R package 'stats' version (R Core Team, 2012).

3.3.4.1 Differences in Dichelobacter nodosus load and detection frequencies over time

The distribution of the data was tested using Kernel Density plots and the Shapiro-Wilks test. Overall differences in *D. nodosus* load between weeks and according to disease state was tested using 1-way ANOVA or Kruskal Wallis followed by either Tukeys honestly significant difference test (HSD) or Man-Whitney pairwise comparison test. Statistical differences in detection frequency were calculated using Chi-square test. Fishers exact test was used when observations had a frequency of <5.

3.3.4.2 Kaplan Meier survival curve (Study 1)

The dependent variable was the time-point at which *D. nodosus* ceases to be detectable in a sample. The data was left and right censored to create intervals, as the exact time point

at which a sample became negative was unknown. The survival curves give the probability (P) of survival up to a time point or in this case, time interval. Samples where no *D. nodosus* was detected in week 1 were excluded from the analysis. Samples that became negative at a certain time point were assumed to remain negative for the remaining study period. The survival analysis package calculates the non-parametric maximum likelihood estimate for the distribution from the interval-censored data. The associated survival distribution generalizes the Kaplan-Meier estimate to interval-censored data. Disease status was not included in the survival analysis due to the low sample number of sheep with ID and SFR lesions in week 1.

3.3.4.3 Correlations and associations between variables (Studies 1 and 2)

In order to identify pairs of variables that contain the same information, correlations between all continuous variables were evaluated using Spearman's rank correlation coefficient. Dependence of categorical variables were calculated using chi-square test and correlations between categorical and continuous variables were tested using either as Man-Whitney U test or a Kruskal-Wallis followed by a Man-Whitney pairwise comparison test when variables had more than two levels in the factor.

3.3.4.4 Binomial mixed effects regression model (Studies 1 and 2)

A Binomial mixed effects logistic regression model was used to estimate univariable and multivariable associations with the dichotomous (binomial 1:0) outcome variable (Y); in this case whether *D. nodosus* was present or absent on feet in the gingival cavity or in soil. A logit transform of the probability of the outcome was performed and modeled as a linear function of a set of predictor variables (X) (Dohoo *et al*., 2003). Predictor variables were classed as fixed effects whereas "sheep" and "foot" were classed as random effects (r). Incorporation of these random effects accounts for clustering of feet within sheep and therefore acknowledges the lack of independence between the two variables. Variables with less than two data points were excluded from the models. Variables were added into the model using stepwise forward selection (Dohoo *et al*., 2003). Only variables associated with the outcome at a P-value of < 0.2 were added to the model.

All results were classed as significant at $p < 0.05$. Strength of associations between the outcome and the predictor variables were evaluated by calculating the odds ratios (OR) from the coefficient returned by the model. The outcome variables were the probability of *D. nodosus* being present on feet (study 1 and 2), in the oral cavity (study 1) and in soil

samples (study 2). Predictor variables were lagged by one week (study 1) and one and two weeks (study 2). The model used in study 1 is shown in Equation 3.2.

Equation 3.2: Study 1: Binomial mixed effects logistic regression model with outcome variable predictor variable and incorporated random effects

$$Y\sim + x1 + x2 + (1|r1/r2)$$

Where:

Y = Response variable (dependent, outcome)

X = Explanatory variable/covariates

r = Random effects

For study 2 only, the time variable "week" was standardized, in order to take into account that it may not have a linear relationship with the outcome. The standardized variable was then squared, cubed and calculated to the 4th power and included in the model (Equation 3.3). Equation 3.3 shows the model structure.

Equation 3.3: Study 2: Binomial mixed effects logistic regression model with outcome variable, predictor variable, standardized week variables and incorporated random effects

$$Y\sim w^1 + w^2 + w^3 + w^4 + x1 + x2 + (1|r1/r2)$$

Where:

Y= response variable (dependent, outcome)

X =explanatory variable/covariates

r = random effects

w^1=standardized week variable

w^2=standardized week variable squared

w^3=standardized week variable cubed

w^4=standardized week variable to the fourth power

Presence and load data for *F. necrophorum* was obtained for both studies originating from a concurrently run project (unpublished data) and included in the model build.

3.4 Study 1: Results

3.4.1 Disease status of the study group

The data for the study group and treatments administered over time is presented in Table 3.4. Two lambs were sent to slaughter before the end of the study; hence no data were obtained for week 7. Locomotion was not scored in this trial as the study focus was on associating FR lesions with bacterial load. In addition, some lameness in the trial was attributable to the presence of foot abscesses.

On 5 occasions feet had both ID and SFR, on 2 occasions feet had SFR only and 9 occasions ID only; 152 feet observations were healthy. One ewe and 1 lamb never became diseased. ID was present in the flock every week that sheep were observed.

An increase in FR occurred in week 5 (Figure 3.6, A), when 8 feet from 6 sheep presented with FR. More severe lesions were observed on lambs, especially in week 5 (Figure 3.6, C). Feet were treated with antibiotic spray on 22 occasions, including 3 occasions were the foot was sprayed due to foot abscesses when FR was not present. Fifty percent of occasions where a foot was sprayed occurred in week 5, when 10 feet were treated due to ID and SFR lesions and 1 foot was treated for a foot abscess.

Table 3.4: Disease status of ewes and lambs (n=10) and treatment given throughout the study period.

Ewe ID	Foot	Week 1	Week 3	Week 5	Week 7
Ewe 1544	LF	H*	H	H	FR1
	RF	H	H	H	H
	LR	H	H	ID3, FR1, S	H
	RR	ID2, lame, S*	H	H (ID1)	H (ID1)
Ewe 13	LF	H	H	H	H
	RF	H (ID1)	H	H (ID1), S	ID2
	LR	H	H (ID1), S, T*	H (ID1)	ID2
	RR	H (ID1)	H (ID1), S, T	H (ID1)	H (ID1)
Ewe 2667	LF	H	H	H (ID1)	H
	RF	H	H	H	H
	LR	H	H	H	H
	RR	ID4, lame, S, I*	A***, S	H, A, S	H, A, S
Ewe 3647	LF	H	H (ID1)	H	H
	RF	H (ID1)	H (ID1)	ID2, S	H (ID1)
	LR	H	H	H	H
	RR	H	H	H	H (ID1)
Ewe 5582	LF	H (ID1), Healing**	Healing, T	****	H
	RF	H	H	H	H
	LR	H	H	H (ID1), S	H
	RR	H	H	H	H
Lamb ID	**Foot**	**Week 1**	**Week 3**	**Week 5**	**Week 7**
Lamb 1	LF	H (ID1)	H (ID1)	H (ID1)	No data
	RF	H (ID1)	H (ID1)	H (ID1)	No data
	LR	H	H (ID1)	ID3, S	No data
	RR	ID4, FR3, lame, S	H (ID1), S	H (ID1)	No data
Lamb 2	LF	H	H	H	H
	RF	H	H	H	H
	LR	H	H	H	H (ID1)
	RR	H	H	H	H (ID1)
Lamb 3	LF	H	H	ID3, FR1, S	No data
	RF	ID1, A, lame, S	H	Healing FR1, T	No data
	LR	H	H	H	No data
	RR	H	H	H	No data
Lamb 4	LF	H (ID1)	H (ID1)	H	H (ID1)
	RF	H	H (ID1)	H	H (ID1)
	LR	H (ID1)	ID2, A, S, T,	ID4, FR3, S	ID1, FR3
	RR	H	H (ID1)	H	H (ID1)
Lamb 5	LF	H	H (ID1)	ID2, S	H (ID1)
	RF	H	H (ID1)	H (ID1), S	H (ID1)
	LR	H	H (ID1)	ID2, S	H (ID1)
	RR	H	H (ID1)	H (ID1)	H (ID1)

***H**=Healthy, **S**=Antibiotic spray administered, **I**=antibiotic injection given, **T**= foot was trimmed, ** Healing FR lesion, **A**=Abscess present, ***Granulomatous infected ID tissue and discharging abscess on the lateral claw, ****Medial claw swollen and clumpy, disease scores for ID and SFR are shown as ID1-4 and SFR1-4 respectively. Feet that scored ID 1 were classed as healthy, but score is shown in the table in parentheses.

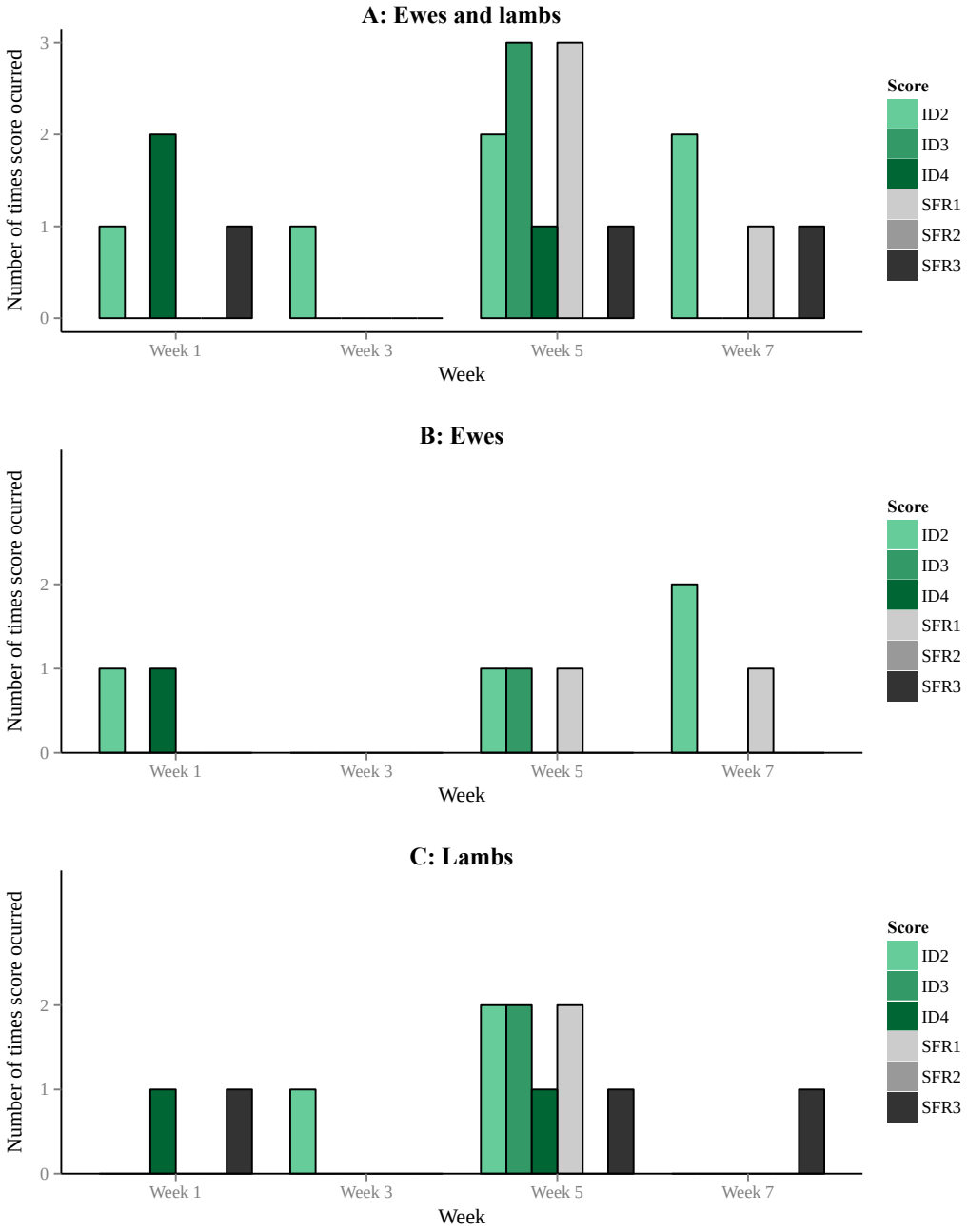


Figure 3.6: Frequency of interdigital dermatitis (ID) and severe footrot (SFR) by severity score by week. Data are the frequency of each severity score (ID 2-4, SFR 1-4) by feet/week **(n=40, week 7: n=32) (A)**, ewes only **(n=20) (B)**, and lambs only **(n=20, week 7: n=12) (C)**.

3.4.2 Climate during study 1

Climate data are presented in Figure 3.7. Average ambient temperatures ranged from 12.2 °C on the first day of sampling (May 28th) to 17.6 °C on the last day (July 9th). Total rainfall during the 7 days prior to the fist sampling was 48.0 mm; 4.6 mm of rain fell on the first day of sampling (week 1). The first visit was characterized by wet and muddy conditions, whereas the ground was dry and temperature had increased at all subsequent visits.

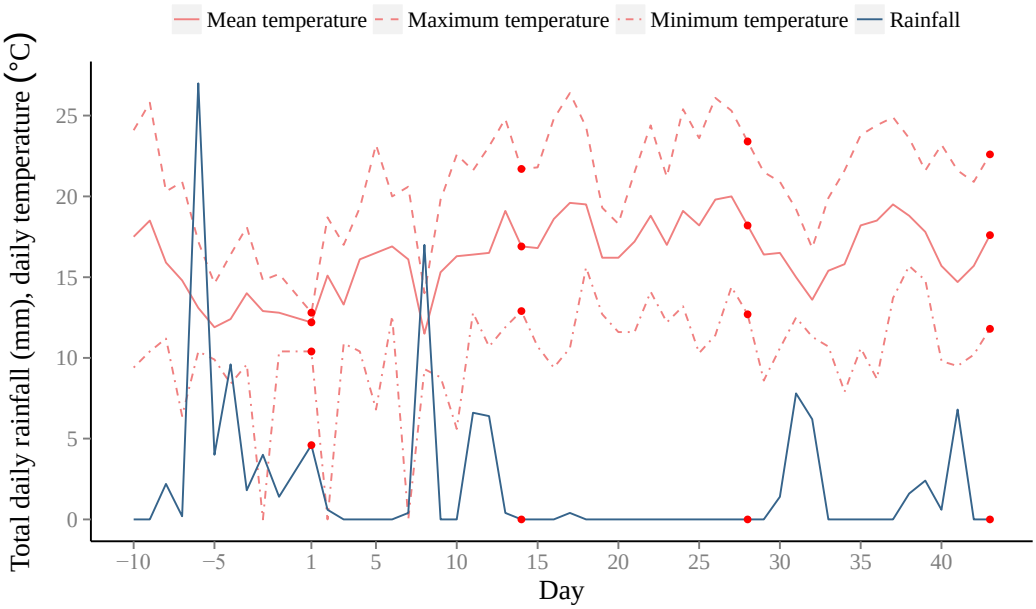


Figure 3.7: Daily temperature (Mean (−), Minimum (•-•-•), Maximum (- - -) temperature [°C]) and total daily rainfall (mm) from 10 days prior to the start of the study (-10) to the last day of the study (day 43). Red data points (•) indicate the days' sheep were sampled: Day 1 (week 1), day 14 (week 3), day 28 (week 5), day 43 (week 7).

3.4.3 Detection and quantification of Dichelobacter nodosus in all sample types

D. nodosus was detected by qPCR in 36/88 (41.9%) of samples. It was detected in all sample types but not all samples of each type (Table 3.5). For the first time *D. nodosus* was detected on grass (10/24). *D. nodosus* was also detected in 16/44 soil samples taken at 0-1 cm depth and in 20/44 soil samples taken at 4-5cm depths as well as high (17/44) and low (19/44) traffic areas. It was detected on the surface and in the core of faecal samples and on feet with all disease states including healthy. The number of positive foot swabs ($P<0.01$) and grass swabs ($P<0.05$) was greater than expected by chance.

The minimum, maximum and average *D. nodosus rpoD* genome copies detected is presented in Appendix 1. *D. nodosus* frequencies and loads varied by week in all sample types. Results are summarized in Table 3.5 and Figures 3.8-3.9. The only site where *D. nodosus* was detected consistently and in high loads was the feet of sheep. Overall, *D. nodosus* was most frequently detected in week 1 and detection frequencies declined throughout the study (Table 3.5), even though disease prevalence increased in week 5.

D. nodosus loads were higher on diseased feet than on healthy feet and higher in the gingival cavity when sheep had ID, but not SFR (Appendix 2). No statistical analysis for load differences was conducted due to the small number feet that had ID and SFR.

Table 3.5: Mean $\log_{10}$ *D. nodosus rpoD* genome copies detected throughout the study period per swab/sample and number of samples where *D. nodosus* was detected/week.

		Week 1		**Week 3**		**Week 5**		**Week 7**	
	Total number/% *D. nodosus* positive	Median load	Number/ % positive	Median Load	Number/ % positive	Median load	Number/ % positive	Median load	Number/ % positive
Feet (n=152, 40/week*)	97/63.8								
All feet		3.71^{a}	$40/100^{a}$	3.39^{ac}	$26/65^{b}$	0.00^{bc}	$18/45^{b}$	0.00^{c}	$15/47^{b}$
Positive feet		3.71		4.14		4.35		3.15	
Mouth (n=38, 10/week*)	13/32.5								
All mouth		0.00	4/40	0.00	1/10	0.00	3/30	0.00	2/25
Positive mouth samples		3.82		4.89		2.66		2.78	
Faeces (n=40, 10/week)	10/25								
All faeces samples		0.00^{a}	$4/40^{ab}$	1.26^{ab}	$5/50^{a}$	0.00^{ac}	$3/30^{ab}$	ND^{c}	$0/0^{b}$
Positive faecal samples		2.61		3.26		3.99		ND	
Soil (n=88, 22/week)	36/41.9								
All soil samples		3.25^{a}	$19/86^{a}$	0.00^{b}	$9/41^{b}$	0.00^{b}	$5/23^{b}$	0.00^{b}	$3/14^{b}$
Positive soil samples		3.39		3.84		2.89		3.27	
Grass (n=24, 6/week)	10/41.7								
All grass samples		3.00^{a}	4/67	2.55^{a}	5/83	ND^{**b}	0/0	0.00^{ab}	1/17
Positive grass samples		3.22		3.66		ND		2.39	

*n=32 and n=8 for foot and mouth samples respectively in week 7 due to two missing sheep, ****ND**=not detected. Superscripts that are different from each other indicate a significant statistical difference. No significant differences found in rows without superscripts. Bacterial loads are presented as $\log_{10}$ *D. nodosus rpoD* genome copies per swab or sample.

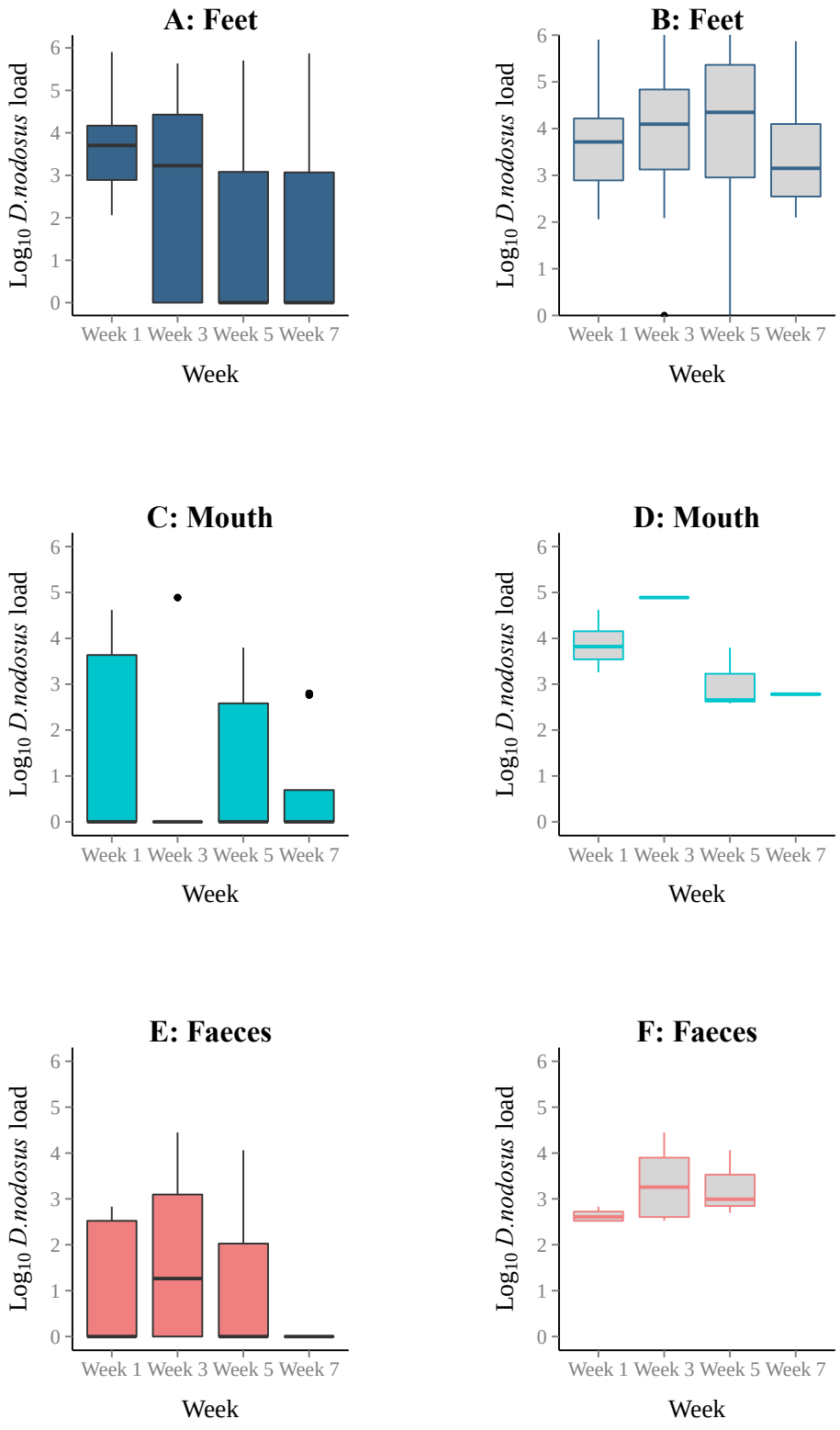


Figure 3.8: Log_{10} *D. nodosus* load on clinical swabs from feet, from the oral cavity and in faecal samples, collected fortnightly over the study period (Weeks 1-7). **A, C, E**: *D. nodosus* load on foot swabs **(A) (n=152)**, oral cavity swabs (**C) (n=38)**, and faeces **(n=40)**, including negative samples **B, D, F**: *D. nodosus* load including only positive samples on foot swabs **(B) (n=97**) in the oral cavity **(D) (n=10)** and in faeces **(F) (n=10).** Bacterial loads are presented per swab/sample.

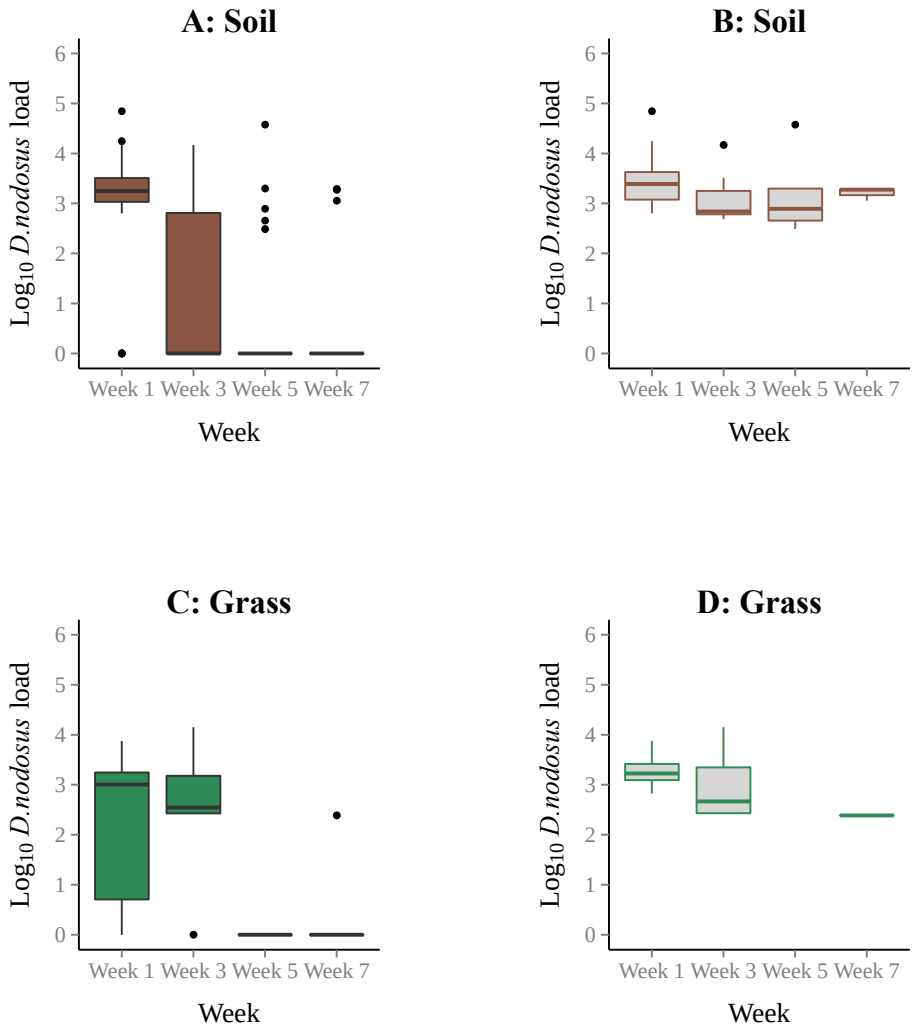


Figure 3.9: Log_{10} *D. nodosus* load in soil and grass at two-week intervals over the study period (Weeks 1-7). **A, C**: Log_{10} *D. nodosus* load including negative samples in soil **(A) (n=88)** and grass **(C) (n=25)**. **B, D**: *D. nodosus* load including only positive samples in soil **(B) (n=36)** and grass **(D) (n=10)**. Bacterial loads are presented per [wet wt] sample.

D. nodosus load in faecal core samples was significantly higher than the load in the outer layer that had become in contact with the pasture (P<0.05) (Figure 3.10). There was no significant difference between soil loads and detection frequencies in HTA's and LTA's, as well as in surface and core samples (Appendix 3).

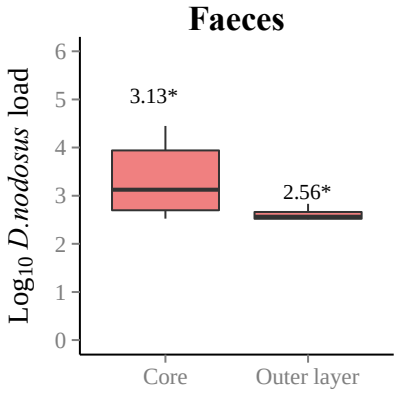


Figure 3.10: Differences of Log_{10} bacterial load in *D. nodosus* positive faecal samples collected from the core **(n=8)** and the outer layer of the material **(n=4).** Bacterial loads are presented per faecal [wet wt] sample. *Median shown on top of each box.

Although ewes had less severe lesions, *D. nodosus* load on feet overall was higher in ewes than in lambs (P<0.05) when all samples were taken into account. Loads on ewes were higher in week 1 compared to any other week, disregarding the number of samples included in the analysis. Ewes also presented with a larger range of loads (Figure 3.11, A, B, statistical differences: Appendix 4).

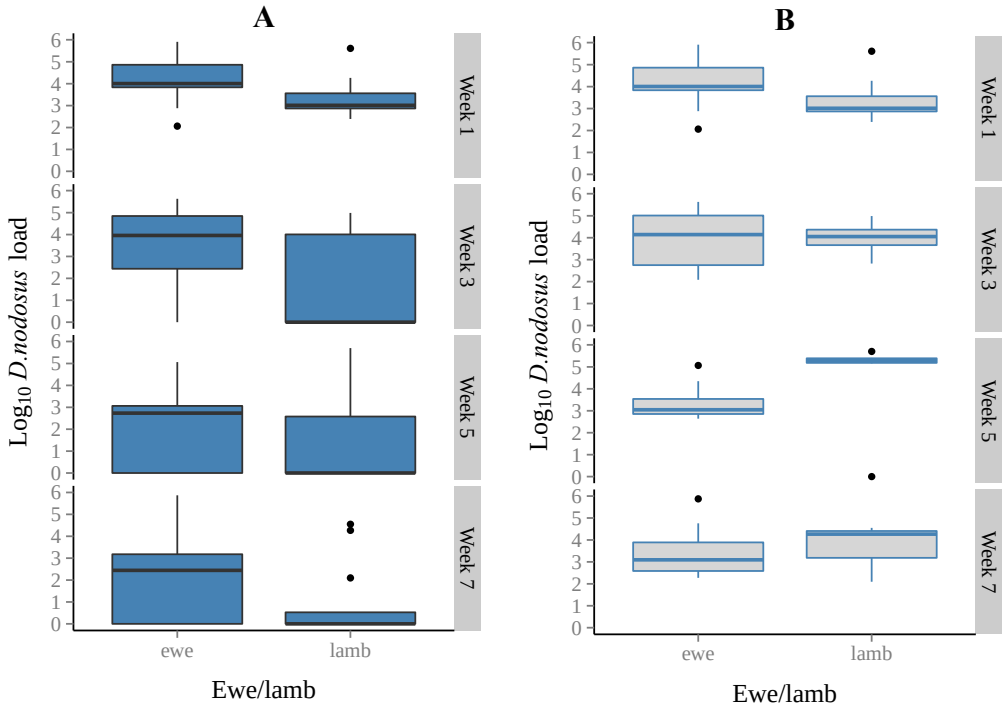


Figure 3.11: Differences of Log_{10} *rpoD* genome copies/swab of *D. nodosus* positive samples collected from the feet of ewes (n=20) and lambs (n=20) by week. **A**: Data includes negative samples **(n=20)**. **B**: Data includes positive samples only: **Week 1: Ewes n=20, lambs n=20. Week 3: ewes n=17, lambs n=10. Week 5: Ewes n=12, lambs n=6. Week 7: Ewes n=12, lambs n=3**.

3.4.4 Kaplan-Meier survival curve

The Kaplan-Meier survival curve (Figure 3.12, A) shows that the probability of *D. nodosus* survival was highest on the feet of sheep. There was no difference in survival probability between soil collected from HTA's and LTA's as well as 0-1 cm and 4-5 cm (Figure 3.12, B, C).

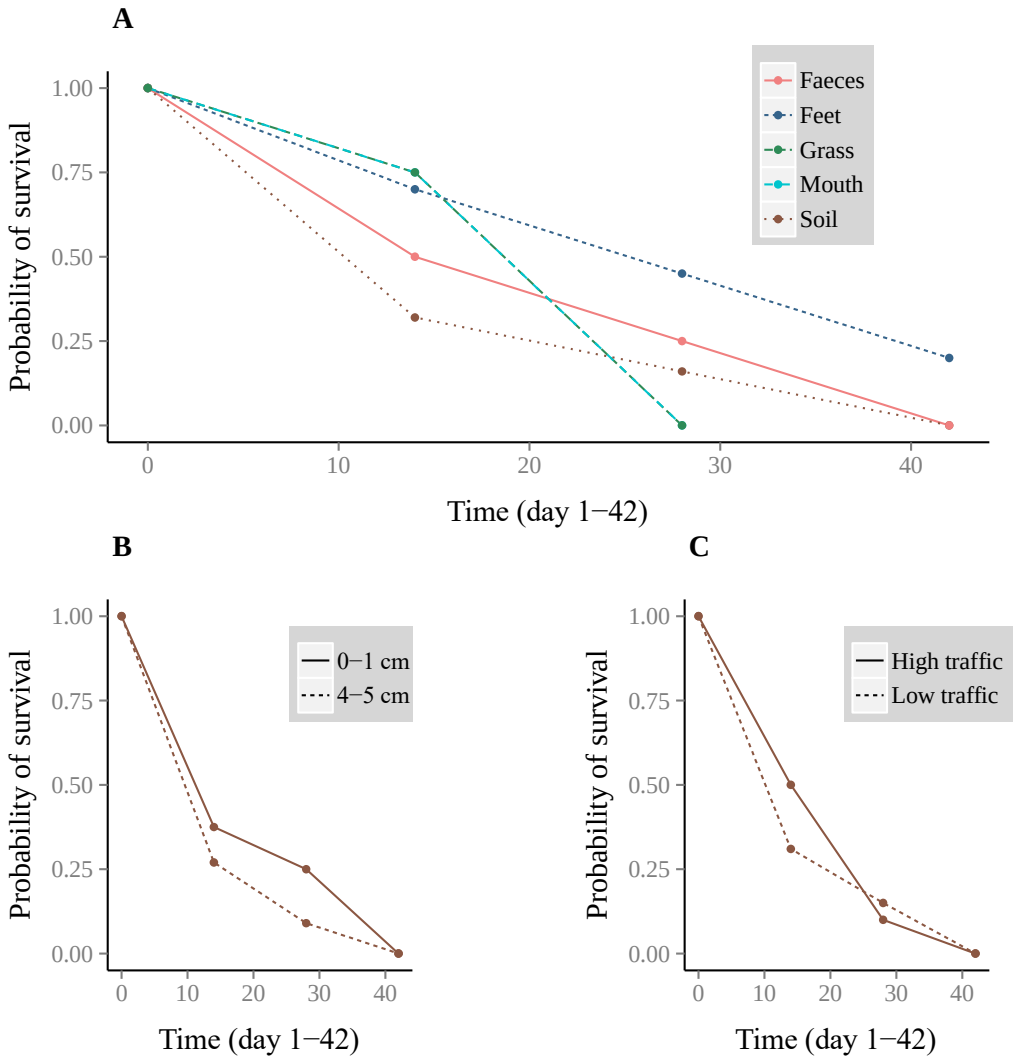


Figure 3.12: Kaplan-Meier survival curve of presence of *D. nodosus* by sample type; feet (n=40), gingival cavity, (n=4), grass (n=4), faeces (n=4), and soil (n=19) [**A**] including samples taken at 0-1 and 4-5 cm [**B**]) and samples from High traffic areas (HTA's) and low traffic areas (LTA's) [**C**]. Markers represent the day of sampling (Day 1= week 1, day 14= week 2, day 28= week 5, day 42= Week 7). The distances between markers represent time intervals (Day 0-14, 14-28, 28-42). Note data for grass and mouth are identical, hence lines are overlapping in the figure [**A**].

3.4.5 Binomial mixed effects logistic regression model

Tables 3.6-3.11 below show a summary all variables tested in the binomial model. No model for *D. nodosus* presence/absence in the environment was created due to the limited number of time points (weekly data). Variables that were excluded from univariable associations by the outcome are shown in Appendix 5.

Table 3.6: Univariable associations of continuous weekly variables by the outcome (*D. nodosus* is present on feet [Foot model] or *D. nodosus* is present in the mouth [Mouth model]).

Weekly continuous variables	P-value (Foot model)	n (Foot model)	P-value (Mouth model)	n (Mouth model)	Median	1st Qu	3rd Qu	Min.	Max.
Number of *D. nodosus* positive feet t^{-1}	F**	3	0.35	3	26.00	18.00	40.00	18.00	40.00
Total number of diseased feet t^{-1}	0.56	3	0.97	3	3.00	1.00	8.00	1.00	8.00
Number of *D. nodosus* positive mouth samples t^{-1}	**0.05**	3	0.32	3	3.00	1.00	4.00	1.00	4.00
***D. nodosus* load in soil t^{-1}**	**0.01**	3	***NA	NA	4.92	4.68	5.45	4.68	5.45
Number of *D. nodosus* positive soil samples t^{-1}	F	3	0.57	3	9.00	5.00	19.00	5.00	19.00
***D. nodosus* load on grass t^{-1}**	0.21	3	NA	3	4.36	0.00	4.54	0.00	4.54
***D. nodosus* load in faeces t^{-1}**	**0.02**	3	NA	3	4.42	3.56	4.89	3.56	4.89
Number of *D. nodosus* positive faecal samples t^{-1}	0.91	3	0.75	3	4.00	3.00	5.00	3.00	5.00
***F. necrophorum* load on feet t^{-1}**	**0.01**	3	NA	3	53.83	49.27	152.83	49.27	152.83
Total rain (mm)*, 7 days prior sampling	**<0.01**	4	0.45	4	30.80	0.00	48.00	0.00	48.00
Total rain (mm), 14 days prior sampling	F	4	0.62	4	37.40	0.40	50.20	0.40	50.20
Total rain (mm), 28 days prior sampling	**<0.01**	4	0.89	4	79.60	37.80	90.80	27.20	90.80
Mean temperature* (°C), 7 days prior sampling	**<0.01**	4	0.24	4	15.98	13.13	18.59	13.13	18.59
Mean temperature (°C), 14 days prior sampling	**<0.01**	4	0.30	4	15.59	14.67	18.14	14.67	21.71
Mean temperature (°C), 28 days prior sampling	**<0.01**	4	0.42	4	15.10	13.20	17.00	13.20	17.40
Mean min. temperature (°C), 7 days prior sampling	**<0.01**	4	0.95	4	8.44	7.87	12.46	7.87	12.45

Table continued below

Weekly continuous variables (continued)	P-value (Foot model)	n (Foot model)	P-value (Mouth model)	n (Mouth model)	Median	1st Qu	3rd Qu	Min.	Max.
Mean min. temperature (°C), 14 days prior sampling	**<0.01**	4	0.86	4	10.05	8.26	12.19	8.26	12.46
Mean min. temperature (°C), 28 days prior sampling	**<0.01**	4	0.96	4	8.32	7.81	10.40	7.81	11.66
Mean max. temperature (°C), 7 days prior sampling	**<0.01**	4	0.53	4	20.72	16.74	23.93	16.74	23.93
Mean max. temperature (°C), 14 days prior sampling	**<0.01**	4	0.73	4	19.71	19.48	23.15	19.48	23.15
Mean max. temperature (°C), 28 days prior sampling	**<0.01**	4	0.67	4	19.64	18.00	21.21	18.00	22.46

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}). Exceptions: For climate variables (rainfall and temperature) the average value of 7,14 and 28 days before sampling where taken (*rolling mean). N represents the number of unique values in each row of the dataset. **F=model failed. ***NA=not analyzed: Variables were not suitable to be used in either the mouth or foot model.

Table 3.7: Univariable associations of categorical weekly variables by the outcome (*D. nodosus* is present on feet [Foot model] or *D. nodosus* is present in the mouth [Mouth model]).

Weekly categorical variables	P-value (Foot model)	n (Foot model)	P-value (Mouth model)	n (Mouth model)	Number positive	% Positive
Week of study (categorical 1, 3, 5, 7)	**<0.01**	3	0.68	3	-	-
***D. nodosus* present on grass t^{-1} (1=present)**	**0.18**	3	0.79	3	10	41.67
***D. nodosus* present in faeces t^{-1} (1=present)**	**0.21**	3	0.83	3	10	25

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}). N represents the number of unique values in each row of the dataset.

Table 3.8: Univariable associations of continuous variables (by sheep) by the outcome (*D. nodosus* is present on feet [Foot model] or *D. nodosus* is present in the mouth [Mouth model]).

Variables by sheep (continuous) (n=38)	P-value (Foot model)	n (Foot model)	P-value (Mouth model)	n (Mouth model)	Median	1st Qu	3rd Qu	Min.	Max.
***F. necrophorum* load in the mouth t^{-1}**	0.52	28	0.71	28	3.47	2.35	4.43	0.00	6.03
***D. nodosus* load in the mouth t^{-1}**	0.21	28	1	28	0.00	0.00	0.00	0.00	4.89

Variables are lagged by one time-point (t^{-1}). N represents the number of unique values in each row of the dataset.

Table 3.9: Univariable associations of categorical variables (by sheep) by the outcome (*D. nodosus* is present on feet [Foot model] or *D. nodosus* is present in the mouth [Mouth model]).

Variables by sheep (categorical) (n=38)	P-value (Foot model)	n (Foot model)	P-value (Mouth model)	n (Mouth model)	Number positive	%Positive
***D.nodosus* present in the mouth t^{-1} (1=present)**	**0.15**	28	*NA	28	10	26.32
***F. necrophorum* present in the mouth t^{-1} (1=present)**	0.34	28	NA	NA	30	79.95
Sheep had FR t^{-1} (1=diseased)	0.58	28	0.43	28	13	34.21
Sheep had FR (Day of sampling) (1=diseased)	0.28	38	**<0.001**	28	13	34.21
Lamb or ewe (age) (1=ewe)	**0.03**	28	1.00	28	Lamb: 18	-
Body condition score t^{-1}	0.28	28	0.31	28	-	-

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}). Exceptions: Disease state of the sheep/foot and whether the animal was a lamb or an ewe (0=healthy, 1= diseased) is also presented non-lagged. N represents the number of unique values in each row of the dataset. *NA=not analyzed: Variables were not suitable to be used in either the mouth or foot model.

Table 3.10: Univariable associations of continuous variables (by foot) by the outcome (*D. nodosus* is present on feet [Foot model] or *D. nodosus* is present in the mouth [Mouth model]).

Variables by foot continuous (n=152)	P-value (Foot model)	n (Foot model)	P-value (Mouth model)	n (Mouth model)	Median	1st Qu	3rd Qu	Min.	Max.
***D. nodosus* load on feet t^{-1}**	**0.09**	112	*NA	NA	3.30	0.00	4.35	0.00	7.24
***F. necrophorum* load on feet t^{-1}**	**0.10**	112	NA	NA	2.52	0.00	3.61	0.00	7.92

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}). N represents the number of unique values in each row of the dataset. *NA=not analyzed: Variables were not suitable to be used in either the mouth or foot model.

Table 3.11: Univariable associations of categorical variables (by foot) by the outcome (*D. nodosus* is present on feet [Foot model] or *D. nodosus* is present in the mouth [Mouth model]).

Variables by foot categorical (n=152)	P-value (Foot model)	n (Foot model)	P-value (Mouth model)	n (Mouth model)	Number positive	% Positive
***F. necrophorum* present on feet t^{-1}**	**0.10**	112	NA	NA	76	50.00
Foot had FR lesions t^{-1} (1=diseased)	**0.13**	112	NA	NA	13	8.55
Foot had FR lesions (Day of sampling) (1=diseased)	0.83	152	NA	NA	13	8.55
Antibiotic spray given t^{-1} (1=yes)	**0.01**	112	0.82	28	24	15.79
Foot trimmed t^{-1} (1=yes)	0.47	112	0.56	28	4	2.62

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}). Exceptions: Disease state of the sheep/foot (0=healthy, 1= diseased) and whether the animal was a lamb or a ewe is also presented non-lagged. N represents the number of unique values in each row of the dataset. NA=not analyzed: Variables were not suitable to be used in either the mouth or foot model.

D. nodosus was 1.4 (95% CI: 1.05, 1.89) more likely to be present on the foot if the bacterial load increased by 1 log the previous week, less likely to be present if a foot had been treated with antibiotic spray the previous week (OR 0.02, 95% CI:0.02, 0.37) and more likely to be present on feet of ewes than lambs (Table 3.12). There was no effect of time in the final model and the model was not improved by including the variable "week".

The only variable associated with *D. nodosus* detected in mouths was whether the sheep had FR or not (binomial 1-0 [FR-Healthy) (Table 3.13). *D. nodosus* is 24 times (95% CI: 4.30, 221.23) more likely to be present in the gingival cavity of sheep that had ID or SFR. The disease status and *D. nodosus* load of the sheep the previous week was not significant (p=0.43) (Table 3.9).

Table 3.12: Final model (Model 1), factors associated with *D. nodosus* presence on feet (binomial 1-0).

Predictor variables	n	% positive	Coef	s.e.	P	OR	CI (2.5%)	CI (97.5)
***D. nodosus* load on feet (t^{-1})**	152	63.80	0.35	0.14	<0.05	1.42	1.05	1.89
Foot treated w. antibiotic spray t^{-1} (1=yes)	152	15.79	-2.21	0.66	<0.001	0.12	0.02	0.38
Ewe versus lamb (1=ewe)	5	-	-1.39	0.59	< 0.05	0.25	0.04	0.82

Coef=Coefficient, s.e. = Standard error, OR = Odds ratio, CI= Confidence interval.

Table 3.13: Final model (Model 2), factors associated with *D. nodosus* presence in the gingival cavity (binomial 1-0)

Predictor variables	n	% positive	Coef	s.e.	P	OR	CI (2.5%)	CI (97.5)
Sheep had FR (present week)	38	34.21	3.18	0.96	<0.01	24.0	4.30	221.23

Coef=Coefficient, s.e. = Standard error, OR = Odds ratio, CI= Confidence interval.

3.4.6 Correlations and of predictor variables

Complete correlation tables are listed in Appendix 6. Correlations of the final model variables with other variables considered for the model are shown in Tables 3.14-3.16. There was a high correlation between the load of *D. nodosus* on feet and *D. nodosus* load in the environment (Table 3.14). *D. nodosus* load was positively correlated with presence of *F. necrophorum* on feet (Table 3.15). Loads where higher in ewes than in lambs and lower when antibiotic spray was administered (Table 3.15) which corresponds with the results of the model.

Table 3.14: Correlation of continuous predictor variables with final continuous model variables.

Continuous predictor variables	***D. nodosus* load on feet**
Weekly variables	
Total number of *D. nodosus* positive feet	**0.38**
Total number of *D. nodosus* positive mouth samples	**0.18**
Total number of positive grass samples	**0.29**
Total number of *D. nodosus* positive soil samples	**0.38**
D. nodosus load in soil	**0.32**
D. nodosus load in grass	**0.26**
D. nodosus load in faeces	**0.17**
Number of *D. nodosus* positive faecal samples	**0.27**
Total rain (mm) 7 days*	-0.15
Mean temperature (°C), 7 days*	**0.39**
Variables by sheep	
D. nodosus load in the mouth	0.04
F. necrophorum load (mouth)	0.03
Variables by foot	
F. necrophorum load	0.15

R^2 values are shown: **Bold** R^2 values are statistically significant. *Rolling mean. Maximum and minimum temperatures, as well as temperatures after 14 days prior sampling and 28 days prior are not presented as they are highly correlated with other temperature variables (Appendix 6).

Table 3.15: Correlation of categorical predictor variables with final continuous model variables.

Categorical predictor variables	***D. nodosus* load on feet**
Weekly variables	
Week (categorical 1,3,5,7)	**<0.01 (**-)
D. nodosus present on grass (1= present)	0.06
D.nodosus present in faeces (1= present)	**<0.01** (+)
Variables by sheep	
Sheep had FR (1=diseased)	0.85
D.nodosus present in mouth (1= present)	0.78
F. necrophorum present in the mouth	0.86
Age (1=ewe, 0=lamb)	**0.01** (+)
Variables by foot	
Foot had FR lesions (1=diseased)	0.05 (+)
D.nodosus present on feet (1= present)	**<0.01** (+)
F. necrophorum present on feet (1=present)	**0.02** (+)
Age (1=ewe, 0=lamb)	**0.01** (+)
Antibiotic spray given (1=yes)	**<0.01** (+)

Significant P-values are shown in **bold**. Maximum and minimum temperatures, as well as temperatures after 14 days prior sampling and 28 days prior are not presented as they are highly correlated with other temperature variables (Appendix 6). ^The direction of the effect in indicated in parentheses after the significant p-value: The plus symbol (+) indicates that the median is significantly higher in binomial 1 variable, the minus symbol (-) indicates that the median is significantly higher in binomial 0 variable.

Table 3.16: Associations of continuous and categorical predictor sheep variables with final categorical model variables.

Continuous predictor variables	**AB* spray(1=yes)**	**Age (1=ewe)**	**Sheep had FR(1=diseased)**
Weekly variables			
Number of *D. nodosus* positive feet	0.63	0.63	0.69
Number of *D. nodosus* positive mouth samples	0.44	0.76	**<0.01**(+)
Variables by sheep			
D. nodosus load in the mouth	0.71	0.91	**0.03** (+)
F. necrophorum load in the mouth	0.88	**<0.01**(+)	**0.03** (+)
Variables by foot			
F. necrophorum load on feet	0.60	**0.01** (-)	0.10
Categorical predictor variables			
Antibiotic spray given	1.00		
Lamb or ewe	0.87	1.00	
Overall disease state (sheep)	0.14	0.33	1.00
Variables by sheep			
D.nodosus present in mouth (1= present)	0.93	0.84	**<0.01**
Variables by foot			
Foot had FR lesions (1=diseased)	**<0.01**	0.05	**<0.01**
D.nodosus present on feet (1= present)	0.23	0.01	0.30
F. necrophorum present on feet (1= present)	0.66	0.73	0.14

Significant P=values are shown in **bold**. ***AB**=antibiotic spray. Maximum and minimum temperatures, as well as temperatures after 14 days prior sampling and 28 days prior are not presented as they are highly correlated with other temperature variables (Appendix 6) ^The direction of the effect in indicated in parentheses after the significant p-value: The plus symbol (+) indicates that the median is significantly higher in binomial 1 variable, the minus symbol (-) indicates that the median is significantly higher in binomial 0 variable.

3.5 Study 2 results

3.5.1 Disease status of the study group and animal selection for analysis

Of the 80 sheep examined, 12 had SFR, 9 had ID and 2 sheep had both disease presentations. Thirty out of 40 sheep from the study group had ID or SFR lesions during the study period and 10 sheep remained healthy. Sixteen sheep were excluded from laboratory analysis because they either presented with lesions at only one time point, they had "scattered" scores throughout the trial with no clear sign of disease progression, or presented lesions consecutively in the same sheep but on different feet. Ultimately, 7 sheep were selected for laboratory analysis. Two sheep that had no ID or SFR lesions were experimental controls and 5 diseased sheep with disease progression on the same foot for a period of at least 3 weeks (Figure 3.13), this included 2 sheep that presented with only SFR lesions. One sheep was treated with an antibiotic injection to treat FR in week 9.

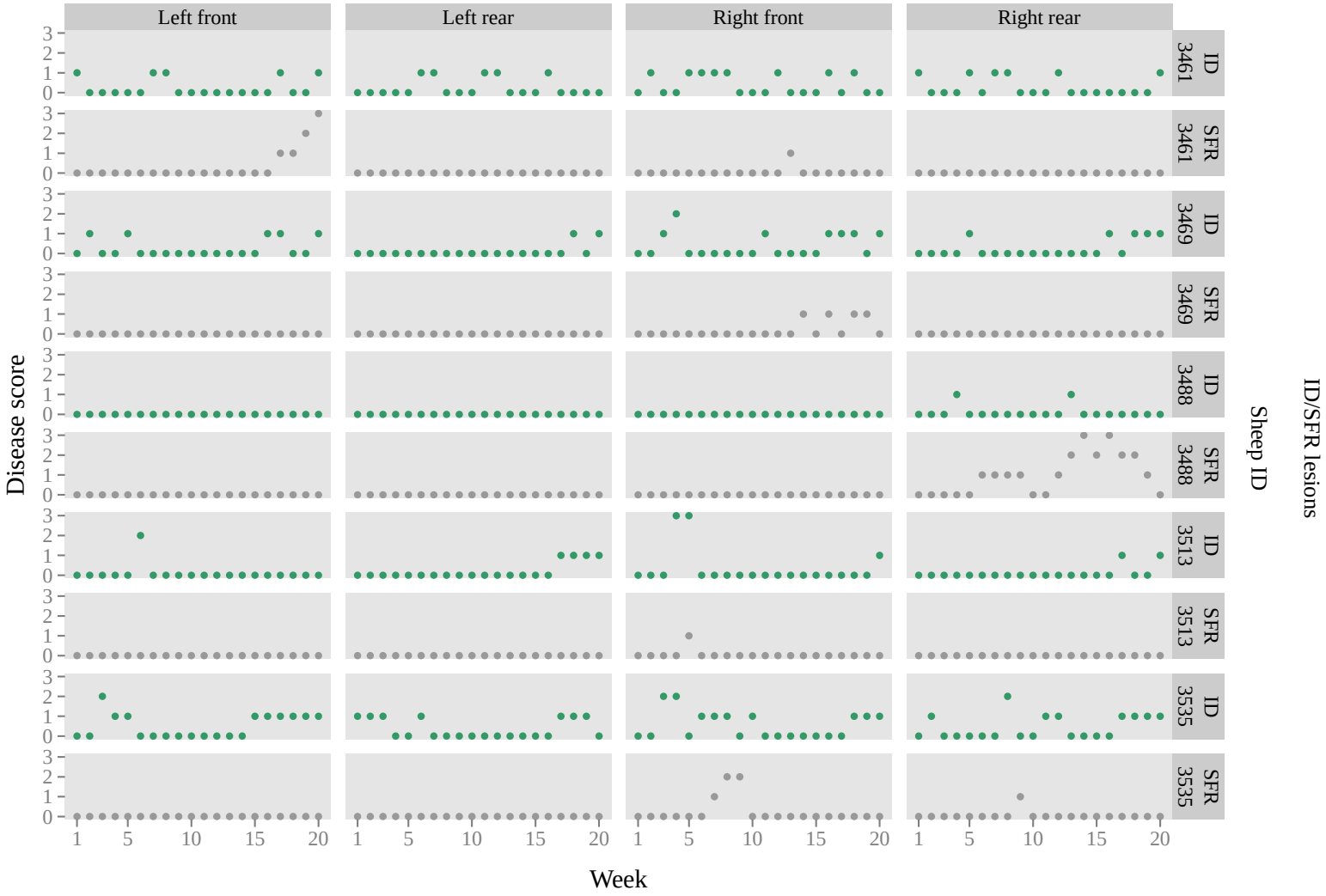


Figure 3.13: Severe footrot (SFR) and interdigital dermatitis (ID) scores of the 5 diseased sheep selected for laboratory analysis. SFR scores are presented in **grey** and ID scores in **green.**

For each sheep, 1 sample from each foot, 1 from the oral cavity and one faecal sample, were taken. Samples from control sheep were analysed every 4th week. Samples from the 5 diseased sheep were analysed each week from 2 weeks before occurrence of disease until 1 week without disease or trial end. A total of 232 foot swabs, 58 oral cavity swabs and 58 faecal samples were analysed (Table 3.17). An additional 342 samples were analysed from sheep in weeks 1-3 (228 foot swabs, 57 mouth swabs and 57 faecal samples), including the 7 sheep above and 14 randomly chosen animals (Table 3.18). All 640 samples from the pasture were analysed.

Table 3.17: Samples from 7 sheep analyzed, including swabs from feet and mouth and faecal samples (Weeks and number of samples analyzed).

Sheep ID	Type	Weeks	Number of samples
3464	Control	1,5,9,13,17	30
3246	Control	1,5,9,13,17	30
3535	Diseased	1-11	66
3488	Diseased	4-20	102
3461	Diseased	15-20	36
3469	Diseased	12-20	54
3513	Diseased	2-6	30
			348

Table 3.18: Additional samples analyzed, including swabs from feet and mouth and faecal samples (Weeks and number of samples analyzed).

Sheep ID	Type	Weeks	Number of samples
3464	Control	2,3	12
3246	Control	2,3	12
3535*	Diseased	0	0
3488	Diseased	1,2,3	18
3461	Diseased	1,2,3	18
3469	Diseased	1,2,3	18
3513	Diseased	1,3	12
3242	Random	1,2,3	18
3483	Random	1,2,3	18
3487	Random	1,2,3	18
3510	Random	1,2,3	18
3524	Random	1,2,3	18
3527	Random	1,2,3	18
3529	Random	1,2,3	18
3541	Random	1,2,3	18
4052	Random	1,2,3	18
3479	Random	1,2,3	18
3481	Random	1,2,3	18
3492	Random	1,2,3	18
3520	Random	1,2,3	18
3539	Random	1,2,3	18
			342

*Week 1-3 were analyzed in the first group (Table 3.17).

3.5.2 Climate during study 2

Local average ambient temperatures during study 2 ranged from 0.7-26.8 °C and increased throughout the study period (Figure 3.14, A). Approximately 217 mm of rain fell during the trial (starting from day -10 when the baseline pasture was sampled). Spring 2016 was uncharacteristically dry with a period of almost no rainfall between days 47-75 (weeks 7-11, covering the whole month of April) (Figure 3.14, A) with a total rainfall of 4.2 mm). This dry period was reflected by a decline in soil moisture during this period (Figure 3.14, B).

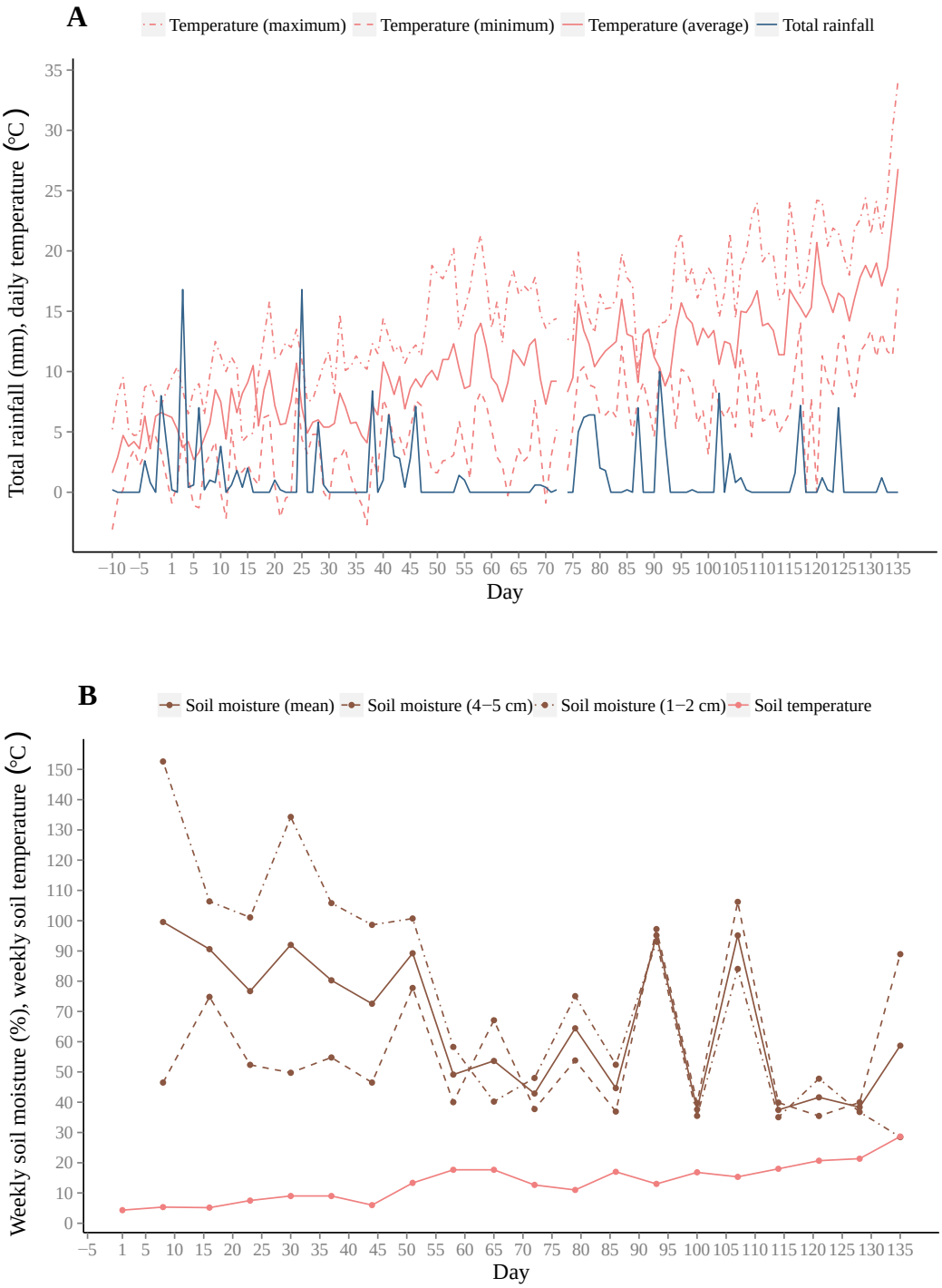


Figure 3.14: Daily temperature, daily rainfall and weekly soil moisture and soil temperature **A**: Daily temperature (Mean [−], Minimum [- - -] Maximum [•−•−•] temperature [°C]) and total daily rainfall (mm) **B**: Weekly soil temperature [−] (°C), soil moisture (mean [−], 0-1 cm [- - -], 4-5 cm [•−•−•]) from samples collected weekly.

3.5.3 Detection and quantification of Dichelobacter nodosus

The only site where *D. nodosus* was detected consistently every week was on the feet of sheep. Loads where higher on feet than at any other site (Table 3.19) which was also the case in study 1.

Table 3.19: Minimum, maximum and median *D. nodosus* $\log_{10}$ *rpoD* genome copies present swab/sample.

Sample type	Minimum	Maximum	Median load (All samples)	Median load (Positive samples)
Feet	1.65	6.48	0.67 (n=690)	3.65 (n=84)
Mouth	2.37	2.37	0.02 (n=115)	2.37 (n=1)
Soil	2.27	4.14	0.11 (n=462)	2.82 (n=19)
Grass	2.24	2.77	0.06 (n=178)	2.53 (n=4)
Faeces	2.27	2.51	0.08 (n=115)	2.39 (n=2)

3.5.3.1 Dichelobacter nodosus detection and quantification on feet, in the gingival cavity and in faeces

Detection of *D. nodosus* by sheep and sample type is shown in Table 3.20. *D. nodosus* was detected in 84/460 (18.3%) foot samples analyzed in the study. It was detected on the feet of control sheep on 5 occasions, but only in week 1. The largest proportion of positive samples originated from 2/5 diseased sheep (3488, 3535) (70.24% of all *D. nodosus* positive foot samples). One sheep that presented FR lesions (3513) had *D. nodosus* on feet on only one occasion, and this did not correspond with feet that were diseased. Two out of 5 sheep that had SFR, had *D. nodosus* on their feet on one (3469) and two (3461) occasions. Only 1 sample from the gingival cavity was positive for *D. nodosus.* The sample was collected from a diseased sheep (3488) in week 5 and the bacterial load was 2.32×10^2 *rpoD* genome copies/swab. *D. nodosus* was detected in 2 faecal samples, indicating for the first time that faecal shedding is possible. Bacterial loads ranged from 1.84×10^2 to 3.20×10^2 cell copies g^{-1}. One *D. nodosus* positive faecal sample originated from sheep 3488 (week 12) with the longest disease period and 1 from a control sheep in week 1 (ID 04326).

Table 3.20: Samples where *D. nodosus* was detected by sheep and sample type.

Sheep ID	Type	Number of samples	Feet (n=460)	Mouth (n=115)	Faeces (n=115)
3464	Control	42	4	0	0
3246	Control	42	1	0	1
3535	Diseased	66	20	0	0
3488	Diseased	120	39	1	1
3461	Diseased	54	2	0	0
3469	Diseased	72	1	0	0
3513	Diseased	42	1	0	0
3242	Random	18	1	0	0
3483	Random	18	0	0	0
3487	Random	18	0	0	0
3510	Random	18	1	0	0
3524	Random	18	1	0	0
3527	Random	18	1	0	0
3529	Random	18	1	0	0
3541	Random	18	0	0	0
4052	Random	18	2	0	0
3479	Random	18	1	0	0
3481	Random	18	4	0	0
3492	Random	18	0	0	0
3520	Random	18	3	0	0
3539	Random	18	**1**	0	0
	Total	**690**	**84**	**1**	**2**

3.5.3.2 Detection and quantification of Dichelobacter nodosus in soil and grass samples

D. nodosus was detected in 4.1% of soil samples (19/462). Fifty-eight percent (11/19) and 42% (8/19) of positive soil samples originated from samples taken from 0-1 cm depth and 4-5 cm depth respectively. Forty-seven per cent (9/19) of soil positives were taken from HTA's and 53% (10/19) from LTA's. There were no statistical differences in detection frequencies. Bacterial loads ranged from 3.74 $x10^2$ to 8.24 $x10^3$ cell copies g^{-1}.

D. nodosus was detected on 2.2% (4/178) grass samples collected from the pasture. It was only detected on the baseline pasture and not on grass throughout the trial when sheep were present. The bacterial load on grass samples ranged from 8.65 x 10^2 to 2.96 $x10^3$ cell copies g^{-1}.

Interestingly *D. nodosus* was present on only 1 grass sample collected from the pasture 10 days before start of the trial. Ten days later, before sheep from the study group had been turned onto the pasture, it was present in 4 soil samples at 4-5 cm depth and 3 grass samples (2 LTA's, 1 HTA), showing that the *D. nodosus* can be detected on unoccupied pasture for 10 days. Baseline pasture bacterial loads were low, ranging from 1.73 $x10^2$ to 7.90 $x10^2$ cell copies g^{-1}.

D. nodosus loads were higher on feet with ID and SFR than on healthy feet (Appendix 7), coinciding with results from study 1. It is unknown whether these differences are significant due to the small number of feet with ID and SFR lesions.

3.5.4 D. nodosus detection on lesion-free feet

D. nodosus was detected on the healthy feet of 2 sheep that became diseased during the study. Sheep 3488 (Figure 3.15) carried *D. nodosus* on healthy all 4 feet until the right rear (RR) foot became the diseased foot in week 6. *D. nodosus* was detected on the RR from week 3.

D. nodosus was detected on all feet of Sheep 3535 in week 1 (Figure 3.16). *D.nodosus* was detected on the RF in week 2 and in week 3 at which point it presents with ID and the episode of disease commences.

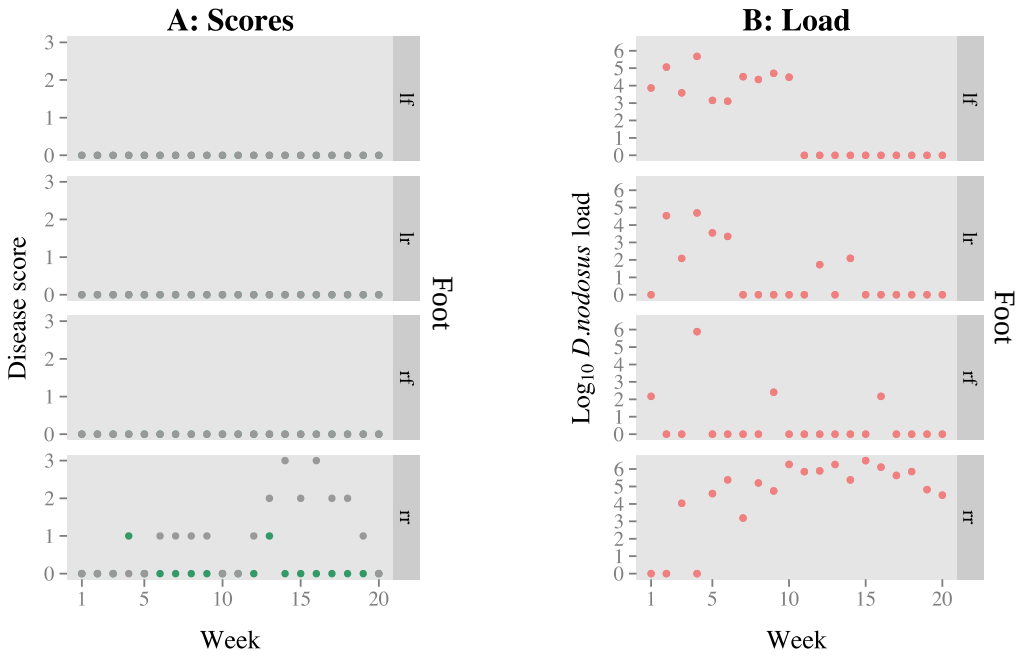


Figure 3.15: Lesion scores and bacterial loads of sheep 3488**. A**: Disease scores of the right rear foot (ID +SFR), no other foot presented lesions throughout the trial. SFR scores are presented in grey (•) and ID scores in green (•). **B**: Log_{10} *D. nodosus* bacterial load on 4 feet throughout study 2. **LF**=left front foot**, RF**= right front foot, **LR**=left rear foot, **RR**=right rear. foot.

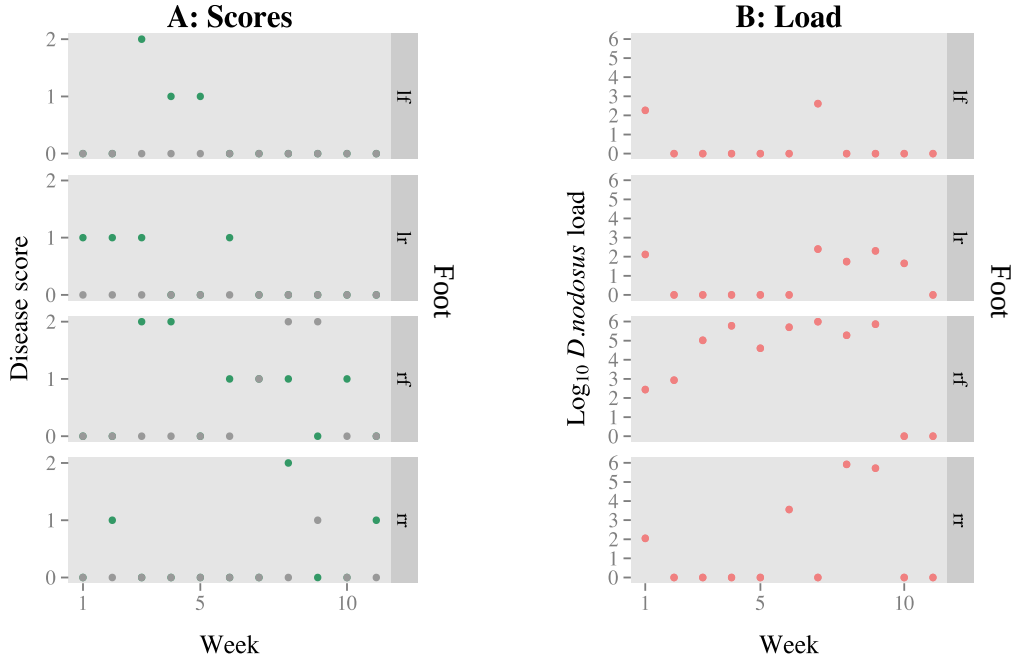


Figure 3.16: Lesion scores and bacterial loads of sheep 3535**. A**: Disease scores on all feet from week 1-10. SFR scores are presented in grey (•) and ID scores in green (•) **B**: *D. nodosus* log_{10} bacterial load on all feet from week 1-10. **LF**=left front foot, **RF**= right front foot, **LR**=left rear foot, **RR**=right rear foot.

3.5.5 Dichelobacter nodosus load on feet from week 1-3

D. nodosus was significantly more frequently detected in week 1 than in week 1 and 3 (P<0.01, Appendix 8). The 2 sheep that carried *D. nodosus* on their feet in week 2 and 3 were the sheep that became diseased during the study. Bacterial loads increased in both sheep after week 1 and additional feet became positive, indicating that colonization took place (Table 3.25, Figure 3.17).

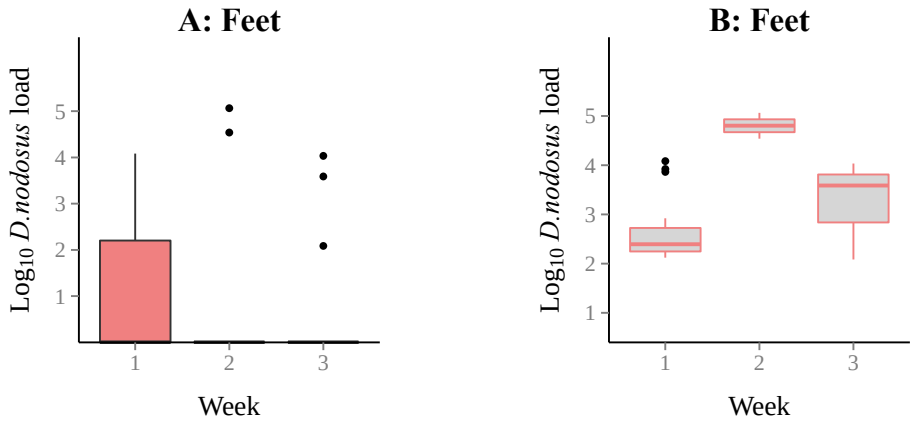


Figure 3.17: Log_{10} *D. nodosus* load on clinical swabs from all feet from week 1-3. **A**: *D. nodosus* load on foot swabs/week **(A) (n=84)** including negative samples **B**: *D. nodosus* load including only positive samples on foot swabs/week **(B) (n=34).** *D. nodosus* was detected on 13 sheep and 27 feet in week 1 and on 2 sheep in week 2 and 3 (3 and 4 feet respectively).

3.5.6 The effect of climate on disease scores and Dichelobacter nodosus detection

The period of uncharacteristically low rainfall (day 47-75) was followed by a period where no ID lesions ≥ 2 were scored (day 51-100, week 8-15) (Figure 3.18). ID 1 lesions were scored throughout the study, but were noticeably less prevalent from day 58 (week 9) (Figure 3.19). A reduced prevalence of SFR lesions during the dry period was less noticeable, with lesion prevalence being lower from day 65-86 (week 10-13). It was not possible to determine with certainty whether *D. nodosus* loads were affected by the dry period, as there was only one sheep (ID 3488) where *D. nodosus* was continuously detected during this period. This sheep had no lesions in week 10 and 11 (Figure 3.20), whereas *D. nodosus* loads remained unaffected.

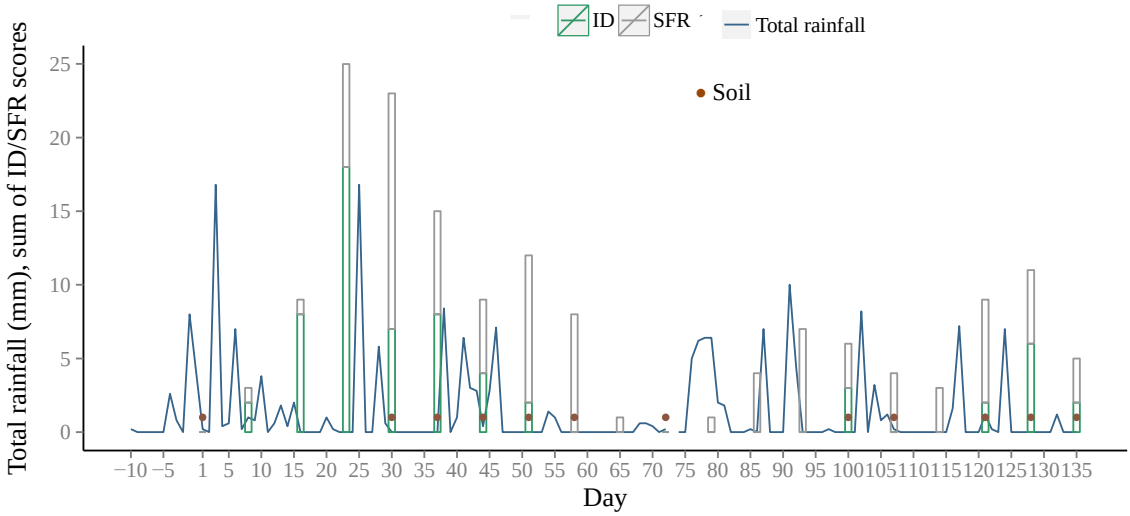


Figure 3.18: Interdigital dermatitis **(**ID) and severe footrot (SFR) lesions of the study group (40 sheep) during study 1 and daily total rainfall (mm). Circular data points (•) indicate weeks where *D. nodosus* was detected in soil. Daily total rainfall data commences at day -10 when the baseline pasture was first sampled.

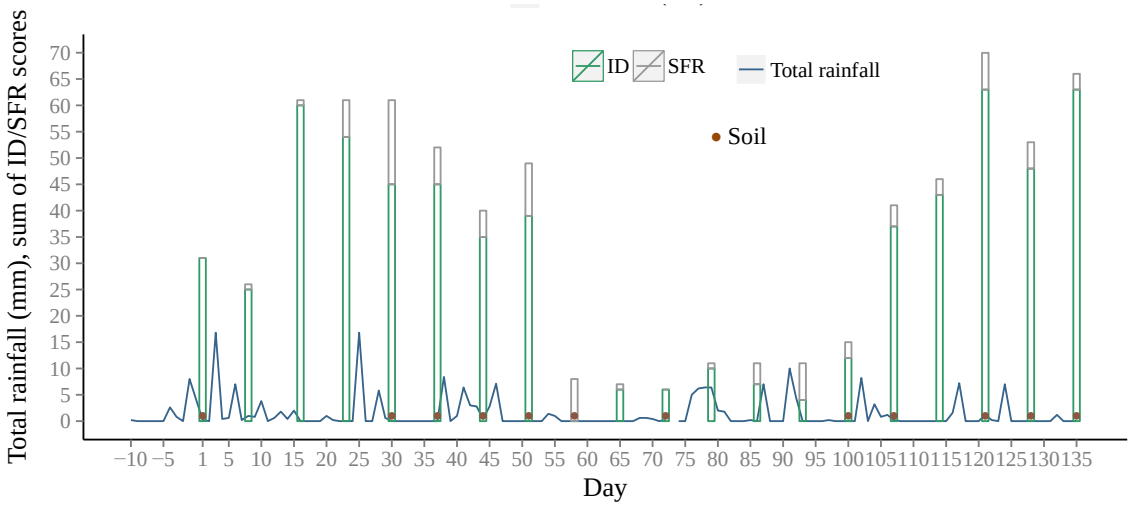


Figure 3.19 Interdigital dermatitis **(**ID) and severe footrot (SFR) lesions of the study group (40 sheep) during study 1 and daily total rainfall (mm). ID 1 scores are included. Circular data points (•) indicate weeks were *D. nodosus* was detected in soil. Daily total rainfall data commences at day -10 when the baseline pasture was first sampled.

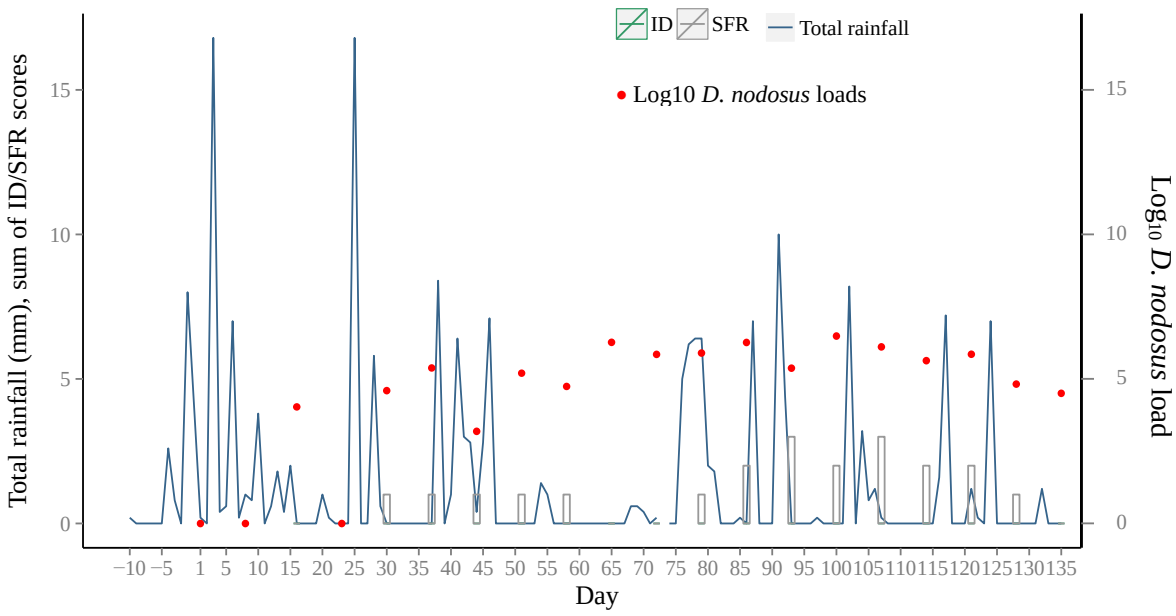


Figure 3.20: Interdigital dermatitis **(**ID) and severe footrot (SFR) lesions (diseased foot) and disease scores of sheep 3488 and daily total rainfall (mm). Log_{10} *D. nodosus* load of sheep 3488 are included (•). Daily total rainfall data commences at day -10 when the baseline pasture was first sampled.

3.5.7 Binomial mixed effects logistic regression model

Tables 3.21 - 3.25 below show a summary all variable considered for the binomial model. No model for *D. nodosus* presence in the gingival cavity was created due only one positive sample. In addition, no model with the outcome whether soil was present or absent was created because there were no significant univariable associations. Variables that were excluded from univariable association are shown in Appendix 5.

Table 3.21: Univariable associations by the outcome (*D. nodosus* is present on feet [Foot model] or *D. nodosus* is present in soil [Soil model]) with all predictor variables.

Weekly continuous variables	P-value (Foot model)	n (Foot model)	P-value (Soil model)	n (Soil model)	Median	1st Qu	3rd Qu	Min.	Max.
Number of *D. nodosus* positive feet t^{-1}	0.50	19	0.26	19	4.00	3.00	5.00	1.00	11.00
Number of *D. nodosus* positive feet t^{-2}	0.92	18	0.81	18	4.00	3.00	5.00	1.00	11.00
Total number of diseased feet t^{-1}	0.85	19	0.77	19	1.00	0.00	1.00	0.00	2.00
Total number of diseased feet t^{-2}	0.62	18	0.25	18	1.00	0.00	1.00	0.00	2.00
***D. nodosus* load in soil (week) t^{-1}**	**0.05**	19	0.20	19	2.48	0.00	2.71	0.00	4.14
***D. nodosus* load in soil (week) t^{-2}**	0.97	18	0.20	18	2.45	0.00	2.71	0.00	4.14
Number of *D. nodosus* positive soil samples t^{-1}	**0.15**	19	0.25	19	1.00	0.00	2.00	0.00	4.00
Number of *D. nodosus* positive soil samples t^{-2}	0.21	18	0.39	18	1.00	0.00	2.00	0.00	4.00
Sum of *F. necrophorum* load on feet t^{-1}	0.47	19	0.50	19	9.22	6.25	17.11	0.00	68.54
Sum of *F. necrophorum* load on feet t^{-2}	0.64	18	0.85	18	9.22	6.25	17.11	0.00	68.54
Total rain (mm)*, 7 days prior sampling	0.94	20	0.94	20	10.30	1.20	17.80	0.00	25.20
Total rain (mm), 14 days prior sampling	0.51	20	0.85	20	21.60	12.20	25.40	1.80	36.60
Total rain (mm), 28 days prior sampling	0.53	20	0.50	20	43.60	30.80	48.20	14.30	59.80
Mean temperature (°C), 7 days prior sampling	0.94	20	0.26	20	7.97	6.77	10.73	4.76	18.80
Mean temperature (°C), 14 days prior sampling	0.25	20	0.19	20	7.57	6.43	10.66	3.59	17.35
Mean temperature (°C), 28 days prior sampling	**0.19**	20	0.47	20	7.10	6.15	10.11	3.23	16.17
Min. temperature (°C), 7 days prior sampling	**0.09**	20	0.30	20	3.66	1.81	6.74	0.83	12.10

Continued on next page

Weekly continuous variables (continued)	P-value (Foot model)	n (Foot model)	P-value (Soil model)	n (Soil model)	Median	1st Qu	3rd Qu	Min.	Max.
Min. temperature (°C), 14 days prior sampling	0.45	20	0.74	20	2.71	2.13	4.04	0.85	11.16
Min. temperature (°C), 28 days prior sampling	0.68	20	0.09	20	2.39	1.98	3.80	0.13	8.89
Max. temperature (°C), 7 days prior sampling	0.31	20	0.27	20	12.16	10.19	16.57	3.44	24.07
Max. temperature (°C), 14 days prior sampling	**0.18**	20	0.14	20	11.59	10.19	15.69	6.43	22.56
Max temperature (°C), 28 days prior sampling	0.68	20	0.13	20	10.64	9.04	15.09	6.39	21.36
Soil moisture (%) t^{-1}	0.41	19	0.86	19	76.71	49.13	92.00	37.46	99.55
Soil moisture (%) t^{-2}	0.49	18	0.89	18	78.50	52.52	90.94	37.46	99.55
Soil moisture 0-1 cm (%) t^{-1}	0.27	19	0.70	19	93.08	46.59	105.80	35.05	134.26
Soil moisture 0-1 cm (%) t^{-2}	0.62	18	0.44	18	95.85	47.95	102.25	35.05	134.26
Soil moisture 0-5 cm (%) t^{-1}	0.74	19	0.61	19	52.35	40.01	74.80	35.45	152.60
Soil moisture 0-5 cm (%) t^{-2}	0.51	18	0.68	18	53.07	44.88	75.54	35.45	152.60
Soil temperature (°C) t^{-1}	0.26	19	0.96	19	9.00	5.33	16.83	4.33	21.33
Soil temperature (°C) t^{-2}	**0.01**	18	0.80	18	9.00	5.33	15.33	4.33	21.33
Soil temperature (°C) t^{-4}	0.56	16	1.29	16	9.00	5.33	15.21	4.33	21.33

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}) and 2 time-points (t^{-2}). Exceptions: For climate variables (rainfall and temperature) the average value of 7,14 and 28 days before sampling where taken (*rolling mean). N represents the number of unique values in each row of the dataset.

Table 3.22: Univariable associations of categorical variables (weekly variables) by the outcome (*D. nodosus* is present / absent on feet [Foot model] or *D. nodosus* is present in soil [Soil model]).

Weekly categorical variables	P-value (Foot model)	n (Foot model)	P-value (Soil model)	n (Soil model)	Number positive	% Positive
Week of study (categorical 1,3,5,7)	0.68	20	0.26	20	-	-
***D. nodosus* present in soil t^{-1} (1=present)**	**0.06**	19	0.15	19	14	3.34
***D. nodosus* present in soil t^{-2} (1=present)**	0.85	18	0.22	18	14	3.54

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}) and 2 time-points (t^{-2}). N represents the number of unique values in each row of the dataset.

Table 3.23: Univariable associations of categorical variables (by sheep) by the outcome (*D. nodosus* is present / absent on feet [Foot model] or *D. nodosus* is present in soil [Soil model]).

Variables by sheep (categorical)	P-value (Foot model)	n (Foot model)	P-value (Soil model)	n (Soil model)	Number positive	% Positive
Sheep had FR t^{-1} (1=diseased)	0.95	34	NA	NA	19	55.88
Sheep had FR t^{-2} (1=diseased)	0.26	31	NA	NA	17	54.84
Sheep had FR (Day of sampling) (1=diseased)	**0.11**	37	NA	NA	23	48.94
Body condition score t^{-1} (Scale from 1-5)	0.56	34	0.91	34	-	-
Body condition score t^{-2} (Scale from 1-5)	0.38	31	0.20	31	-	-

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}) and 2 time-points (t^{-2}). Exceptions: Disease state of the sheep/foot and whether the animal was a lamb or an ewe (0=healthy, 1= diseased) is also presented non-lagged. N represents the number of unique values in each row of the dataset). NA=not analyzed. Variables that were not suitable to be used in either the mouth or foot model.

Table 3.24: Univariable associations of continuous variables (by foot) by the outcome (*D. nodosus* is present / absent on feet [Foot model] or *D. nodosus* is present in soil [Soil model]).

Variables by foot (continuous)	P-value (Foot model)	n (Foot model)	P-value (Soil model)	n (Soil model)	Median	1st Qu	3rd Qu	Min.	Max.
***D. nodosus* load on feet t^{-1}**	**<0.01**	136	NA	NA	0.00	0.00	3.66	0.00	6.48
***D. nodosus* load on feet t^{-2}**	**<0.01**	124	NA	NA	0.00	0.00	4.11	0.00	6.48
***F. necrophorum* load on feet t^{-1}**	**<0.01**	136	NA	NA	0.00	0.00	2.45	0.00	7.47
***F. necrophorum* load on feet t^{-2}**	0.43	124	NA	NA	0.00	0.00	2.39	0.00	7.47

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}) and 2 time-points (t^{-2}). N represents the number of unique values in each row of the dataset. NA=not analyzed. Variables that were not suitable to be used in foot or mouth model.

Table 3.25: Univariable associations of categorical variables (by foot) by the outcome (*D. nodosus* is present / absent on feet [Foot model] or *D. nodosus* is present in Soil [Soil model]).

Categorical variables (by foot)	P-value (Foot model)	n (Foot model)	P-value (Soil model)	n (Soil model)	Number positive	% Positive
***F. necrophorum* present on feet t^{-1}**	**<0.01**	136	NA	NA	34	25.00
***F. necrophorum* present on feet t^{-2}**	**0.02**	124	NA	NA	30	24.19
Foot had FR lesions t^{-1} (1=diseased)	**0.02**	136	NA	NA	76	55.88
Foot had FR lesions t^{-2} (1=diseased)	0.66	124	NA	NA	68	54.84
Foot had FR lesions (Day of sampling) (1=diseased)	**<0.01**	148	NA	NA	80	54.05

P-values < 0.2 are highlighted in **bold** and represent variables that were included in the model. Variables are lagged by one time-point (t^{-1}) and 2 time-points (t^{-2}). Exceptions: Disease state of the sheep/foot and whether the animal was a lamb or an ewe (0=healthy, 1= diseased) is also presented non-lagged. N represents the number of unique values in each row of the dataset. NA=not analyzed. Variables that were not suitable to be used in mouth or foot model.

Similar to study 1, *D. nodosus* was more likely to be present on feet with increasing load the previous week (OR 1.85, 95% CI: 1.38, 12.52). The likelihood of *D. nodosus* presence also increased 7.7 times with detection of *F. necrophorum* the previous week (95%, CI: 1.62, 47.96). There was no effect of time. *D. nodosus* was more likely to be present when the number of soil positives increased the previous week (OR 2.62, 95%, CI:1.07, 7.08) (Table 3.26). *D. nodosus* was less likely to be present on feet when soil temperature increased 2 weeks previously (OR 0.71, 95%, CI: 0.52, 0.93). The robustness of this model is reduced due the low number of sheep included the model. In addition, FR is present only during certain time periods. It is therefore difficult to evaluate the effects of soil temperature and number of *D. nodosus* positive samples with certainty. These variables remained in the model, as their removal caused an increase in the Akaike information criterion (AIC) value, indicating that the fit of the model deteriorated.

Table 3.26: Final model variables (Foot model), where the outcome is whether *D. nodosus* is present on feet (binomial 1-0).

Predictor variables	n	% Pos.	Coef	s. e	P	OR	CI (2.5%)	CI (97.5 %)
Week (generalized)	20	-	2.01	1.63	0.22	7.44	0.32	205.70
$Week^2$	20	-	0.67	1.03	0.52	1.95	0.26	15.27
$Week^3$	20	-	-1.42	1.39	0.30	0.24	0.02	3.66
$Week^4$	20	-	0.40	0.57	0.48	1.49	0.49	4.69
D. nodosus load on feet (t^{-1})	136	41.91	0.62	0.14	<0.01	1.85	1.38	12.52
F. necrophorum present on feet (t^{-1})	136	25.00	2.04	0.84	0.01	7.70	1.62	47.96
Soil temperature (°C) (t^{-2})	20	-	-0.34	0.15	0.02	0.71	0.52	0.93
Number of *D. nodosus* positive soil samples (t^{-1})	462	4.10	0.96	0.73	0.04	2.62	1.07	7.08

% Pos.= Samples that were *D. nodosus* positive (%). Coef= Coefficient, s.e. =Standard error, OR=Odds ratio, CI=Confidence intervals.

3.5.8 Correlations and associations of predictor variables

Correlation and associations between final model variables and all predictor variables considered for the model are presented in the tables below (Tables 3.27-3.29). There was no correlation of *D. nodosus* load on feet with climate and soil moisture variables. There was a significant negative correlation of soil temperature (°C) and number of *D. nodosus* positive soil samples (Table 3.27). The number of soil samples were *D. nodosus* was detected decreased with increasing soil temperature. There was a positive correlation between soil temperature and all ambient temperature variables and a negative correlation between soil temperature and rainfall and soil moisture. A significant positive correlation was present between *D. nodosus* and *F. necrophorum* loads on feet (Table 3.27).

Table 3.27: Study 2: Correlation of continuous predictor variables with final continuous model variables.

Continuous predictor variables	**1**	**2**	**3**
1. *D. nodosus* load on feet	1.00		
2. Mean weekly soil temperature (°C)	0.05	1.00	
3. Number of positive soil samples (week)	-0.03	**-0.22**	1.00
Weekly variables			
Number of *D. nodosus* positive feet	0.06	**-0.54**	**0.36**
D. nodosus load in soil	0.09	**0.18**	**0.63**
Mean temperature 7 days *(°C)	0.02	**0.92**	**-0.24**
Minimum temperature 7 days (°C)	-0.01	**0.65**	**-0.26**
Maximum temperature 7 days (°C)	0.06	**0.93**	**-0.39**
Total rain 7 days ^ (mm)	-0.04	**-0.44**	0.13
Mean weekly soil moisture (%)	0.00	**-0.70**	-0.12
Mean weekly soil moisture 4-5 cm (%)	-0.06	**-0.30**	-0.42
Mean weekly soil moisture 0-1 cm (%)	0.06	**-0.62**	**0.26**
Mean weekly soil moisture LTA (%)	0.02	**-0.59**	0.08
Mean weekly soil moisture HTA (%)	0.00	**-0.97**	**-0.22**
Variables by feet			
F. necrophorum load (feet)	**0.51**	0.05	0.10

R^2 values are shown: **Bold** R^2 values are statistically significant *Rolling mean was used for all temperature and rainfall variables. Maximum and minimum temperatures, as well as temperatures after 14 days prior sampling and 28 days prior are not presented as they are highly correlated with other temperature variables (Appendix 9).

Table 3.28: Associations of categorical predictor variables with final continuous model variables.

Categorical predictor variables	*D. nodosus* load on feet	Mean weekly soil temperature (°C)	Number of positive soil samples (week)
Weekly variables			
D.nodosus present in soil (1=present)	0.29	**0.02** (+^)	**<0.01** (+)
Variables by sheep			
Sheep had FR (1=diseased)	0.44	**<0.01** (+)	0.30
Variables by foot			
Foot had FR lesions (1= diseased)	**<0.01** (+)	0.10	0.62
D.nodosus present on feet (1=present)	**<0.01** (+)	0.99	0.42
F. necrophorum present on feet (1=present)	**<0.01** (+)	0.54	**0.03** (+)

Significant P-values are shown in **bold**, ^The direction of the effect in indicated in parentheses after the significant p-value: The plus symbol (**+**) indicates that the median is significantly higher in binomial 1 variable, the minus symbol (**-**) indicates that the median is significantly higher in binomial 0 variable.

Table 3.29: Associations of continuous and categorical predictor variables with categorical model variables.

Continuous predictor variables	*F. necrophorum* present on feet (1=present)
Weekly variables	
D. nodosus load soil (week)	0.07
F. necrophorum load (feet)	**<0.01** (+) ^
Mean temperature 7 days^ (°C) *	0.71
Minimum temperature 7 days (°C)	0.12
Maximum temperature 7days (°C)	0.58
Total rain 7 days (mm)	0.86
Mean weekly soil moisture (%)	0.99
Mean weekly soil moisture 4-5 cm (%)	0.23
Mean weekly soil moisture 0-1 cm (%)	0.28
Mean weekly soil moisture LTA (%)	0.35
Mean weekly soil moisture HTA (%)	0.82
Categorical predictor variables	
Weekly variables	
D.nodosus present in soil (1= present)	0.10
Variables by sheep	
Sheep had FR (1= diseased)	0.12
Variables by foot	
Foot had FR lesions (1= diseased)	**<0.01**
D.nodosus present (1=present)	**<0.01**

Significant P-values are shown in **bold**, ^The direction of the effect in indicated in parentheses after the significant p-value: The plus symbol (+) indicates that the median is significantly higher in binomial 1 variable, the minus symbol (-) indicates that the median is significantly higher in binomial 0 variable. * Rolling mean was used for all temperature and rainfall variables. Maximum and minimum temperatures, as well as temperatures after 14 days prior sampling and 28 days prior are not presented as they are highly correlated with other temperature variables (Appendix 9).

3.6 Discussion

The overall aim of the studies conducted was to investigate whether *D. nodosus* persists on the sheep, on the pasture, or both. We used real-time PCR to investigate variations of bacterial load within and between sample types and developed a binomial mixed regression model for both studies to elucidate variables that drive the presence of *D. nodosus*, taking into account climatic changes.

The results of study 2 show that *D. nodosus* was carried from a flock were FR was present into a lesion-free flock when the study group was moved onto a separate pasture and that *D. nodosus* could be detected on healthy feet up to 5 weeks before onset of ID and SFR.
D. nodosus has been detected on healthy feet on a number of occasions (Moore *et al*., 2005; Calvo-Bado *et al*., 2011; Stäuble *et al*., 2014), but generally at lower loads than on diseased feet (Stäuble *et al*., 2014). Whereas this is true for both studies in this chapter, *D. nodosus* was load was as high as 4.75×10^5 cells before onset of disease, indicating that colonization took place and supporting previous evidence suggesting that *D. nodosus* plays an important role in the initiation of disease (Witcomb *et al*., 2014). This is further supported by the results of the two-binomial mixed effect foot models, that showed that presence of *D. nodosus* on feet was driven by *D. nodosus* load (and *F. necrophorum* presence) the previous week.

D. nodosus was detected in the gingival cavity in 25% of samples in study 1, whereas it was detected in only 1 sample in study 2. This is possibly attributable to the lower disease prevalence in study 2. Witcomb (2012) detected *D. nodosus* in 74% of all gingival cavity swabs, but disease prevalence in the study flock was high. The high likelihood of *D. nodosus* being present in the gingival cavity when a sheep had FR, shows that disease status of the sheep drives *D. nodosus* presence the gingival cavity rather than load on feet. These results suggest that *D. nodosus* transiently populates the gingival cavity when disease prevalence is high, but it is less likely that it persists.

For the first time, we show that faecal shedding of *D. nodosus* may be possible, as *D. nodosus* was detected in faecal samples directly collected from the sheep in study 2. Witcomb (2012) did not find *D. nodosus* in samples directly obtained from sheep, and did not link detection in faeces to disease status of the foot. In this study, *D. nodosus* was found in the faeces of the most diseased sheep in the flock and in the faeces of a non-diseased sheep in week 1. In this week *D. nodosus* was found in a larger proportion of

sheep, including healthy sheep, compared to any other week. Similar to *D. nodosus* presence in the gingival cavity, it is possible that *D. nodosus* presence in faeces is linked to the disease prevalence in the flock. The low quantity of *D. nodosus* detected suggests that it could be passaging through the digestive system, without colonizing. It may be beneficial to investigate faecal shedding a flock were disease prevalence is high.

For the first-time *D. nodosus* was detected in grass samples. This support existing evidence that *D. nodosus* is able to adhere to the environment at least for a short amount of time (Muzafar *et al*., 2015; Witcomb 2012). Hence grass is likely to act as a transitional medium for *D. nodosus* before it is passed to a new host.

D.nodosus has previously been detected in soil in both *in vitro* and farm-based studies (Witcomb 2012; Cederlof *et al*., 2013; Muzafar *et al*. 2015; Muzafar *et al*., 2016). Here we report for the first time that *D. nodosus* was detected in soil samples taken at an increased depth (4-5 cm) in addition to more superficial samples and that there were no statistical differences in detection frequency and *D. nodosus* load. It could be that the deeper layers of soil constitute a suitable environment for *D. nodosus*. There is however no evidence that suggests that the deeper layer of soil constitute a better environment for the survival of *D. nodosus*. Muzafar (2016) suggested that *D. nodosus* does not thrive in the outer layers of soil due to desiccation. Recent evidence indicates that *D. nodosus* may be able to survive at lower temperature than expected (Cederlof *et al.*, 2013; Muzafar *et al.*, 2016) and Smith *et al*. (2014) showed that transmission of FR is possible at temperatures below 10 °C. Deeper soil layers are colder than the soil surface, which absorbs more heat (Pepper and Gentry, 2015). Survival of *D. nodosus* in the soil would be more likely than survival on grass to due to the anaerobic nature of the bacterium (Rood *et al*., 2005) especially during wet weather when saturated soil is deprived of oxygen (Pepper and Gentry 2015). Whether deeper soil layers are exposed to the host, as a source of infection is unknown, but this may be possible during wet weather when pasture can be poached (heavily damaged by feet). Whether *D. nodosus* survives in the deeper layers of the soil and if so, for how long, is unknown.

D. nodosus was detected in soil and grass samples from the pasture after it had been empty for 10 days. The pasture had previously been occupied by a group of ewes with some lameness (observations only, lesions were not assessed) and it is possible that *D. nodosus* survived on the pasture for 10 days as research suggests that it may survive on pasture from 7-10 days (Beveridge, 1941; Whittington, 1995). Bacterial loads on the pasture where low and given that *D. nodosus* loads on healthy feet where higher than

loads on pasture, it is more likely that the *D. nodosus* that was introduced from the original flock caused disease in this study.

The only time *D. nodosus* was detected frequently and at high loads in the environmet was during the first sampling visit of study 1, when the weather was wet and ground was muddy. After the first week of sampling, temperatures increased and the soil became very dry (authors observation), which was coupled with decreasing detection frequencies and decline of *D. nodosus* loads in soil, grass and faecal samples, which was not affected by the increase of disease prevalence in week 5. In week 5, *D. nodosus* was detected almost exclusively on feet that were diseased, but at higher loads. This could indicate that the wet weather in week 1 facilitated spread and subsequent colonization of *D. nodosus* on some feet. This coincides with results from study 2 where *D. nodosus* could be detected in high loads on the feet of a diseased sheep, but detection of *D. nodosus* in soil was reduced between week 9 and 16 following the dry period lasting from week 8-14.

It is possible that *D. nodosus* does not thrive in drier environments. The optimum conditions for *D. nodosus* survival have been described as warm and damp (Beveridge, 1941, Whittington, 1995). Smith *et al*. (2014) found that the incidence rate of FR increased with increasing rainfall in the previous weeks, suggesting that moisture may play a role in preventing death of *D. nodosus*. It is however difficult to disentangle the contribution of different climatic factors to *D. nodosus* presence in the two studies based on the statistical model and associations between variables alone. There were only 4 sampling days in study 1 and the correlations between *D. nodosus* load and detection frequencies in all sample types are probably attributable to the high detection frequency of *D. nodosus* in week 1 and subsequent decline. In study 2, the effects of climate variables and soil moisture on *D. nodosus* presence and load on feet did not become clear due to the low number of sheep in the model. However, the lack of associations between soil moisture variables (and rainfall, as this was highly correlated) and *D. nodosus* presence or load on feet is maybe not surprising given that seemingly only FR lesions were affected by the dry period. However, the suggestion that *D. nodosus* loads are not affected by dry weather is tentative.

As ambient temperature and soil temperature increased, the number of *D. nodosus* positive soil samples decreased and decreased rainfall and soil moisture was associated with a decrease in *D. nodosus* positive soil samples. This is not surprising, as temperatures increased steadily throughout the trial, whereas detection in soil decreased

during the dry weather period. Although there is a high positive correlation between soil moisture and rainfall, it is more likely that the dry ground affected survival of *D. nodosus* in soil.

The results of model 1 in study 1 show that feet were less likely to carry *D. nodosus* when the foot was treated with antibiotic spray 2 weeks previously. This coincides with recent findings showing that *D. nodosus* load decreased significantly 1 week and 2 weeks after a foot had been treated with antibiotics (Willis, 2017, unpublished data).
The results also show that ewes were more likely to have *D. nodosus* on their feet than lambs. Muzafar *et al*. (2015) found that population loads of *D. nodosus* were significantly higher in on the feet of ewes than on the feet of their lambs and also reported a larger strain diversity in ewes. In Muzafar *et al*. (2015) lambs were only 5-13 hours, whereas in this study lambs were several months old and had been part the main flock for several weeks. It may be possible that age plays a part in the composition of the bacterial community on the feet of sheep and older animals that may have had FR previously may be more susceptible to *D. nodosus* colonization.

Whereas the dry climate in spring 2015 provided the opportunity to gain some insights into the peristence of *D. nodosus,* the combination of unusual climate and creation of a closed group of lesion-free sheep led to low disease prevalence and incidence and only 3 diseased sheep were included in the model (Study 2). To increase robustness of the data it would be beneficial to analyze additional samples from sheep, this was not within the scope of this projest due to practical restrictions.

Although FR disease patterns and disease prevalence varied between studies, the results suggest that *D. nodosus* is more likely to persist on the feet of sheep than other sheep sites (mouth and faeces) and in the environment, due to its transient presence at those sites and decreased detection in dry weather. It could also be possible that soil moisture is an important factor in *D. nodosus* survival, while rainfall may be associated with increased transmission.

CHAPTER 4

Optimization and validation of a multiple locus variable number tandem repeat analysis for differentiation of *Dichelobacter nodosus* strains from mixed DNA samples

4.1 Introduction

An effective molecular typing system is essential to identify the genetic diversity of an organism, so that it can be classified into subtypes and strains when isolated at different times and from different locations. It can be used for disease surveillance, outbreak detection and response (Nadon *et al*., 2013) and research.

A number of strain typing methods have been used to strain type *D. nodosus*, including pulse-field-gel electrophoresis (PFGE) (Zakaria *et al*., 1998; Buller *et al*., 2010) and polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) (Ghimire and Egerton, 1999). However, these methods are labour intensive, expensive, require culture and lack reproducibility between laboratories. In addition, strain discrimination is more challenging when a species of bacteria has high genetic homogeneity. The genome of *D. nodosus* is highly conserved with more than 95% sequence similarity between isolates (Kennan *et al*., 2014).

Tandem repeats are increasingly recognized as markers for genotyping pathogens, facilitated by the availability of whole genome sequences that open the possibility for their systematic evaluation. Tandem repeats are created through replication errors, or slipped strand mispairings that generate diversity in the number of tandem repeats among strains of the same species (Nadon *et al.,* 2013), which permits discrimination of bacterial isolates. The rapid evolution of tandem repeats within a species is thought to contribute to the phenotypic flexibility of pathogens (Le Fleche *et al*., 2001). Multiple locus variable number tandems repeat analysis (MLVA) is based on a comparison between strains of the number of tandem repeats at suitable loci within the genome to get a unique code to differentiate strains. It performs well against several criteria such as discriminatory power, robustness, portability, objectivity, throughput and marker stability (Lindstedt 2005; Hyytia-Trees *et al.*, 2007; Van Belkum *et al.,* 2007).

MLVA has been used in epidemiological studies of bacterial diseases of domestic and wild animals in recent years with the aim to investigate geographical relatedness between strains or genotypic variation of pathogenic bacteria between populations, species or sample types (Vranckx *et al*., 2011; Agdestein *et al*., 2014; Arguello *et al*., 2014; Dugat *et al*., 2014; Biffa *et al*., 2014; Russell *et al*., 2014; Ceglie *et al*., 2015; Koizumi *et al*., 2015; Dugat *et al*., 2016; Gyuranecz *et al*., 2016; Ma *et al*., 2016). MLVA assays are commonly developed for use with bacterial isolates, hence requiring the need to culture (Van Belkum *et al*., 1997, Noller *et al*., 2003, Le Fleche *et al*., 2006; Agdestein *et al*., 2014; Russell *et al*., 2014; Biffa *et al*., 2014; Koizumi *et al*., 2015; Gyuranecz *et al*., 2016; Eisenberg *et al*., 2016; Ranjbar *et al*., 2016; Helldal *et al*., 2017; Liu *et al*., 2017).

The use of MLVA on mixed DNA samples investigating bacterial disease in animals has been described on a number of occasions (Vranckx *et al*., 2011; Dugat *et al*., 2014, Ceglie *et al*., 2015; Muzafar *et al*., 2015; Dugat *et al*., 2016). An MLVA that can be used without the need to culture may be more time efficient particularly when using organisms that are challenging to culture such as *D. nodosus*. It may provide useful information on bacterial communities present at the time of sampling. Information on the proportion and abundance of strains present may be obtained and strains may be compared between different sample types and over time.

Existing MLVA schemes/assays used 3-16 VNTR loci (For example: Van Belkum *et al*., 1997, Noller *et al*., 2003, Le Fleche *et al*., 2006, Agdestein *et al*., 2014; Russell *et al*., 2014; Ceglie *et al*., 2015; Liu *et al*., 2016; Eisenberg *et al*., 2016; Ranjbar *et al*., 2016; Helldal *et al*., 2017; Liu *et al*., 2017). A suitable MLVA typing assay relies on a combination of markers that can provide a relevant clustering when used together. Helldal *et al*., (2017) found that an increase in the number of loci led to greater discrimination between strains of *Eschericia coli*. Van Belkum (1997) reported that an MLVA using 4 VNTR loci successfully typed *Haemophilus influenzae* strains and Vranckx *et al*. (2011) used 4 loci to discriminate between strains *of Mycoplasma hyopneumoniae* from mixed DNA samples.

Russell *et al*. (2014) developed an MLVA assay for *D. nodosus,* with four polymorphic loci (DNTR02, 09, 10, 19), designed for use as a strain-typing tool on *D. nodosus* isolated through culture. Recently this assay was used by Smith *et al*., (2017) to investigate within-flock *D. nodosus* population dynamics. The use of the MLVA assay to analyze the *D. nodosus* communities in DNA extracted from swabs was reported by Muzafar *et al*. (2015), who aimed to investigate *D. nodosus* communities on the interdigital skin surface

of healthy and diseased sheep. Only two loci were used in the analysis; DNTR09 was omitted due to poor amplification and DNTR02 due to non-specific amplification.

In order to use the MLVA assay developed for *D. nodosus* by Russell *et al*. (2014) on swabs and environmental samples that may contain more than one *D. nodosus* strain, it required optimization and validation. Therefore, the first aim of this study was to test the suitability of the MLVA assay for use on DNA extracted from swabs and environmental samples, by optimization of previously established protocols and by conducting additional experiments to identify possible uses and limitations of the assay. The second aim of the study, was to investigate whether *D. nodosus* strains vary between sheep and environmental sites and whether strains may be shared between sheep and over time. In addition, a novel approach to identify individual strains in a sample is described and evaluated.

For clarity, *a bacterial strain is differentiated from another if the number of repeats varies at one or more locus. An isolate is a sample obtained from bacterial culture where presence of a single strain is assumed, although presence of more than one strain is possible*.

We hypothesized that *D. nodosus* MLVA assays can provide information about individual strains in a mixed DNA sample and improve understanding of strain influence on disease progression, persistence and pathogenicity.

4.2 Materials and Methods

4.2.1 Assay optimizations and cycling conditions

Due to previous reports of minor peaks in the PCR amplification of DNTR09 and non-specific products in DNTR02 (Muzafar *et al.,* 2015) the MLVA assay developed by Russell *et al*. (2014) was optimized to increase sensitivity. This included changes in assay composition (change of Mastermix and increased primer quantity) and thermal cycling conditions (increased number of cycles). This resulted in the following final set-up for all four *D. nodosus* tandem repeat (VNTR) loci: All PCR reactions had a final volume of 25 µl containing 12.5 µl Mastermix (MyTaq™ Red Mix, Bioline, London, UK), 1 µl of each primer (10 mM) and 1 µl bovine serum albumin (20 mg ml^{-1}) (Sigma Aldrich, Dorset, UK). One µl DNA template was added to each PCR reaction, which were carried out using the following conditions: 1 cycle of 95 °C for 2 minutes, 40 cycles of 95 °C for 1 minute, 59 °C for 30 seconds and 72 °C for 1 minute with a final extension of 72 °C for 2 minutes.

4.2.2 Amplification of Dichelobacter nodosus from swabs, faeces and environmental samples

Russell *et al*.'s (2014) MLVA assay for *D. nodosus* was originally developed to analyze DNA obtained from isolates (from culture). Muzafar *et al*. (2015) had used a partial MLVA assay to analyze swabs collected from sheep feet, but other sample types were not tested.

To test effective amplification of all 4 target *D. nodosus* VNTR loci, 10 samples from foot swabs (using samples from study 1) with amplified DNTR02 and DNTR09 amplicons and 9 samples with amplified DNTR10 and DNTR19 amplicons were submitted for sequencing following the protocol described in section 2.5 (Chapter 2). In addition, MLVA PCR products from 1 gingival swab sample was also submitted for sequencing where amplification was observed.

In order to test the assays capacity to amplify *D. nodosus* from soil, grass and faecal samples, 6 pasture samples were collected from a field where a flock of sheep with footrot were grazing in October 2013 and tested for the presence *of D. nodosus* using the specific qPCR as described previously (Section 2.9, Chapter 2). The soil samples were

collected from a hoof print and a high traffic area (HTA) (defined in chapter 3, section 4.2.4.4). Two faecal samples were randomly collected from the pasture and 2 grass samples, one collected from a location where a sheep just had stood and another from a HTA. Samples that tested positive in the *D. nodosus* specific qPCR were submitted to the MLVA assay to test the ability of the assay to amplify products from these sample types. VNTR amplicons were submitted for Sanger sequencing as described in section 2.5 (Chapter 2) as an additional test of successful amplification.

4.2.3 Fragment analysis

VNTR amplicons produced by the MLVA PCR assays were purified following the protocol described in section 2.4.1 (Chapter 2) and submitted for fragment analysis (DNA Sequencing and Services, University of Dundee, Scotland). All PCR-products originating from *D. nodosus* isolate DNA were diluted 1:100 and all products originating from mixed DNA samples were diluted in either 1:20 or 1:100, depending in the intensity of the bands. VNTR amplicons were submitted for fragment analysis separately as pooling PCR products resulted in failed analysis. 1200 Liz dye (Applied Biosystems, Warrington, UK) was used as a size standard and data obtained was analyzed with Peak Scanner™ Software (Applied Biosystems, Warrington, UK). The threshold level determined in section 4.2.7 (this chapter) was applied to all samples tested.

4.2.4 Determination of repeat sizes

Russell *et al*. (2014) calculated the number of repeats by determining the size of the sequenced VNTR amplicons based on the *D. nodosus* strain 1703A (GenBank Accession number CP000513). As throughout this project fragment analysis (based on capillary electrophoresis) was used to size VNTR amplicons, 1 µl of DNA from *D. nodosus* strain 1703A was submitted for fragment analysis. In addition, VNTR amplicons were submitted for sequencing. A comparison of the size of the loci obtained from fragment analysis with the available VCS 1703A sequences of the 4 polymorphic *D. nodosus* VNTR loci (Genebank Accession numbers KC676717, KC676718, KC676719, and KC676720 for DNTR02, 09, 10, and 19 respectively) was used to provide information on the accuracy of the assay.

4.2.5 MLVA specificity for Dichelobacter nodosus

Muzafar *et al*. (2015) had previously reported a case of non-specific amplification at the DNTR02 locus, which led to the exclusion of this locus from analysis. Here the specificity of the primers targeting the 4 VNTR loci was tested by performing the MLVA assay on a series of DNA samples isolated from target and non-target organisms. Non-target organisms previously found on sheep feet or present in soil and faeces were chosen (Chapter 2, Section 2.1).

4.2.6 MLVA sensitivity to Dichelobacter nodosus load

No published information on the detection limit for this assay was available. Following the assay optimizations described above, a set of spiking experiments was set up to determine the detection limit (DL).

Soil and grass from one urban garden and one ovine faecal sample were selected for the experiment. All samples had tested negative for the presence of *D. nodosus* using qPCR. *D. nodosus* strain 4303LBV was cultured on Eugon agar as previously described (Chapter 3, Section 2.2.1) and harvested in 1 ml of PBS pH 7.4. Suspended cells were quantified using a Petroff-Hausser counting chamber (Hausser Scientific, PA, USA) and serially diluted resulting in dilutions ranging from 10^{0} to 10^{-6} containing approximately $1.07x10^{6}$ to 1.07 *rpoD* copies per sample. Tubes containing either 0.5 g of soil, 0.1 g of faeces, 0.2g of grass, or a sterile swab were then spiked with 50 µl of undiluted and serially diluted culture. DNA was then extracted as previously described (Purdy, 2005). An additional PEG precipitation step was applied to all environmental samples (Chapter 2, Section 2.3). Non-inoculated grass and soil samples and blank swabs were included in all extractions as negative controls. DNA concentration was measured (NanoDrop™ 2000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA USA) and A260/280 and 260/230 ratios were recorded. Samples were the screened for *D. nodosus* using the MLVA assay as described above. To test whether detection could be improved further all samples were submitted to a second round of the MLVA PCR assay using the same cycling conditions. As environmental DNA can contain contaminants such as humic acids (Tsai and Olson, 1992), soil DNA was diluted to 1:10, 1:20 and 1:50 and grass DNA was diluted to 1:10 and 1:20 to test whether dilution improved detection.

4.2.7 MLVA profiles of Dichelobacter nodosus isolates and determination of the fragment analysis threshold level

Some users reported the occurrence of multiple minor peaks for each locus after fragment analysis. In order to assess this and test possible interference with a community analysis, DNA from 16 *D. nodosus* isolates was analyzed with the MLVA assay and fragment analysis as described previously: 8/16 samples were tested using only primers targeting the DNTR09 and DNTR10 loci. The other 8 samples were tested using all 4 loci. There was evidence of minor peaks in MLVA analysis of isolates which would suggest that this is either a consistent anomaly within the *D. nodosus* MLVA analysis or a biological feature of *D. nodosus* strains. Therefore, to avoid this "noise" confounding the community analysis, a relative fluorescence unit (RFU) threshold was set below which peaks were assumed to be technical noise and not a minor community. Bin range was set to 4bp (therefore fragment size was determined with an error of ±2 bps) and minimum fragment length cut off values were determined to be 500, 500, 400, and 550 bp for DNTR02, 9, 10, and 19 respectively, based on the minimum length of each fragment without repeats.

4.2.8 Testing recovery of Dichelobacter nodosus communities through the creation of model communities

To test the applicability of the assay on mixed DNA samples, two bacterial communities (community 1 and 2) were constructed *in vitro* and submitted for MLVA analysis. Each model community comprised DNA from 4 isolates from a total of 8 isolates with differing MLVA profiles. All samples were standardized to a working concentration of 15ng/µl and 5 µl of each sample were used to create a suspension in a 1:1:1:1 ratio. A third model community was created identical to community 1 with the exception of the *D. nodosus* 1703A strain diluted 1:5 to evaluate the ability of the assay to recover non-dominant strains from mixed DNA samples and to assess the fragment analysis' ability to size RFU peaks proportionately according to concentration. Samples were submitted to the MLVA assay followed by fragment analysis.

4.2.9 Assessment of the MLVA assay on mixed DNA samples in two contrasting field studies (Studies 1 and 2)

4.2.9.1 Farms, animals and sample collection

The DNA samples used in this chapter originate from the samples collected in study 1 and 2. Details on the farms chosen, animals and data collection procedures are presented in section 3.3 (Chapter 3). Briefly, study 1 was conducted between May and August 2014 on a commercial farm located near Kenilworth, Warwickshire. The flock had a history of footrot and the disease was present at the time of the study. Five North Country Mules and 5 lambs (North-country mule x Texel) were selected for the study. The lambs were unrelated to the ewes. Sheep that were both symptomatic and asymptomatic for ID and SFR were chosen. The farm was visited every two weeks on 4 occasions. The sheep remained in the same field throughout the study. Study 2 was conducted from February to July 2015. The study pasture was vacated 10 days prior to commencement of the study and soil and grass samples were taken to determine baseline level of *D. nodosus*. Forty non-lame ewe lambs with no visible lesions of footrot were selected from a study population of 120 ewe lambs and transferred onto the study pasture where they remained throughout the duration of the study. All sheep were sampled according to the protocols outlined in chapter 3. Additional swabs for culturing *D. nodosus* were taken during study 1. The rationale behind selecting sheep and samples for processing in the laboratory can be found in sections 3.3.3 and 3.5.1 (Chapter 3).

4.2.9.2 Laboratory analysis

DNA was extracted from all samples according to Purdy *et al*. (2005) following the protocol described in 2.3.1 (Chapter 2). Samples collected from sheep in field study 1 were also cultured for strain isolation as described in section 2.2 (Chapter 2). All mixed DNA samples and all *D. nodosus* samples isolated through culture were submitted for MLVA assay and fragment analysis as described in sections 4.2.1 and 4.2.3.

4.2.9.3 Data analysis

The maximum number of strains present in each sample was calculated using the decision table below. Strains were allocated a strain identification number and allocated to be either "present" or "possibly present" (Table 4.1). *Strains are defined as "present" either when only one locus variant was detected at all 4 loci, or when variants were found in only one out of four loci. The number of strains present is then reflected in the number of variants present at the variable locus. Strains are defined as "possibly present" when a number of variants are detected in more than one locus*. In these cases, exactly how the loci variants are combined in actual strains cannot be determined from this analysis, therefore the strains are only "possibly present". The minimum number of strains in a sample equals the number of variants at the most variable locus; the maximum number of strains the product of the number of variants at each locus.

Table 4.1: Calculation of the maximum number of *D. nodosus* strains present in a sample.

	Number of strains					
Combinations	Identified strains*	Min/max number of strains**	Example: Products present in base pairs at each locus			
			DNTR02	DNTR09	DNTR10	DNTR19
1: 1 variant present at each locus	1	n/a	555	985	693	1019
2: 2 variants present at the same locus	2	n/a	550, 555	985	693	1019
3: 3 variants present at the same locus	3	n/a	550, 555,565	985	693	1019
4: 2 variants present at 2 loci	n/a	2/4	555,560	985	693	851,1019
5: variants present at 3 loci	n/a	2/8	555, 560	789, 985	693	851, 1019

* All strains in the sample are identifiable, **The minimum and maximum number of strains present in the sample, n/a: Not applicable. Combination 1-3: All strains can be identified. Combinations 4 and 5: The maximum number of strains present in the sample is 4 and 8 respectively, but exactly how the loci variants are combined in actual strains cannot be determined from this analysis, therefore the strains are only "possibly present".

4.3 Results

4.3.1 Dichelobacter nodosus MLVA PCR specificity

To ensure that the MLVA PCR amplifications were specific for *D. nodosus*, amplified products were submitted for sequencing. All sequenced amplified loci from foot swabs and the gingival cavity were from *D. nodosus*. DNTR19 did not amplify in the sample from the gingival cavity (Table 4.2).

Table 4.2: Sequencing results for DNA samples from foot swabs of the first sampling visit.

Locus	**Number of samples submitted**	***D.nodosus* sequences**	**Sequence similarity**	**Other matches**
Foot swabs				
DNTR02	10	Yes	99-100%	No
DNTR09	10	Yes	99-100%	No
DNTR10	9	Yes	99-100%	No
DNTR19	9	Yes	99-100%	No
Mouth swabs				
DNTR02	1	Yes	95-97%	No
DNTR09	1	Yes	96%	No
DNTR10	1	Yes	97%	No
DNTR19	1	/	/	/

None of the environmental and faecal samples tested yielded an amplified product for all 4 loci after the MLVA PCR, but at least one locus from every sample did amplify. Sequenced PCR products showed a high similarity (92-99%) to known *D. nodosus* sequences with no other matches (Table 4.3).

Table 4.3: Product amplification and sequencing results of soil, grass and faecal samples.

Sample	Locus	*PCR Product obtained*	Sequence similarity to *D. nodosus*
Soil 1*	DNTR02	+	99%
	DNTR09	-	-
	DNTR10	-	-
	DNTR19	-	-
Soil 2**	DNTR02	+	95%
	DNTR09	+	92%
	DNTR10	+	93%
	DNTR19	-	-
Grass ***	DNTR02	+	98%
	DNTR09	+	98%
	DNTR10	+	99%
	DNTR19	-	-
Faeces	DNTR02	+	93%
	DNTR09	-	-
	DNTR10	-	-
	DNTR19	-	-

*Soil sample collected from where sheep had recently stood, **soil sample from high traffic area, ***grass sample from where sheep had just been standing.

The 4 primers targeting the *D. nodosus* VNTR loci where assessed for specificity using a range of target and non-target organisms. There was no visible amplification of non-target organisms and all *D. nodosus* positive control samples were amplified (Figure 4.1). *D.nodosus* amplification was further tested on a selection of *D. nodosus* strains (Table 4.4). The MLVA assay amplified all 4 VNTR loci in all strains tested.

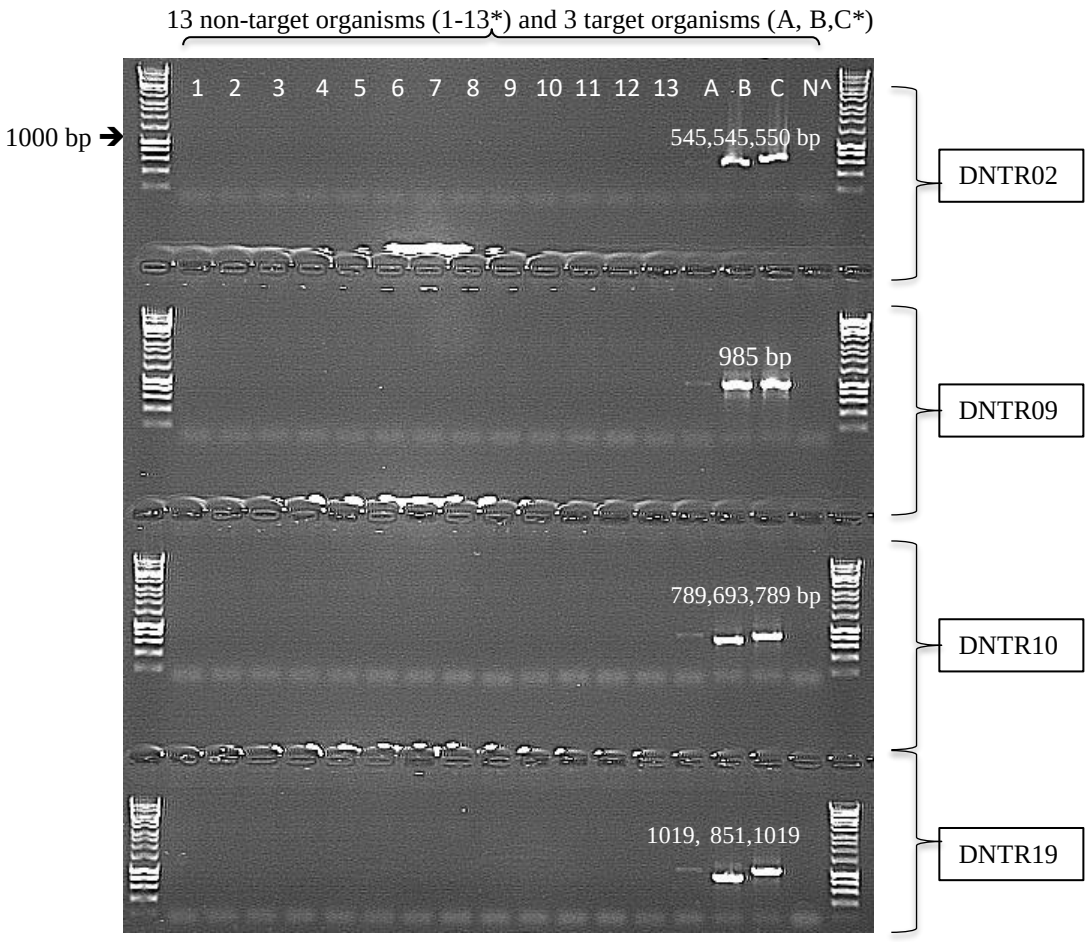


Figure 4.1: Test of non-target organism for the *D. nodosus* MLVA assay shown on 1% (w/v) agarose gel. ^**N**= Sterile water non-template control. Bioline 1 kb DNA ladder with 1000 bp marker indicated. Note that amplification of control strain C305 (lane A) is weak probably due to low DNA concentration in the sample. Legend of organisms: **1** *Staphylococcus uberis,* **2** *Staphylococcus epidermis,* **3** *Staphylococcus intermedius,* **4** *Staphylococcus aureus,* **5** *Staphylococcus hyicus,* **6** *Staphylococcus chromogenis,* **7** *Streptococcus dysgalactidae*, **8** *Streptococcus agalactidae, 9 Mannheimia spp.,* **10** *Fusobacterium necrophorum,* **11** *Pseudomonas aeruginosa*, **12** *Escherichia coli,* **13** *Mycobacterium tuberculosis,* **A** *D. nodosus* strain C305, **B** *D. nodosus strain* VCS1703A, **C** *D. nodosus* 4303 LBV.

Table 4.4: Additional *D. nodosus* strains tested for amplification of all 4 VNTR's.

Strain ID	Country of isolation*	Virulence status	Serogroups	Amplification of VNTR loci
UNE135	Australia	Unknown	Unknown	+
UNE149	Australia	Unknown	Unknown	+
CS101	Australia	Benign	Unknown	+
VCS1690	Australia	Virulent	H	+
13295C	Australia	Unknown	F	+
VCA1001 (A198)	Australia	Virulent	A	+
SP-02-418-C	Spain	Virulent	E	+
SP-02-428-C	Spain	Virulent	E	+
SP-02-473-C	Spain	Virulent	E	+
SP-02-475-C	Spain	Virulent	E	+
SP-02-508-C	Spain	Virulent	E	+
SP-02-520-C	Spain	Virulent	E	+
BS8	UK	Virulent	H	+

*For location of isolation see chapter 2, table 2.1

4.3.2 Determination of repeat sizes for the targeted MLVA loci

Sizing VNTR amplicons using sequence analysis (Russell *et al*., 2014) is not suitable for analysis where multiple strains may be present in a sample. Table 4.5 shows the comparison between MLVA loci repeat sizes based on sequencing (Russell *et al*., 2013) and those from the fragment analysis of the loci amplified from *D. nodosus* strain VCS 1703A (This study). The number of base pairs varied slightly between the amplicon length corresponding to the published VCS1703A sequences (GenBank Accession number CP000513) and the fragment size obtained from fragment analysis. Lengths however vary only by a few base pairs. The number of repeats found in the VCS 1703A sequences obtained from fragment analysis also differs at 2 loci compared to the ones determined by Russell *et al*. (2013).

Table 4.5: Description of the four VNTR loci developed by Russell *et al*. (2014) based on samples submitted for fragment analysis. All data is based on the VCS1703A genome strain (Myers *et al*., 2007).

VNTR locus	Repeat size (bp)	Sequenced amplicon length (bp)* Russell *et al*. (2014)	Number of repeats (n)* Russell *et al*. (2014)	Observed fragment ** size (bp ±2)	Number of repeats (n)**
DNTR02	5	549	6	545	6
DNTR09	108	987	5	985	5
DNTR10	48	704	7	693	6
DNTR19	84	854	3	851	3

* Amplicon length corresponding to the published VCS1703A sequences (GenBank Accession number CP000513) and VNTR amplicon length as published by Russell *et al*. (2014), ** Size of the VNTR amplicon as measured by fragment analysis and number of repeats observed in this study.

4.3.3 Sensitivity of the MLVA assay

The sensitivity of the individual *D. nodosus* MLVA primers had not been investigated previously and was investigated here by a spiking experiment using DNA of strain 4303LBV. The detection limit for the *D. nodosus* loci after one round of MLVA was 10^2, 10^3, 10^2 and 10^3 copies μl^{-1} DNA for DNTR02, DNTR09, DNTR10 and DNTR19 respectively (Figure 4.2). A second round of PCR only improved the DNTR19 locus, increasing the detection limit to 10^2 cell copies μl^{-1}. Detection limits for soil and grass samples are shown in Table 4.6. Submitting environmental samples to a second round of MLVA resulted in multiple bands on the gel; therefore, only results after one round of the assay are shown. Diluting samples did not improve detection (data not shown).

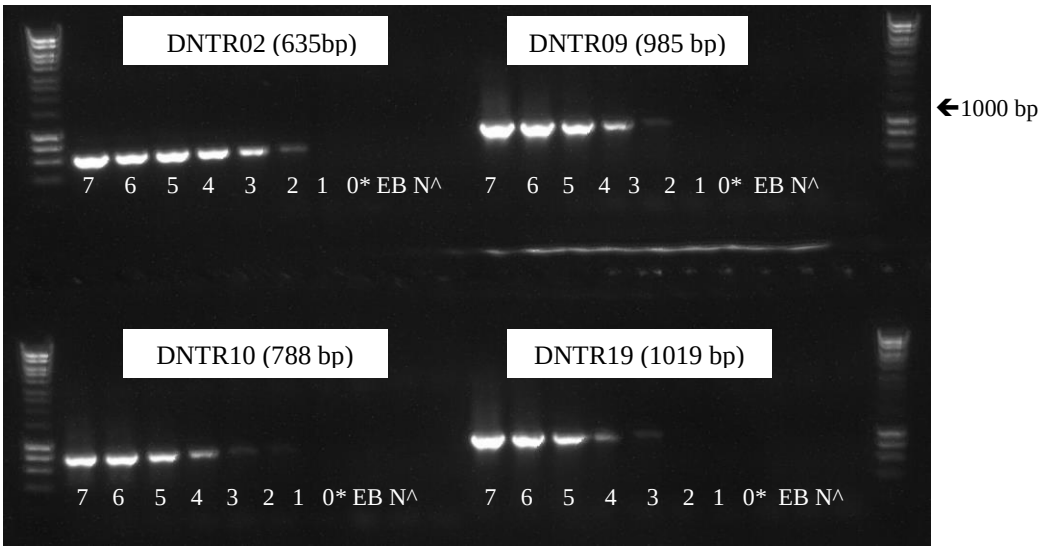


Figure 4.2: MLVA assay performed on DNA extracted from spiked swabs to determine the detection limit shown on 1% (w/v) agarose gel, *Numbers 1-7 represent *D. nodosus* samples ranging from 10^7 (7) cells to 10^0 cells (0). **EB**= Extraction blank of PBS. **^N**= Sterile water non-template control. The same set-up applies to DNTR09, 10 and 19. Bioline 1 kb DNA ladder with 1000 bp marker indicated.

Table 4.6: Detection limit observed for DNTR02, 09, 10 and 19 in cell copies μl^{-1}.

Locus	Soil	Grass	Faeces
DNTR02	10^2	10^2	10^2
DNTR09	10^3	10^3	10^3
DNTR10	10^4	10^3	10^3
DNTR19	10^3	10^3	10^3

4.3.4 Determination of minimum peak size in fragment analysis using the Dichelobacter nodosus MLVA assay

As previously reported fragment analysis revealed multiple peaks in DNA extracted from *D. nodosus* isolate samples (Figures 4.3 and 4.4). Interestingly all isolates from Spain and one from Australia did not show more than one peak per locus (Figure 4.4), indicating that the presence of technical PCR artifact is less probable as isolates were tested using the same MLVA assay and all samples were analyzed in the same run.

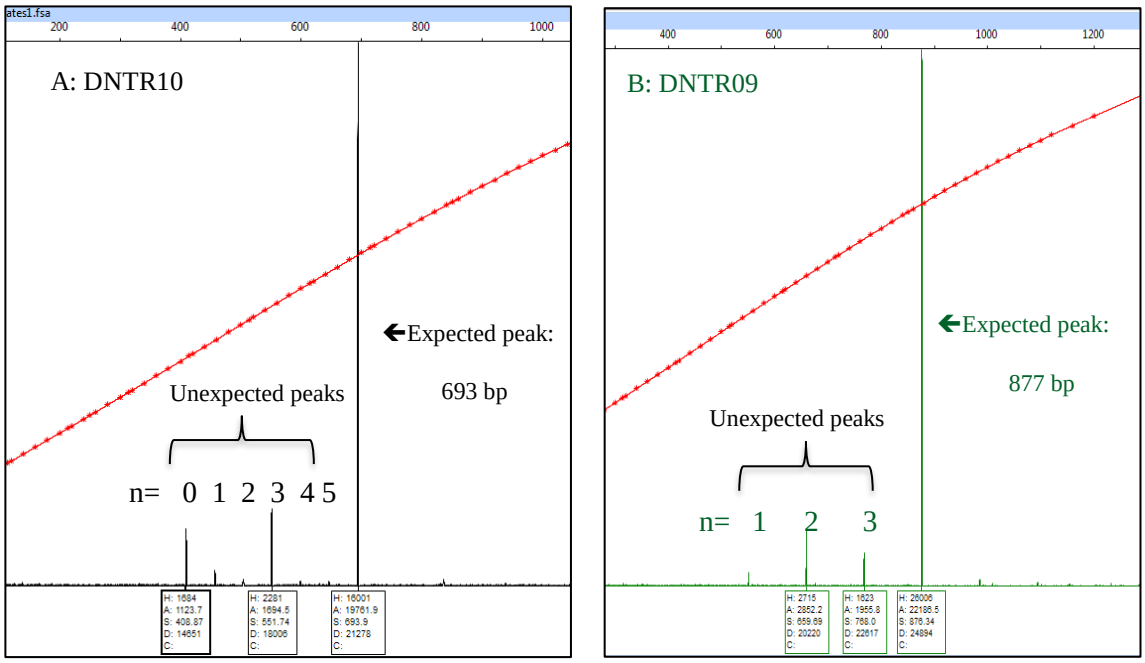


Figure 4.3: Electropherogram of the DNTR10 locus of *D. nodosus* isolate VCS1703A (**A**) and the DNTR09 locus of isolate UNE149 (**B**) after MLVA and fragment analysis. A range of unexpected peaks were observed. All peaks are multiples of 48 bp (DNTR10) and 108bp (DNTR09), corresponding to the sizes of the tandem repeats. In both panels peaks are visible at repeat sizes smaller by single units than the main peak. * **n**= number of tandem repeats that correspond to the peak.

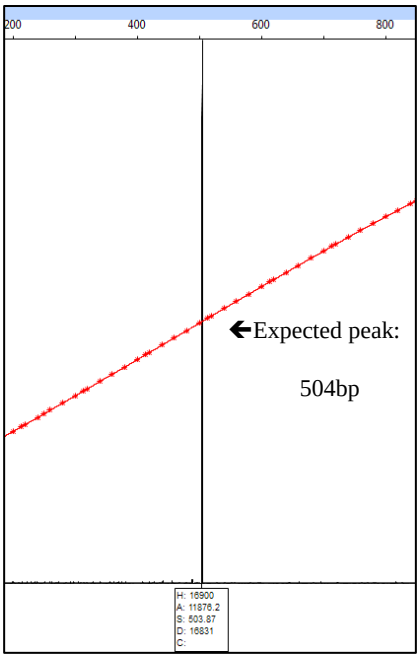


Figure 4.4: Electropherogram of the DNTR10 locus of the Australian *D. nodosus* isolate VCS1690. No unexpected peaks were observed. * **n**= number of tandem repeats that correspond to the peak

As a result of the multiple peak traces, the dominant peaks and the highest secondary peaks of all isolate DNA samples were located in all available electropherograms and sized. The largest secondary peak detected was 20% the peak of the dominant peak. Therefore, a conservative peak height baseline threshold was determined, where secondary peaks below 20% the size of the dominant peak present in the sample were assumed to be "noise" within the analysis and so were not used in the analysis of *D. nodosus* in swab and other samples.

4.3.5 Dichelobacter nodosus model communities to test the recovery of all VNTR amplicons after fragment analysis

In order to test the possible application of this assay on mixed DNA samples, recovery of DNA was tested on two model communities from a range of *D. nodosus* isolates.

Peaks corresponding to the products of the VNTR loci present of the individual isolates present in the model community were observed in all electropherograms. Although all products were recovered, some were below the 20% signal threshold set above and therefore and would have been classed as negative in a field study (Table 4.7). This *in vitro* experiment however shows that recovery of all *D. nodosus* strains in a sample is possible. As a consequence of the established threshold however, it is possible that non-dominant strains present at lower abundances within a mixed DNA sample may not be reliably identified.

Table 4.7: Input and recovery of MLVA PCR amplicons and comparison of peak sizes between those present in individual *D. nodosus* isolates and those present in the model community.

***D.nodosus* isolate**	**Size (bp)**	**Peak size (RFU)***	**SRP* (RFU)**	**Size (bp)**	**Peak Size (RFU)**	**SRP (RFU)**	**Size (bp)**	**Peak size (RFU)**	**SRP (RFU)**	**Size (bp)**	**Peak size (RFU)**	**SRP (RFU)**
Community 1	DNTR02			DNTR09			DNTR10			DNTR19		
VCS 1703A	545	15530	209**	985	11722	2323**	693	9795	182**	851	7855	219**
JIR3918	610	8091	2950	768	28924	16045	835	22117	483**	1019	3692	1621
JIR3919	650	13779	2681	876	18892	11636	646	10793	101**	1019	2078	1621
JIR3350	560	16543	4975	985	23681	12323	505	25910	8144	932	6326	1178
Community 1 (diluted)												
VCS 1703A (1:5)	545	13092	85**	985	11812	1205**	693	5382	138**	851	8624	372**
JIR3918	610	8091	4034	768	28924	16045	835	22117	1158	1019	3692	3384
JIR3919	650	37779	4501	876	18892	11363	646	10793	3786	1019	2078	3384
JIR3350	560	16543	1597	985	23681	1205**	505	25910	3030	932	6326	242**
Community 2												
VCS 1703A	545	15530	150**	985	11812	5194	693	5382	339**	851	8627	129**
JIR3918	610	8091	1874	786	28924	10421	835	22117	2322	1019	3692	872
4303 LBV	635	9853	705**	985	19638	5194	788	/***	687**	1019	2714	872
BS8	555	18255	4529	985	9345	5194	835	3475	2322	933	5191	1814

*Peak size RFU= the size of the peak observed when testing *D. nodosus* isolates individually in relative fluorescent units (relative fluorescent units), SRP=size of recovered product in relative fluorescence units when incorporated into the mixed community, **Recovered products that fall below the established threshold, ***No product size recovered.

4.3.6 Detection of the Dichelobacter nodosus VNTR loci in mixed DNA swabs and environmental samples

4.3.6.1 Study 1

Foot and oral cavity swabs and environmental samples that tested positive for *D. nodosus* qPCR (Chapter 3), were analyzed using the MLVA assay. All 4 VNTR loci were amplified in 53/152 (34.9%) swab samples from sheep feet and 3 VNTR loci amplified in 1/38 (2.63%) samples from the gingival cavity (DNTR19 did not amplify). The DNTR02 locus was amplified in 1 sample originating from soil (LTA, 0-1cm). Fragment analysis of this positive soil sample gave a product of 570 bp (*n*=11), which was not observed in mixed DNA swabs. There was no amplification of any grass and faecal samples. Almost all samples that yielded incomplete DNA profiles or that tested negative for any of the 4 VNTR's had *rpoD* counts at or below the MLVA DL determined (Data not presented).

4.3.6.2 Study 2

All 4 VNTR loci were amplified in 41/460 (8.91%) swab samples from sheep feet. Only 3/41 (7.32%) samples originated from 2/13 additional sheep analyzed from weeks 1-3. 38/41 samples that amplified originated from two diseased sheep. 26/41 samples originated from sheep 3488 and amplification of all 4 loci was achieved for all weeks except week 16. 13/41 positive samples originated from sheep 3535 and ranged from weeks 1-9. Amplification coincided with the period of disease occurrence in both animals. Amplification of the DNTR02 locus alone was achieved in 70/768 samples, probably due to its lower detection limit.

Only 3 soil samples with a bacterial load higher than 50 genome copies μl^{-1}, were submitted for fragment analysis. Interestingly the three samples originated from samples taken from the baseline pasture on the day before the study group entered the field and after the 10-day vacant period. Only the DNTR02 locus amplified in 2 samples showing a product of 555 bp, which coincides with the DNTR02 VNTR amplicon that dominates in the flock and throughout the trial. One sample was taken from a HTA at 4-5 cm and the other from a LTA at 0-1 cm. No amplification was observed in samples originating from the oral cavity or from faeces.

4.3.7 MLVA profile of isolates and mixed DNA samples

Across both studies, there were fewer samples with multi-peak profiles compared to the isolates tested in section 4.2.7 and peaks tended to be smaller. The maximum secondary peak size observed below the 20% threshold size was 18.07% and average was 7.29%. The minimum secondary peak for the sample to be classed as positive for *D. nodosus* was 32.8% of the major peak, but averaging 52.37% of the main peak. This was a clear divide between peaks that are positive and those below the threshold, with few borderline samples. A typical electropherogram showing the size of the VNTR amplicons as measures by fragment analysis can be seen below (Figure 4.5).

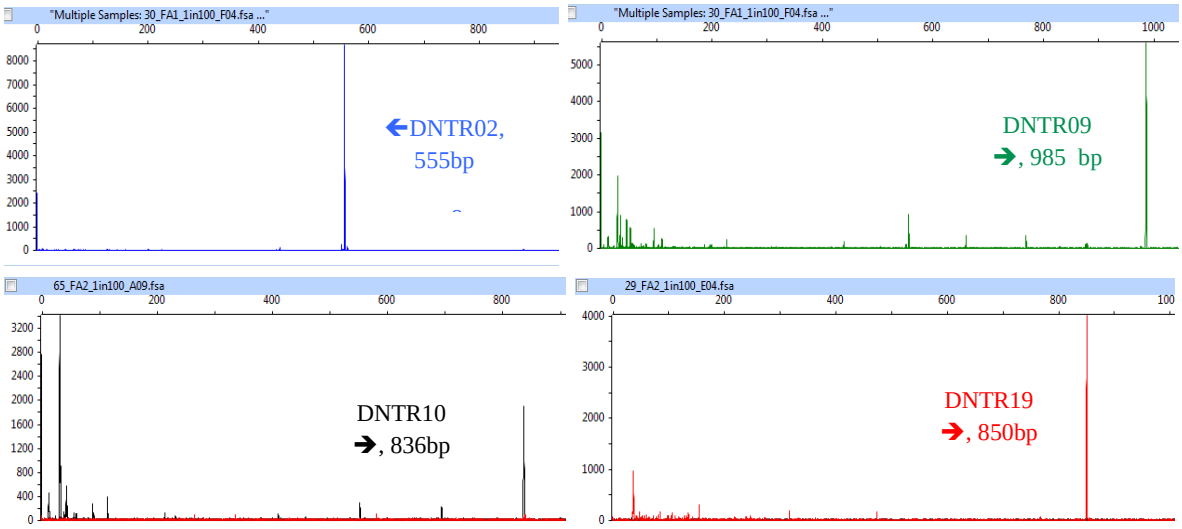


Figure 4.5: Electropherogram showing the peaks and size (bp) of the 4 amplified VNTR loci from a sample taken from an ovine foot (sheep 4388, left front foot, week 4, study 2). Secondary peaks can be seen in the traces. * **n**= number of tandem repeats that correspond to the peak.

4.3.7.1 Study 1

Complete MLVA strain profiles were obtained from 2 feet that had both ID and SFR, 3 feet with ID and 34 feet that were classed as healthy (ID scores ≤ 1 and no SFR). A total of 25 potential strains were identified (Table 4.8). 16/25 strains were classed as "present", whereas the remaining 9 strains were classed as "possibly present" as determined by the classification system outlined in Table 4.1, section 4.2.9.3. *D. nodosus* was successfully isolated and cultured from 7 foot swabs from 4 sheep in week 1 and 3. Two isolates were obtained from gingival swab samples of 2 sheep collected in week 3. Both gingival cavity isolates had an identical MLVA profile, with 2 variants at DNTR02 in both cultures, indicating that neither

were axenic but contained 2 strains. Fragment analysis of the 3 amplified VNTR's of the mixed DNA gingival cavity swab sample revealed the same amplicons. Interestingly the 2 *D. nodosus* strains observed in the gingival cavity correspond to the 2 strains (types 4 and 5) that were cultured from foot swabs (Table 4.9). The isolated strains were the 2nd and 3rd most frequently occurring strains "present" in the mixed DNA samples. The most frequently occurring strain (strain type 3, Table 4.8) was not isolated and varied from strain 4 and 5 by only one (strain type 4) and two (strain type 5) 5 bp repeats at the DNTR02 locus. In 5/7 cases strains isolated from foot swabs corresponded with strains present on the swab from the same foot at the same time (Table 4.9). There were no swab data available for one sample as no MLVA amplification was observed (sheep 13 right rear foot, Table 4.9) and the MLVA strain profile of the swab (sheep 3647 right front foot, Table 4.9) from a second sample did not correspond with the relevant cultured isolate, varying by one repeat at the DNTR02 locus.

Table 4.8: Strains detected on the feet of sheep, highlighting the 3 most frequently occurring stains

MLVA strain type	Strain type is present	Strain type is possibly present	Number of times strain type was isolated
1	1	2	0
2	1	2	0
3	10	12	0
****4**	7	9	2
****5**	5	8	5
6	2	5	0
7	2	8	0
8	5	10	0
9	1	4	0
10	1	1	0
***11**	0	1	0
***12**	0	1	0
13	1	2	0
14	1	1	0
15	1	1	0
***16**	0	1	0
17	1	2	0
18	1	1	0
***19**	0	1	0
***20**	0	1	0
***21**	0	1	0
***22**	0	2	0
23	1	2	0
***24**	0	1	0
25	1	1	0

*It is unknown whether these strains exist. **Strains were isolated after culturing

Table 4.9: MLVA profile of *D. nodosus* isolates obtained through culturing and comparison with MLVA profiles in mixed DNA samples

Sample origin	Sheep	Week	Locus Detected fragment sizes /repeat number				Culture strain type*	Strain type present in swab?
Foot			DNTR02	DNTR09	DNTR10	DNTR19		
RR	13	1	565/10	985/5	789/8	1019/5	5	+
RF	3647	1	565/10	985/5	789/8	1019/5	5	^
LR	3647	1	565/10	985/5	789/8	1019/5	5	+
RF	5582	3	560/9	985/5	789/8	1019/5	4	+
RR	13	3	565/10	985/5	789/8	1019/5	5	^^
LR	13	3	565/10	985/5	789/8	/	5**	+
LR	5	3	560/9	985/5	789/8	1019/5	4	+
Mouth	13	3	560,565/9-10	985/5	789/8	1019/5	4,5	^^
Mouth	3	3	560,565/9-10	985/5	789/8	1019/5	4,5	^^^

*Allocated strain type: ^Swab data indicates presence of strain number 3 only, a 565 bp product was present in the electropherogram but classed as below the detection threshold, ^^no swab data available, ^^^ DNTR19 did not amplify in the gingival cavity swab, but remaining VNTR's are identical, ** DNTR19 did not amplify, but strain number 5 was present in the corresponding swab.

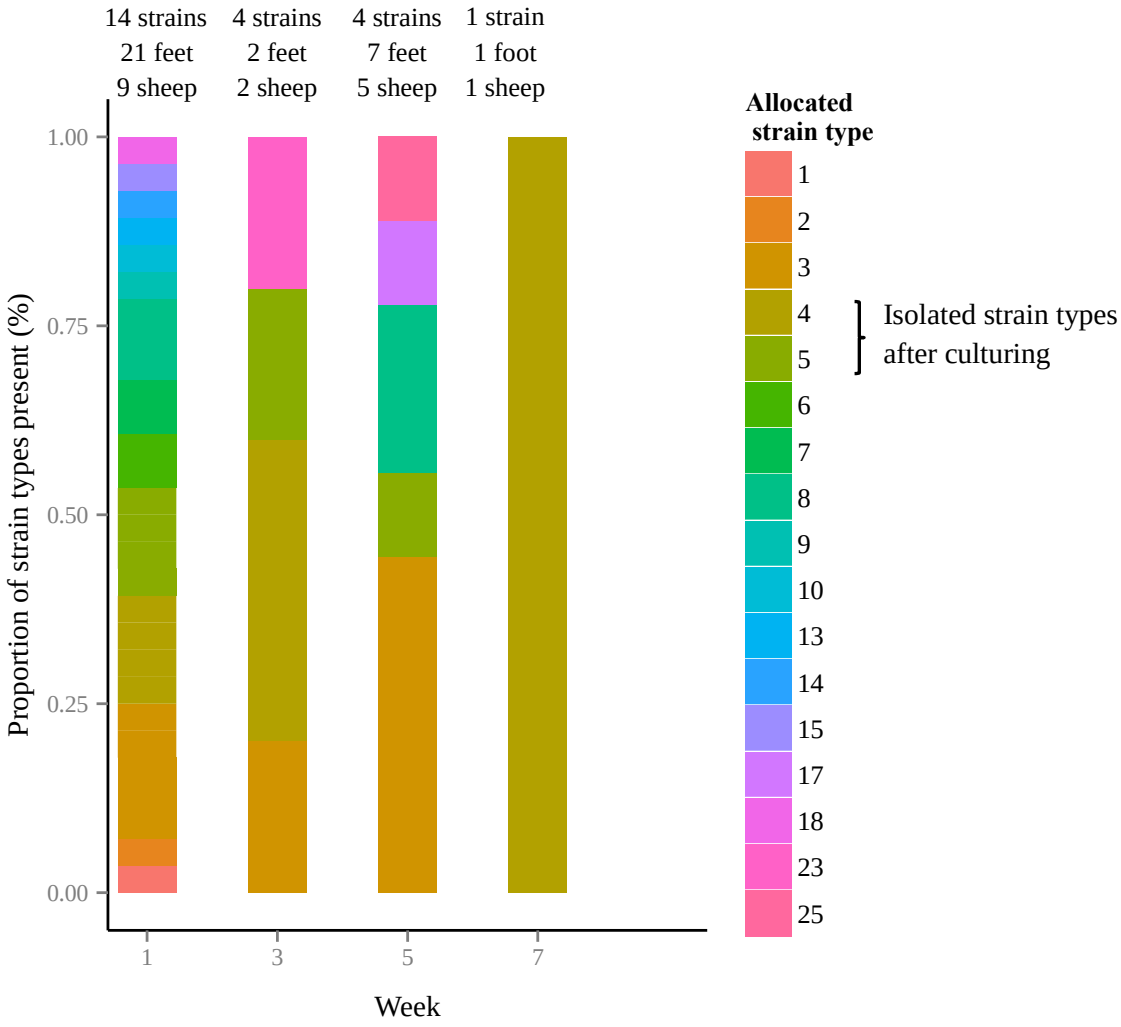


Figure 4.6: Strains classed as "present" on the feet of sheep in study 1 over a 2-month sampling period with fortnightly sampling visits (Weeks 1, 3, 5, 7). Strains that were successfully isolated are indicated. Each bar is labeled within the number of strains present, and the number of feet and sheep where the strains were present.

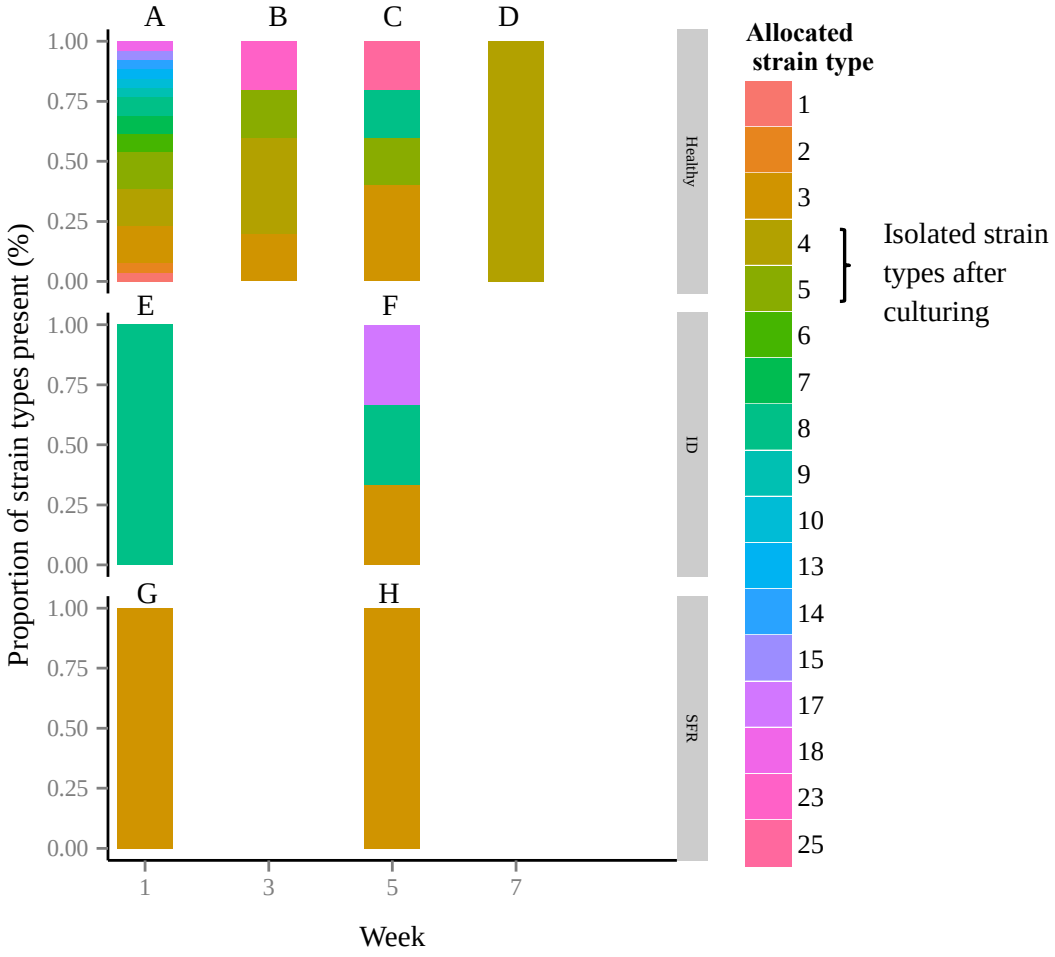


Figure 4.7: Strains classed as "present" on the feet of sheep according to the disease state of the foot (Healthy, ID, SFR). in study 1 over the two-month sampling period with fortnightly sampling visits (Week 1, 3, 5, 7). Strains that were successfully isolated are indicated. Number of strains, feet and sheep at each sampling point: **A**=14 strains/19 feet/ 8 sheep, **B**=4 strains/3 feet/3 sheep, **C**=4 strains/4 feet/4 sheep, **D**=1 strain/1 foot/1 sheep, **E**=1 strain/1 foot/1/1 sheep, **F**=3 strains/2 feet/2 sheep, **G**=1 strain/1 foot/1 sheep, **H**=1 strain/1 foot/1 sheep.

Strains on sheep feet varied by foot, sheep and over time. Most samples that amplified all 4 VNTR loci were obtained in week 1 (Figure 4.6). Strain number 4 and 5 (from which cultured *D. nodosus* isolates were obtained) and strain number 3 were the most frequently present in the community. Only strain 4 was detected on 2 feet from 2 different animals diagnosed with FR (Figure 4.7).

4.3.7.2 Study 2

Forty full MLVA profiles (All VNTR's amplified) and 28 partial MLVA strain profiles (≥ 1 VNTR did not amplify) were obtained from 68 feet in study 2. Thirty-seven full profiles were obtained from the two sheep that became diseased: Samples from sheep 3488 and 3535 yielded complete MLVA strain profiles from week 1-20 and 1-9 respectively. Three additional full profiles were obtained from 3 other sheep in week 1. With the exception of 1 MLVA profile in week 1 which indicates presence of 2 or more strain of *D. nodosus* in the sample, all full profiles (39/40) indicate presence of one single strain of *D. nodosus* (Table 4.10).

Partial MLVA profiles coincide with this result, but their nature also suggests that a larger number of *D. nodosus* strains was present on feet in week 1: The DNTR02 locus was the only locus that was detected in 97.4% of samples (67/68). It was also the only locus that was detected consistently in week 1. Finally, it was the locus that showed the most variation, which is in agreement with Russell *et al*. (2014). This locus is therefore a good indicator of the minimum number of strains present. A minimum of 4 strains were present in the flock in week 1, but only 1 strain was subsequently detected (with one exception) on the two sheep that became diseased (Figures 4.8 and 4.9).

Table 4.10: VNTR amplicon that was amplified by each of the DNTR primers, showing the size of the product and the number of repeats associated.

	DNTR02/ repeats	**DNTR09/ repeats**	**DNTR10/ repeats**	**DNTR10/ repeats**
Product(bp)/repeat number	555 bp/8	985bp/4	836 bp/7	850bp/3

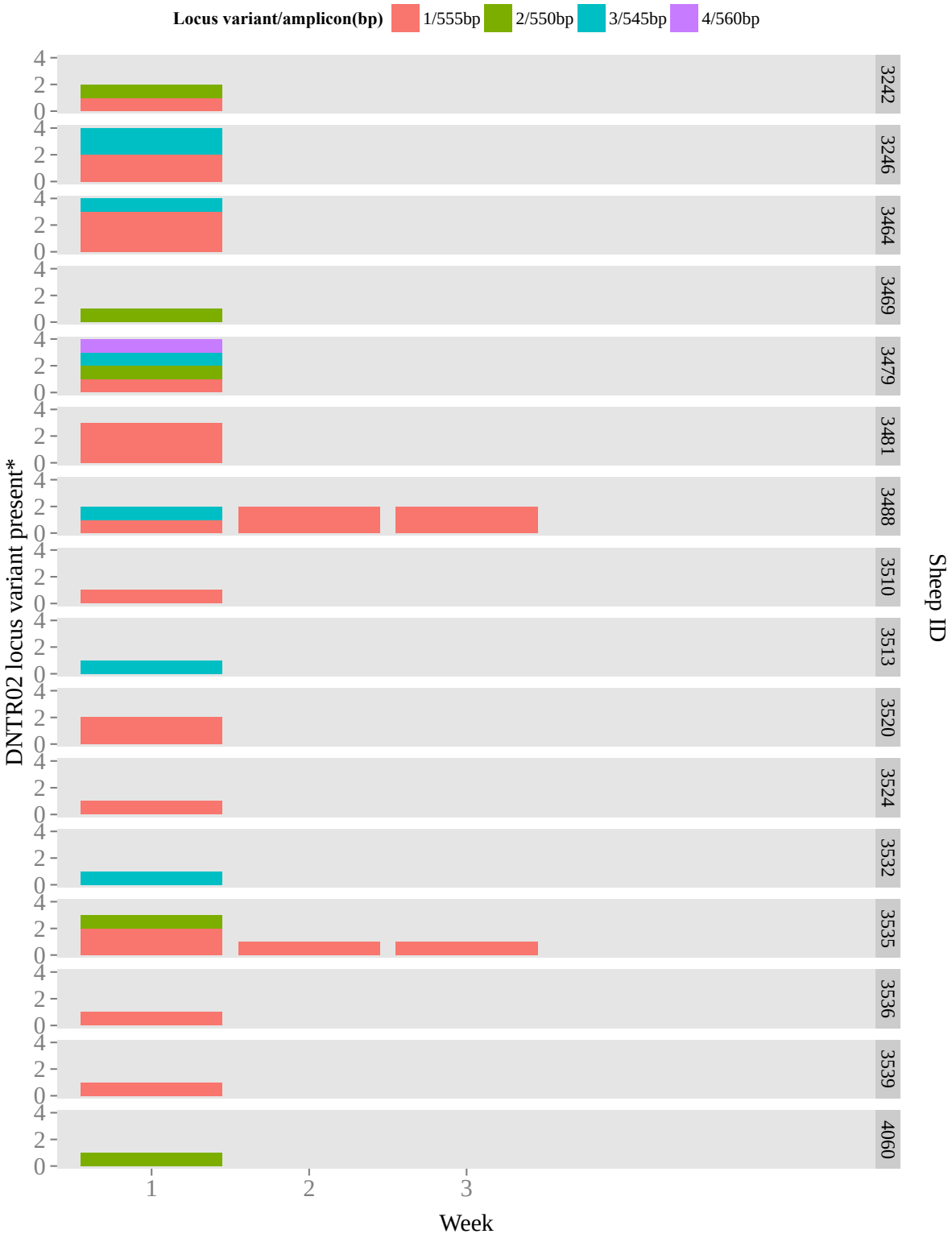


Figure 4.8: DNTR02 locus variants present on the feet of sheep in week 1, 2 and 3. Data is available for all the sheep of study 2 during that time period.

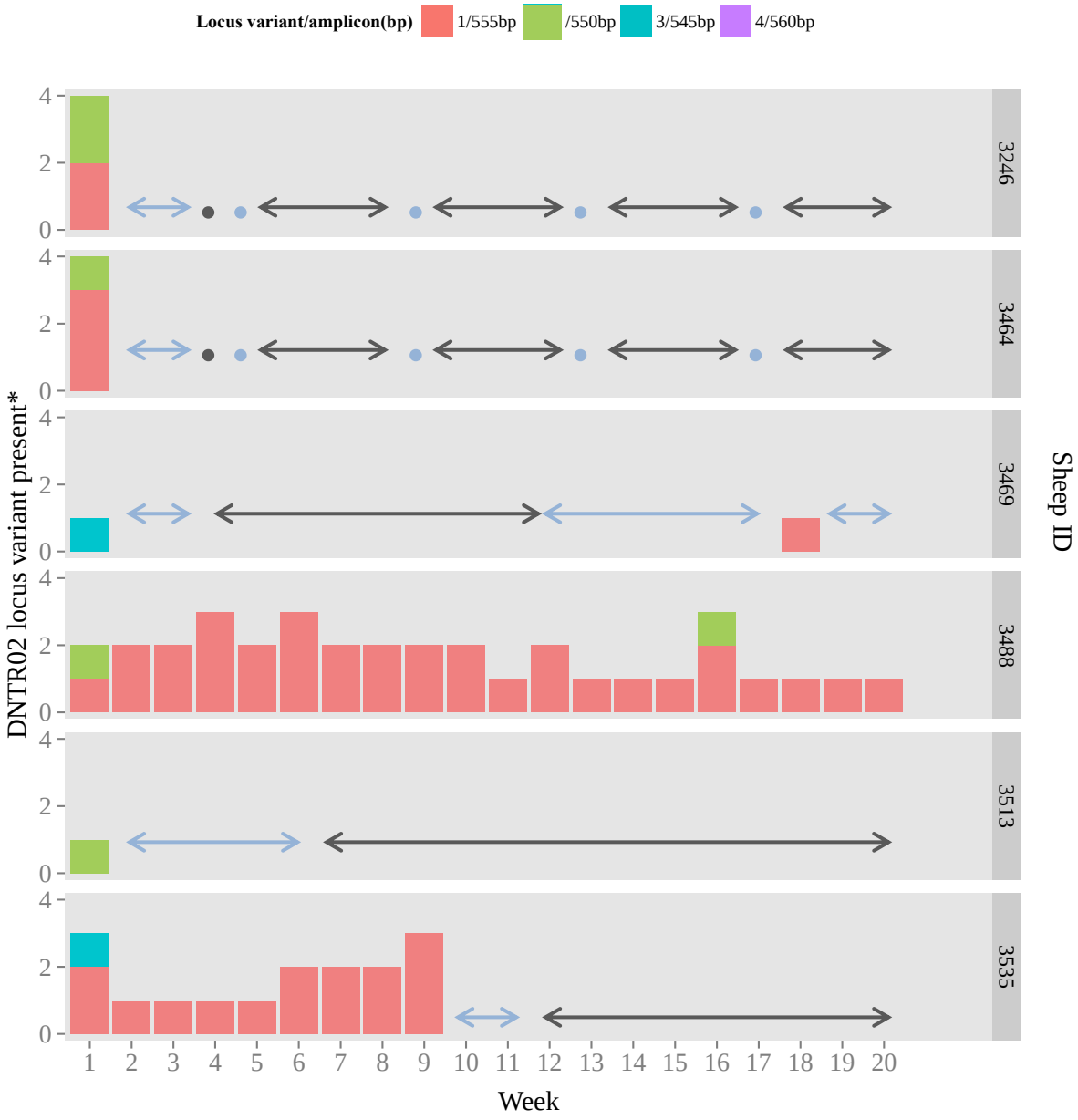


Figure 4.9: DNTR02 locus variants present on the feet of sheep in week 1-20. The figure shows 6/7 sheep that were analyzed over this time period (No amplification was observed for one sheep). Time periods where no data was available (samples not analyzed →) and time periods where the sample was negative (*D. nodosus* not detected →) are indicated.

4.4 Discussion

The aims of this study were to test the suitability of the *D. nodosus* MLVA assay developed by Russell *et al*. (2014) to investigate animal and environmental samples and to test whether the assay can provide information about bacterial persistence of *D. nodosus* when applied to mixed DNA samples collected in a field study.

The use of MLVA on mixed DNA samples to investigate pathogenic bacteria in animals has been described in the literature on a number of occasions. In all cases MLVA profiles were allocated to samples on the basis of the number of repeats present at a locus, providing information on allelic distribution and diversity (Vranckx *et al*., 2011; Dugat *et al*., 2014; Ceglie *et al*., 2015; Muzafar *et al*., 2015; Dugat *et al*., 2016).

Most authors did not focus on persistence, but investigated genotypic variability of pathogenic bacteria between sample types or species, using a limited number of samples, widespread sampling intervals, and did not sample the same animals repeatedly (Vranckx *et al*., 2011; Dugat *et al*., 2014; Ceglie *et al*., 2015; Muzafar *et al*., 2015; Dugat *et al*., 2016). One study investigated within-herd persistence of strains, without repetitive sampling of individuals (Vranckx *et al*., 2011). Recently, Smith *et al*. (2017) used the *D. nodosus* MLVA to investigate the population dynamics of *D. nodosus* in flock of sheep and showed that strains persisted in the flock, although there was only a small number of repetitive sampling events. In addition, Smith *et al*. (2017) used *D. nodosus* isolates from culture and did therefore not provide information of the whole *D. nodosus* community present on the foot at any time. Muzafar *et al*. (2015) aimed to investigate *D. nodosus* communities present in a sample, but only used 2/4 loci. By improving the assay, we were able to use the complete set of 4 *D. nodosus* VNTR loci and we developed a novel approach for a community based analysis that can identify individual strains in a sample and follow them over time.

Here we show for the first time that it is possible to identify individual strains that occur most frequently during an outbreak and/or are the most detectable dominant strains in a sample (studies 1 and 2) and that these strains can be isolated through culturing (study 1). We show that it is possible to track strains within a host overtime, which has been described as one of the main challenges when investigating transmission and pathogenesis in multistrain infections (Balmer and Tanner 2011).

Whereas the results of study 1 show no real consistency with regards of strains over time (likely due to some of the limitations discussed below), study 2 clearly shows that one dominant *D. nodosus* strain detected on the same feet throughout an episode of disease, clearly showing that this strain persists. *D. nodosus* is present on healthy feet in week 1 and then every following week on the healthy feet of two sheep before they became diseased (Chapter 3) and the same strain that later became the dominant strain present on feet at all those time-points.

The results of both study 1 and 2 show that multiple *D. nodosus* strains can be present on the same foot, which has been described by other authors (for example Muzafar *et al*., 2015). In both studies the largest variety of strains was observed during the first sampling visit. Fourteen different strains (classed as "present") were observed in week 1 of study 1. Disease peaked in week 5, but only 4 strains were detected. This may be attributable to the large number of *D. nodosus* positive samples in week 1. It also is possible that the wet climate on the day led to the increased detection of *D. nodosus* in soil and grass samples (see chapter 3) and this may have caused increased transmission of strains of *D. nodosus* between sheep and feet. The results of study 2 suggest that the variety of strains observed in week 1 was attributable to the study group still being part of the study population, suggesting that multiple strains were present in the study population. It is likely that by selecting only healthy animals some strains were not carried over to the study group when located to a new pasture.

The results of study 1 show that the strains isolated from the gingival cavity and from feet (in clinical mixed DNA swabs and isolated through culture) were identical. Although *D. nodosus* was isolated from mouth samples on only 2 occasions (from 2 sheep) it is likely that the oral cavity becomes contaminated with *D. nodosus* originating from the feet. Both strains were isolated from samples originating from week 5 when disease occurrence was highest. This supported by evidence from chapter 3 that suggests that *D. nodosus* is more likely to be present in the gingival cavity when sheep have FR.

Three main limiting factors were identified in terms of the assays suitability. The detection limit of the *D. nodosus* MLVA PCR had not been tested by other users and was the main limiting factor. Although the optimized protocol described in this chapter improved the detection limit, it was not possible to detect all 4 VNTR loci in samples with low *D. nodosus* loads. Only the DNTR02 locus was amplified consistently in foot samples from week 1 of study 2. In order to gain an understanding of the *D. nodosus* communities present in week 1, only this locus was used to identify strains. The DNTR02 locus is however the most variable

locus (Russell *et al*., 2014) and the majority of the variation in samples were all 4 loci amplified was seen at this locus. Therefore, the DNTR02 locus provides a good indication of the minimum number of strains present in the sample.

The high detection limit also presented a challenge when testing low load soil and grass samples and only the DNTR02 locus amplified in some of the soil samples tested. Hence it was not possible to compare strains found in the environment with strains found on the sheep. This was also not possible for faecal samples, as no amplification was observed after the *D. nodosus* MLVA PCR. Sequencing of the amplicons from soil, grass and faecal samples however showed that, although not all VNTR's were detected in the validation assay, amplification was achieved. Therefore, the assay may be more useful when disease prevalence is high in a flock.

Another limit of the assay was the detection of multiple peaks in samples from *D. nodosus* isolates after fragment analysis. As a consequence of the high threshold level set in this study, it is likely that a complete *D. nodosus* community profile was not obtained and that only the most dominant strains present in the sample were detected. Therefore, it cannot be assumed that only one strain was present on the feet of the two diseased sheep. Other strains may have been present, but falling below the threshold and/or detection limit. The presence of such peaks has not been reported in the available literature; probably due to the MLVA being exclusively used on isolates and sizing of the repeats based on gel electrophoresis or sequencing rather than fragment analysis (For example Van Belkum *et al*., 1997; Le Fleche *et al*., 2006; Eisenberg *et al*., 2016; Dugat *et al*., 2014. See also Tables 1.2 and 1.3 in chapter 1). Interestingly secondary peaks were not observed in all isolates even when tested in the same PCR run, suggesting that they are unlikely to be an artifact of the *D. nodosus* MLVA PCR. It is possible that the *D. nodosus* cultures had more than 1 strain or that *D. nodosus* strains may display a fast mutation rate or genomic polymorphism between strains (Lindstedt, 2005; Le Fleche *et al*., 2006). Further investigation of this was however outside the scope of this project.

In both studies strains were allocated as either "present" or "possibly present" when different peak sizes were observed at more than one locus. Therefore, samples without clear evidence of strain presence were excluded from analysis and graphical presentation. This also reduced the tests ability to provide complete strain profiles.

The MLVA assay was optimized by changing the Mastermix and increasing the primer concentration as well as the number of PCR cycles. This improved the sensitivity of the assay considerably. Increasing the number of cycles could have resulted in decreased specificity. However, Sanger sequencing results showed that the assay remained specific to *D. nodosus*.
For future uses of the *D. nodosus* MLVA, optimizing the primers individually could further improve the detection limit. An investigation into the nature of the secondary peaks observed when submitting *D. nodosus* isolates for fragment analysis may be investigated to lead to a reduction of the threshold level. This may provide a more complete strain community profile. In addition, we recommend testing of a larger number of samples to validate the use of the *D. nodosus* MLVA assay on mixed DNA samples further. By using this assay for testing a flock with higher FR prevalence than observed here, it may be possible to investigate strain communities according to the disease state of the sheep. This was not possible here due to the low number of diseased sheep where amplification of all 4 loci was observed. Detection of samples originating from the gingival cavity, faeces and the environment could also be improved this way.

The *D. nodosus* MLVA assay has the potential to be used on mixed DNA samples and allowed detection of strains present on the feet of sheep over time and informed on the *D. nodosus* bacterial community on feet at the time of sampling. Firstly, we have shown that dominant strains can be identified in mixed DNA samples and that these strains can be isolated through culturing. This potentially allows for further molecular analysis of such strains in order to identify characteristics that indicate which *D. nodosus* strains (if any) are more likely to cause disease and may provide valuable information on the pathogenesis of ovine FR. Secondly, we have clearly shown that a strain can persists over time on the feet of sheep. Further optimization of the assay may result in a more complete picture of the *D. nodosus* strain communities present in mixed DNA samples.

CHAPTER 5

Persistence of *Dichelobacter nodosus* during periods of non-transmission in Southern Spain

5.1 Introduction

The UK is characterized by a temperate maritime climate (Kottek *et al*., 2006), where FR prevalence increases during the spring and autumn, but new cases can occur throughout the year (Green *et al*., 2007; Ridler *et al*., 2009). Therefore, FR is endemic in the UK.

In countries with arid, semi-arid and tropical climates the disease pattern of FR is epidemic and highly seasonal and sheep are thought to self-cure during non-transmission periods in hot and dry weather. For example, in some areas of Australia such as New South Wales and Western Australia spread of FR is observed in the spring after periods of high rainfall (Parsonson *et al*., 1967; Graham, 1968; Stewart, 1989). In Brazil, frequent outbreaks of FR are observed in the semiarid southern regions after the rainy season (Aguiar *et al*., 2011) and similarly in Ethiopia FR spread is observed during the wet season from July to September (Chanyalew and Alemu, 2014). In tropical Southern India outbreaks occur in the rainy season from June to December (Sreenivasulu *et al*., 2013). Some studies in Australia investigated the effect of rainfall and ambient temperatures on disease parameters and persistence. They investigated the prevalence of FR lesions over time as opposed to detection and/or quantification of *D. nodosus* over time (Graham, 1968; Abbot and Egerton 2003).

The results from the 2 previous chapters show that *D. nodosus* can be present on healthy feet in high loads, which has been reported by other authors (Moore *et al*., 2005). The results of chapter 3 and 4 indicate also that *D. nodosus* may be more likely to persist on the feet of sheep and suggest that the gingival cavity of sheep, faeces and the pasture may not be significant reservoirs of *D. nodosus* due to its transient presence at these sites. These studies were conducted in the United Kingdom where an extended period of non-transmission is absent due to the endemic nature of the disease. Therefore, it would be informative to sample sheep in a country with a long period of non-transmission.

Ovine FR is an animal health concern in Spain. Most research focuses on *in vitro* investigation of the aetiology and molecular epidemiology of the disease (Piriz *et al*., 1991;

Hurtado *et al*.; 1998, Lacombe-Antoneli *et al*.; 2006, Martin-Palomino *et al*.; 2004, Maboni, 2017). As in other arid countries FR is reported to occur in epidemics during the wet periods (Fernandez *et al*., 1996). The region of Andalucía in Southern Spain is characterized by subtropical mediterranean climate (Kottek *et al*., 2006) and has the highest daily summer temperatures in Europe. Precipitation levels are highest from October to April, peaking in the autumn. It generally does not rain in June, July and August (Agencia Estatal de Meteorología [AEMET], 2017). FR prevalence increases in spring, and although no published scientific evidence exists, farmers anecdotally report no cases of FR during the summer months.

The aim of this study was to elucidate where *D. nodosus* is most likely to persist in arid climates by sampling sheep and the environment on two farms in Southern Spain during a period when sheep are diseased and during a period of non-transmission in the summer.

5.2 Materials and Methods

5.2.1 Research collaboration and ethical approval

This study was conducted with the collaboration of the University of Cordoba (School of Veterinary Science), Spain. Prior to commencement of this study, ethical approval was obtained by from the Spanish authorities (*Consejería de Agricultura, Pesca y Desarrollo Rural, Dirección general de la Producción Agrícola y Ganadera, Junta de Andalucía*, Document number 201645000004208).

5.2.2 Farms and Animals

Two sheep flocks from two different farms in the region of Andalucía, Spain where chosen for the study. Farms were sourced and selected on the basis of the information obtained from the collaborating veterinarian, who confirmed history of footrot and annual re-occurrence of the disease in both flocks. In addition, both farms were visited prior to commencement of the study in order to assess their suitability. Farmers that agreed to participate in the study confirmed that no replacement animals would be purchased throughout the duration of the trial and that sheep movement would be limited within the boundaries of their properties.

All animals were Merino sheep (Farm 1) or Merino Crosses (Farm 2) rearing lambs for meat production. The locks sizes were approximately 950 (Farm 1) and 900 (Farm 2). Farm 1 was located in the hills of northern Andalucía and Farm 2 was located on the outskirts of Cordoba, Andalucía.

5.2.3 Sampling procedure: Sheep

All materials required for sampling sheep were transported to Spain from the UK. Sheep sampling procedures are described in detail in section 3.3.2 (Chapter 3). During the first visit in April all sheep were screened for lameness. Lame sheep where examined for footrot lesions and foot swabs, gingival cavity swabs and faecal samples were obtained from 5 diseased sheep. An additional 30 sheep were screened for footrot lesions in order to assess the disease status of the herd and 5 sheep without foot lesions were sampled as controls. During

the second visit in July the same sheep were identified and sampled. When an animal could not be located, another animal was randomly selected and screened or sampled.

5.2.4 Sampling procedure: Environment

Environmental sampling procedures are described in detail in section 3.3.2. (Chapter 3) On both visits (April and July) soil samples were collected at 0-1 cm and 4-5 cm from low traffic areas (LTA) and high traffic areas (HTA) and grass samples were collected if present. Three HTA's and one LTA were identified on each farm with the help of the farmers' knowledge of sheep movements. HTA's were defined as "gate", "trough", and "paddock 1" on Farm 1 and "tree", "stream" and "paddock 2" on Farm 2 (Figure 5.1, B-D). Paddock 1 and 2 were the area where sheep where handled on the day of sampling. LTA 's were defined as "field" (Figure 5.1, A) and "hill" on F1 and F2 respectively. During the second visit in July the same areas were sampled.

A

B

C

D

Figure 5.1: Example of high traffic areas (HTA's) and low traffic areas (LTA's) selected. **A**: LTA named "Hill" on farm 2 sampled in this study. **B-D**: HTA's chosen for the study. **B**=Bank of a stream where sheep travel for drinking, **C**: Paddock next to the main farm, **D**: Tree next to the paddock were animals seek shade.

5.2.5 Sample storage and shipping

All samples were stored at the veterinary department of the University of Cordoba. Environmental samples and faecal samples were kept at -20 °C and swabs were kept at -80 °C. The day prior to the planned shipping day environmental samples and faecal samples were weighed (0.5g of soil, 0.2 g of grass, 0.1 g of faeces) and transferred into 1.5 ml sample tubes filled with 0.5g of micro glass beads, ready to be processed for DNA extraction. Samples were shipped on dry ice to the School of Life Sciences, University of Warwick using the DHL 24-hour medical express delivery service and arrived frozen.

5.2.6 Collection of climate data

Information on ambient temperature and rainfall was collected from the Spanish Meteorology Institute (Agencia estatal de Meteorología-Gobierno de España) in the form of published monthly climate summaries, providing average temperature and total rainfall data for cities in Andalucía, including Córdoba. Climate deviations from normality were also provided by comparing monthly temperature and rainfall with national averages originating from a reference period between 1981 and 2010. According to this climate was classed as being "very humid", "humid", "dry" and "very dry" on the basis of total rainfall and "very cold", "cold", "hot" and "extremely hot" on the basis of average ambient temperatures.

5.2.7 Laboratory analysis

DNA extraction was performed according to the protocol described in section 2.3 (Chapter 2) and qPCR was performed as a measure of detection and quantification on all samples following the protocols described in section 2.9 (Chapter 2). Samples that tested positive for *D. nodosus* were also submitted to the MLVA PCR and fragment analysis following the protocols in section 2.11 (Chapter 2).

5.3 Results

5.3.1 Climate in Córdoba from November 2015 to April 2016

The climate in Cordoba in April 2016 was very humid with temperatures averaging 16.2 °C and a total rainfall of 122.7 mm which is 223% above the regional average. Whereas precipitation levels were indicated as “normal” in March and January, the months of February, December and November where classed as “dry” or “very dry”, with total rainfall being 68 % (53.1 mm), 18 % (12.3 mm) and 50 % (55.3 mm) of the city’s average respectively. Farmers on both study farms described the preceding winter as unusually warm with little rainfall.

5.3.2 Climate in Córdoba from May 2015 to July 2015

May 2016 was described as an extremely humid, but “cold” month with temperatures averaging 19.3 degrees and a precipitation level of 271 % (106.2 mm) of the regional average. No rainfall was reported in June and July and climate was classed as “hot” and “extremely hot” respectively.

5.3.3 Farm 1

5.3.3.1 Disease status of the flock on Farm 1 in April

The collaborating veterinarian confirmed an outbreak of FR during the week preceding the sampling date. Diseased sheep had received antibiotic injection treatment one week prior to the sampling day. Only 5 lame sheep were observed in the flock and subsequently sampled. All 5 sheep had received an antibiotic injection the previous week. Three sheep presented with active SFR lesions. Of the 30 sheep that were screened for lesions only, 9 were lesion-free and classed as healthy. Eighteen sheep presented with SFR1 lesions and one sheep presented with ID2 lesion on one foot.

5.3.3.2 Disease status of the flock on Farm 1 in July

Out of the 10 sheep sampled in April, 9 sheep were located and sampled in July. Out of the 30 animals screened for FR lesions in April 25 were located and re-screened. Five additional randomly selected animals were screened to compensate for animals that could not be located. None of the 30 screened animals presented with ID or SFR lesions and no lameness was observed. Two of the animals that had been sampled and were diseased in April were lame. One animal was lesion-free, however, the other presented with extensive damage to the hoof with an active lesion in the interdigital skin consistent with myiasis (parasitic larvae infestation) as diagnosed by the veterinarian.

5.3.3.3 Dichelobacter nodosus bacterial loads and communities on sheep and in the farm environment in April and July

In April, out of the 40 samples collected from the feet of the 10 selected sheep, 3 samples (7.5%) from 2 diseased sheep tested positive for *D. nodosus*, with bacterial loads ranging from 8.54×10^6 to 1.02×10^7 *rpoD* genome copies per swab. MLVA detected presence of a single strain, without evidence of a mixed community of *D. nodosus* and none of the secondary peaks discussed in chapter 3 were observed (Figure 5.2). The strain observed had a 504 bp product at the DNTR10 locus, which was not observed in studies 1 and 2. No *D. nodosus* positive foot samples were detected in July.

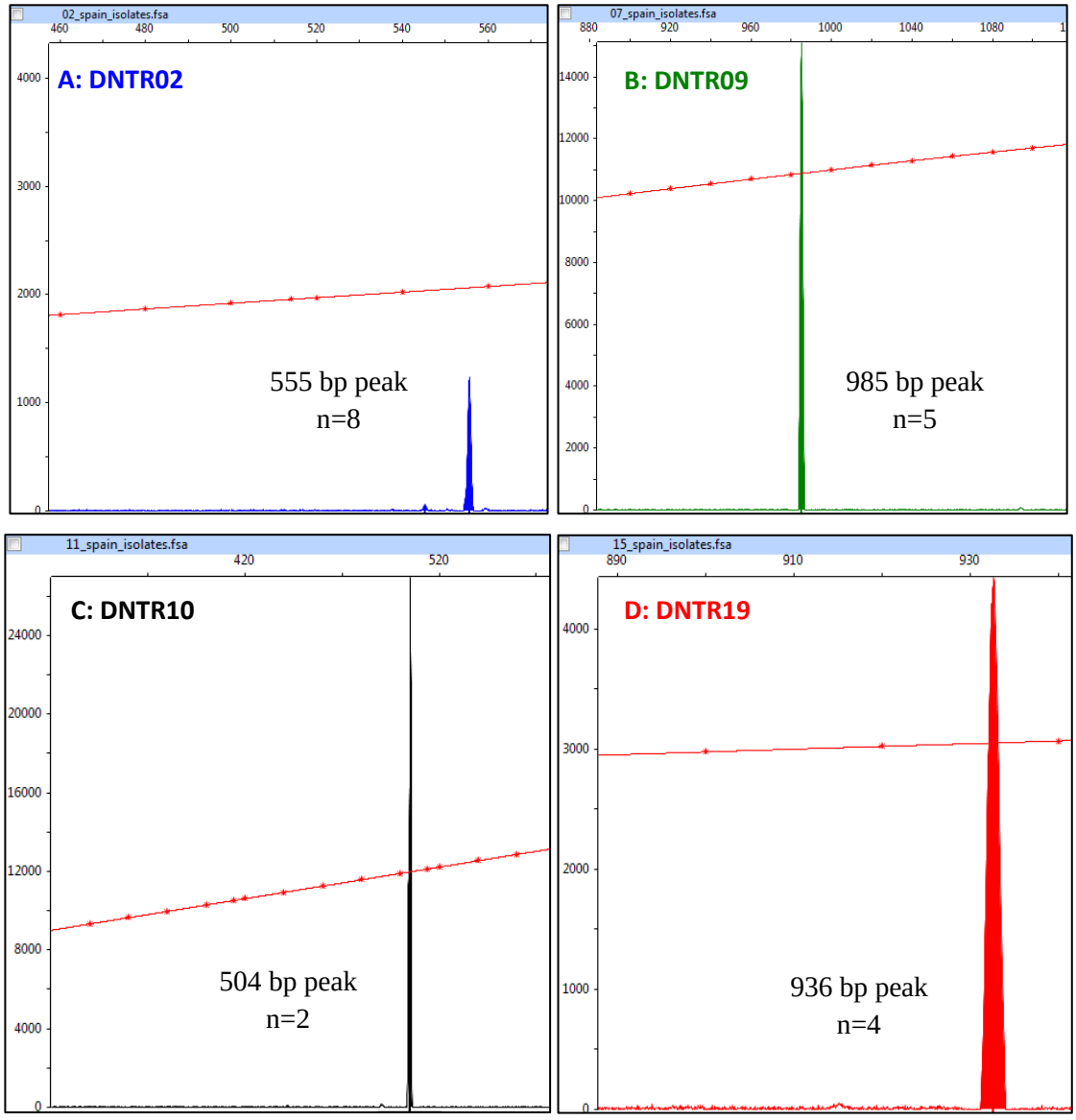


Figure 5.2: *D. nodosus* MLVA strain profile of the right front foot of sheep 40895. **A**: DNTR02/555 bp. **B**: DNTR09/985 bp. **C**: DNTR10/504 bp, **D**: DNTR19/936 bp, **n** indicates the number of repeats present at each locus.

D. nodosus was not detected in the gingival cavity, soil or grass samples during the first sampling visit in April. One faecal sample was positive for *D. nodosus* with load of 1.13 x 10 *rpoD* genome copies/sample from sheep 14237 (1.13 x 10^3 g^{-1}), which had been classed as diseased (score SFR 1, left front foot), although *D. nodosus* was not detected on the feet.

In July, 1 soil sample was positive with a count of 3.68 x 10^2 *D. nodosus rpoD* gene copies g^{-1}.

5.3.4 Farm 2

5.3.4.1 Disease status of farm 2 in April

The collaborating farmer did not report occurrence of lameness or FR during the winter and spring preceding the first sampling visit in April 2016. He described the climate as unusually dry. On the day of sampling there was no case of ID (score >1) and SFR and no lame sheep were observed. ID score 1 was given to 41/160 feet samples. Five "susceptible" ewes were sampled because there were no sheep with foot lesions. The susceptible sheep, were identified by the farmer as those that tended to become lame after a period of rainfall and had thickened hoof horn (Figure 5.3). Only data from April are available for Farm 2, because it could not be re-visited in July due to sheep management challenges.

Figure 5.3: Example of the hoof conformation of the "susceptible" sheep where *D. nodosus* was detected. The figure shows the left rear foot of ewe 14847. The foot has a "clumpy" appearance and the interdigital skin is narrow due to the thickened hoof-horn. This conformation was seen in 4/5 animals that were described as "susceptible" by the farmer.

5.3.4.2 Dichelobacter nodosus bacterial loads and communities on sheep and in the farm environment

D. nodosus was detected in 3 samples originating from feet (7.5%). A low load of *D. nodosus* was detected in one sample from a control, lesion free, sheep. One sheep (ewe 14847) classed as "susceptible", but without FR lesions (Figure 5.3) had high *D. nodosus* loads on two feet (Left rear foot: 1.73×10^5 *rpoD* copies/swab. Right rear foot: 5.86×10^6 *rpoD* copies/swab). MLVA revealed the presence of a single strain of *D. nodosus* on both feet (Figure 5.4). No amplification was observed after the *D. nodosus* MLVA PCR in the sample originating from the control sheep. The strain seen differs from the strain observed on Farm 1 at 2 loci.

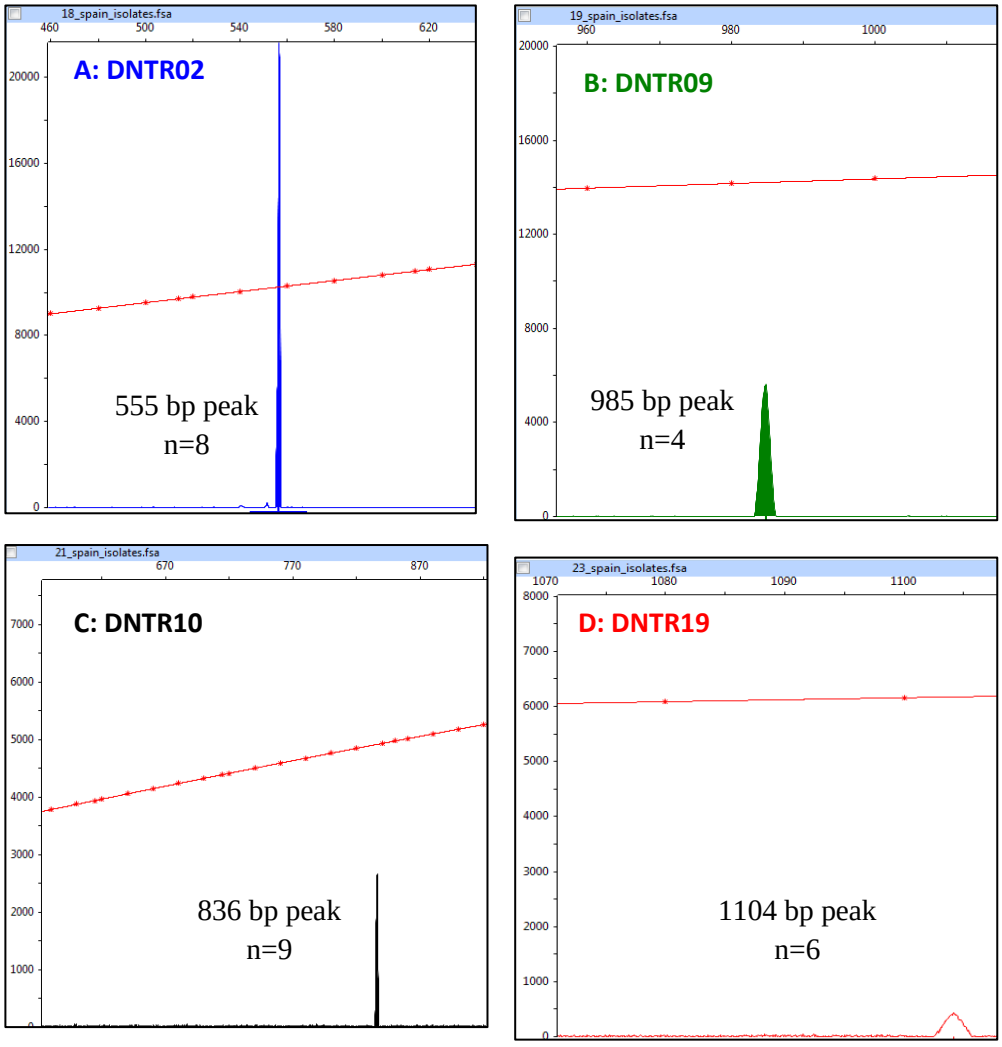


Figure 5.4: *D. nodosus* MLVA strain profile of the feet of sheep 14847. **A**: DNTR02/555 bp. **B**: DNTR09: 985 bp. **C**: DNTR10/836 bp. **D**: DNTR19/1104 bp, **n** indicates the number of repeats present at each locus.

5.4 Discussion

The aim of the study described in this chapter, was to investigate whether and where *D. nodosus* persists in a flock during a period transmission followed by a period of zero-transmission in two sheep flocks in Southern Spain.

FR was present and *D. nodosus* was detected on farm 1 in April. In July *D. nodosus* was detected in one soil sample. This does indicate that *D. nodosus* was still present on the farm, on sheep and/or in the environment during the hot summer non-transmission period. As *D. nodosus* was not detected in the environment in April, when disease was present in the flock, it appears to be more likely that *D. nodosus* persisted on feet and is transiently present in the environment. Similarly, to the results of study 1 and 2 the only site were *D. nodosus* was detected in significant quantities was on the feet of sheep and even though *D. nodosus* was detected in soil in July, bacterial loads were low and a clear site of persistence in the farm environment was not observed.

The unusual dry climate in the 5-month preceding April reported by the farmers of the study farms may explain the lack of footrot on Farm 2. This presented an opportunity to sample sheep that were described as "susceptible" by the farmer. High loads of *D. nodosus* were detected on a healthy foot of a "susceptible" animal on farm 2 where no lameness was observed in the preceding winter and spring. The poor foot conformation observed has been previously described in relation to FR. Beveridge *et al*. (1941) and Egerton *et al*. (1989) defined FR as chronic when characteristic FR lesions were accompanied by thickened, misshapen or overgrown hoof horn and reported that sheep with such lesions can be lame for several months. The results obtained from this farm support the results of study 1 and 2 were *D. nodosus* was detected on several occasions including in high loads on healthy animals that later became diseased. Moore *et al*. (2005) also detected *D. nodosus* on healthy feet and suggested that some sheep may act as carriers and Ghimire and Egerton (1996) suggested that relapse of subclinical cases of FR may cause disease in the flock. Maboni *et al*. (2016) detected *D. nodosus* on a large proportion of interdigital skin biopsies collected from post-slaughter healthy feet and suggest that sheep had subclinical FR with the possibility subsequent lesion development. There is a possibility that the sheep that carried *D. nodosus* was in the initial stages of FR, as research has shown that *D. nodosus* loads increase before

onset of ID (Witcomb *et al*., 2014). It was however not possible to obtain this information. The farmer did however not report an outbreak of FR in the following months.
It is also interesting that one sheep on farm 1 that had been diseased in April was lame in July, but did not have any FR lesions or other visible foot conditions. *D. nodosus* was however not detected on the feet of that sheep. Information on the disease status of the flock and the individual sheep where *D. nodosus* was detected in subsequent weeks would be informative, but this information was not available.

D. nodosus was not detected in the gingival cavity. One *D. nodosus* positive low load faecal sample was detected in April on a sheep with SFR lesions, which supports the hypothesis of faecal shedding described in chapter 3. The low rate of detection of *D. nodosus* at all sites except on the feet of sheep, is comparable to the results from studies 1 and 2, where it was only detected in larger quantities in the environment (visit 1, study 1) when wet weather and muddy ground were coupled with a high prevalence of disease. On both Spanish farms the ground was dry on the day of sampling in April although it had rained the previous week. In addition, no lesions were observed on Farm 2 and the prevalence of disease on Farm 1 was low.

Antibiotic treatment of the flock on Farm 1 the previous week is likely to have contributed to the low disease prevalence in April. Research has shown that 65% and 78% of sheep that receive antibiotic treatment recover from lameness and lesions respectively within 5 days (Kaler *et al*., 2010) and *D. nodosus* load significantly reduces within 7 days of receiving treatment (Willis, 2017, unpublished data). Antibiotic treatment of the flock on Farm 1 may also have contributed to a reduced strain diversity, as antibiotics have been shown to alter the composition of bacterial communities (Langdon *et al*., 2016). A single strain of *D. nodosus* was observed on both Spanish farms. This bears some similarity to study 2, where only 1 dominant strain was observed throughout the study when prevalence of disease was low.

The number of sheep analyzed as well as the number of samples taken from the environment was small compared to the number of sheep present in the flock and the geographical size of the terrain sampled. It is therefore, possible that FR lesions and/or *D. nodosus* were present on sheep that were not screened or that *D. nodosu*s was present on sites in the environment that were not sampled. Therefore, conducting a second study with a larger sample size may provide additional information. It may also be of interest to further study *D. nodosus* presence

and populations over time on sheep with a foot conformation that has been described by some authors in relation to chronic illness and possible persistence.

CHAPTER 6

General discussion, conclusions and future research

6.1 Key findings

1. *Dichelobacter nodosus* persists on the feet of sheep
2. Healthy feet carry *D. nodosus* for at least 5 weeks before disease establishes
3. *D. nodosus* can be detected on grass samples and the deeper layers of soil, which indicates that it may be possible to persist at these sites for a limited amount of time
4. It is unlikely that *D. nodosus* persists in environmental reservoirs during long periods of non-transmission when the ground is dry.
5. Periods of dry weather and reduced soil moisture were accompanied by a reduction of footrot lesions, whereas bacterial loads on one diseased sheep remained constant
6. *D. nodosus* can be detected in the flock environment in Southern Spain during a prolonged non-transmission period when temperatures exceeded 40 °C and no rain had fallen for at least 6 weeks
7. *D. nodosus* was detected in high loads from a sheep with hoof conformation that has been linked with subclinical disease, in a flock that has been disease free for at least 2 months.
8. *D. nodosus* is shed in ovine faeces
9. *D. nodosus* is unlikely to persist in the gingival cavity for longer than 2 weeks.

6.2 Discussion of key findings

The aim of this thesis was to investigate persistence of *D. nodosus* at various sites on sheep and in the environment. The primary hypothesis was that there is at least one site where *D. nodosus* persists when there are no diseased sheep in a flock. The associations between *D. nodosus* presence and persistence at different sites, and levels of disease in flocks over time

were investigated using epidemiological studies of sheep and their environment with a range of molecular tools to detect, quantify and strain-type *D. nodosus*.

A key result of the research conducted is that *D. nodosus* can persist on the feet of sheep for at least 20 weeks, irrespective of the disease state of the sheep (Chapter 3). Detection of *D. nodosus* on feet, in all disease states, has been reported previously (Moore *et al.*, 2005; Calvo-Bado *et al*., 2011 Witcomb, 2012) and led to the suggestion that the feet of sheep may consitute a reservoir of infection. There was, however, no evidence that *D. nodosus* persisted on feet. Some studies have shown that *D. nodosus* can be detected and quantified on the feet of sheep over time during an episode of disease (e.g Witcomb *et al*., 2014), but in order to obtain more relevant information on persistence, information on *D. nodosus* strain identity over time was needed.

We demonstrated that not only can *D. nodosus* be detected for 20 weeks on the feet of sheep irrespective of disease state, but one dominant strain was present on feet over time. Therefore, the MLVA tool provided valuable epidemiological information on persistence of FR that was obtained without the need to culture and will be of use in future studies of *D. nodosus*.

This is the first study that showed that healthy sheep can carry *D. nodosus* for many weeks before initiation of disease. Detection of *D. nodosus* on healthy feet has been reported previously (Moore *et al.*, 2005; Calvo-Bado *et al*., 2011; Witcomb, 2012; Maboni *et al*., 2016) and is in agreement with the results obtained here, as *D. nodosus* was detected on the healthy feet of sheep in all 3 studies. We have shown that *D. nodosus* can be deteted on the healthy feet of sheep up to 5 weeks before sheep became diseased and for 2 weeks on the foot that became diseased (study 2). Not only was *D. nodosus* detected, but the dominant strain identified was present throughout the 5 weeks. This key result and provides novel information on the persistence of *D. nodosus*. In study 3 (Spain, chapter 5), *D. nodosus* was detected in high loads on healthy feet when there had been no FR occurrence in the flock for at least 2 months. This supports previous suggestions that healthy feet or feet with subclinical FR act as carriers of *D. nodosus*. (Beveridge, 1941; Ghimire and Egerton, 1996; Moore *et al*., 2005; Kaler *et al*., 2010b).

The high detection frequency of *D. nodosus* in soil, grass and faecal samples in study 1 was a novel finding, overturning the existing paradigm that *D. nodosus* is mainly associated with the feet of sheep (Beveridge, 1941). Although indirect transmission of FR through pasture has been shown previously, suggesting that *D. nodosus* persists for 7-14 days (Beveridge, 1941;

Woolaston, 1993; Whittington *et al*., 1995), those studies did not investigate detection of *D. nodosus* on pasture. Here, *D. nodosus* was detected in all sample types and was detected for the first time on grass samples and in the deeper layers of soil. This indicates that *D. nodosus* is able to adhere to the external environment at least for a short amount of time. This is in agreement with findings of cross-sectional studies that detected *D. nodosus* in soil and faecal samples collected from the pasture (Witcomb, 2012; Muzafar *et al*., 2015). *D. nodosus* growth in soil is however unlikely from a cell biology perspective, as *D. nodosus* has lost key biosynthetic pathways needed for survival outside the host, as illustrated by its small genome (Meyers *et al*., 2007). Growth, however, does not equal persistence and the facultative anaerobic nature of *D. nodosus* may facilitate dormancy and survival in the environment for a short amount of time, as *D. nodosus* is thought to remain viable under aerobic conditions for at least 10 days on pasture (Whittington *et al*., 1995). *In vitro* studies have shown that *D. nodosus* can remain viable at in soil microcosms between 24 and 40 days (Cederlof *et al*., 2013; Muzafar *et al*., 2016). *D. nodosus* may be able to invade the deeper layer of soil due to its motility, aided by sheep activity churning up the soil. Alternatively, there may be no risk of transmission to sheep, as it may perish before coming in contact with sheep again or may not be present in sufficient quantities to constitute and infectious dose.

Soil moisture plays an important role in *D. nodosus* survival on pasture. Smith *et al*. (2014) reported an increased transmission of FR when rainfall had increased 2-4 weeks previously in the UK. Graham and Egerton (1968) observed FR transmission in Australia when soil was in a saturated state for a long period of time, irrelevant of rainfall, and transmission of FR ceased when the ground dried out. In Muzafar *et al*. (2016) soil moisture was significantly associated with increased survival time with *D. nodosus* surviving longer in constantly moist soil microcosms. We did detect *D. nodosus* in surface and soil core samples when the ground was wet. Therefore, it could be that *D. nodosus* persists in damp soils for at least long enough to facilitate pathogen spread.

The number of *D. nodosus* positive samples on pasture was affected by dry weather in both studies. In study 1, the number of *D. nodosus* positive pasture samples declined as the weather became drier. In study 2, soil moisture levels declined from an average of 87% in the weeks preceding the dry weather period to an average of 50% from week 7-11. This was coupled with a reduction in the number of *D. nodosus* positive soil samples. Dry weather was not associated with change in survival of *D. nodosus* on the feet of sheep. We hypothesized that there is a positive association between *D. nodosus* load on the foot, in the environment

and level of disease in the flock and that environmental loads of *D. nodosus* correlate with disease outbreaks. We found that *D. nodosus* loads in the environment declined with dry weather, irrespective of the disease state of the flock, while *D. nodosus* load on diseased feet remained as high as 10^5 cells per swab. In contrast the host appears to become less susceptible to disease, as FR lesions started to heal in dry weather. No lesions were observed for 2 consecutive weeks on a sheep that had SFR from week 5 and 9. Re-appearance of SFR lesions in week 12 coincided with increasing rainfall. It is possible that dry weather promotes the healing of lesions, while *D. nodosus* remains viable on feet and able to cause disease. Increasing rainfall may soften the interdigital skin which facilitates invasion of *D. nodosus* into the interdigital skin.

It is interesting that *D. nodosus* was found in one soil sample in July (Study 3, Spain) when it had not rained for at least 6 weeks. Although this does not indicate that *D. nodosus* persisted in soil or that no sheep in the flock had FR lesions it is clear that *D. nodosus* can still be detected after a long period of dry and hot weather when sheep are assumed to be lesion-free. As long-term persistence of *D. nodosus* in dry soil is less likely, it is probable that *D. nodosus* was transferred to the soil from the feet of a sheep that was not sampled.

Published evidence coupled with the results from this study indicate that the climatic conditions that favour transmission of *D. nodosus* and survival of *D. nodosus* are not the same. The results of this study suggest that *D. nodosus* spread increases during or shortly after periods of high rainfall. *D. nodosus* may be able to adhere to the moist soil at least for a short amount of time after rainfall ceased and encourage further spread. Therefore, moist soil caused by preceding rainfall seems to be the key factor for transmission and also survival of *D. nodosus* off host. Dry weather may lead to the healing of lesions and a reduction of *D. nodosus* on pasture, but the ability of *D. nodosus* to persist on healthy feet in periods of dry weather shown here may explain why FR re-appears after long periods of non-transmission when it rains. Therefore, it is likely that rainfall plays a key role in initiation of disease, but not persistence of *D. nodosus*. For how long *D. nodosus* can survive on healthy feet in the absence of disease in a flock is unknown.

Some sheep with poor foot conformation (in this case thickened, misshapen or overgrown hoof horn) appear susceptible to repeated bouts of FR. These sheep are most likely to be chronically infected and such sheep were reported by the farmer and sampled in Spain. One sheep had high loads of *D. nodosus*, even though disease had not been observed in the flock

for at least 2 months. Sheep with poor foot conformation have also have been reported by Beveridge (1941) and Kaler *et al*. (2010b), as sheep that harbour *D. nodosus* in micro abscesses and suffer from chronic FR and may be lame for several months, although lameness might be due to lack of treatment (Kaler *et al*., 2012). The farmer in Spain reported that there was a high likelihood of the sheep becoming lame as soon as the weather became wet, without necessarily developing external lesions. If persistence of *D. nodosus* is linked to feet with a characteristic hoof conformation, farmers could improve FR control strategies targeted for treatment or removal from the flock. Control may be more challenging when carriers do not show identifying characteristics, such as poor foot conformation.

This is the first study that investigated *D. nodosus* in the gingival cavity with the disease status of the sheep. It was also the first to attempt to compare strains of *D. nodosus* between the gingival cavity and the feet of sheep. Previous studies detected *D. nodosus* in the gingival cavity without linking presence to disease (Bennett *et al*., 2009; Witcomb, 2012). The results of study 1 show that *D. nodosus* was significantly more likely to be present in the gingival cavity when sheep had FR. This is supported by results from study 2, where *D. nodosus* was detected in the gingival cavity of a sheep with SFR. Study 1 had the highest disease prevalence of all 3 studies. It was the only study were *D. nodosus* was detected in the gingival cavity on more than one occasion (25% of samples). In addition, strains that were present in the gingival cavity were the same strains that were the most dominant strains present on feet, as classed by MLVA. It is likely that *D. nodosus* re-populates the gingival cavity when disease prevalence is high. It is possible that sheep lick lesions on their feet, transferring *D. nodosus* into the gingival cavity or, more likely, that *D. nodosus* is transferred into the mouth from the grass.

Linked with detection of *D. nodosus* in the gingival cavity, we demonstrated that faecal shedding of *D. nodosus* occurs, although it is a rare event (Studies 2 and 3). *D. nodosus* had previously been detected in faeces collected from pasture (Witcomb, 2012) and from faeces on the interdigital skin (Muzafar *et al*., 2015). Presence of *D. nodosus* at these sites could be environmental contamination. Even though colonization of *D. nodosus* in the gingival cavity seems unlikely, it is possible that *D. nodosus* can passage through the digestive system of the sheep.

6.3 Limitations

There are a number of limitations in this study. As discussed in previous chapters there were challenges associated with low disease prevalence and a small number of *D. nodosus* positive samples in Study 2. Therefore, the suggestions above are tentative and require further research. We cannot say with confidence that *D. nodosus* does not persist on pasture due to the low sample size in relation to the size of the area sampled.

It was not possible to obtain a complete MLVA *D. nodosus* community profile, due to the increased threshold level applied when sizing peaks after fragment analysis, hence only the most detectable dominant strains in a sample were identified. As a consequence of the high detection limit it was also not possible to amplify *D. nodosus* in low load samples. This included all samples obtained from the pasture and most gingival cavity samples. We were therefore not able to fulfill the aim of comparing *D. nodosus* strains present in pasture or in faecal samples with strains found on sheep. In addition, it is not possible to obtain the total number of strains present in a sample, due to the strain identification methods used in this study. Another limitation was that the virulence status of the *D. nodosus* detected in the sheep flocks (studies 1 and 2) is unknown. We can therefore not assume that all *D. nodosus* in the study were able to cause disease. Finally, it should be noted that it is unknown whether the *D. nodosus* detected in this study (in particular *D. nodosus* from sources other than the feet of sheep) were viable and able to cause disease. To assess the viability of *D. nodosus*, additional culture experiments should be performed. Whereas this was not within the scope of this study, it should be an important consideration for future work conducted.

6.4 Conclusions

This thesis has contributed to the understanding to the persistence of *D. nodosus* on sheep and in the environment. The combined evidence lead to the identification of the feet of sheep as the primary site of persistence of *D. nodosus*, and suggests that long-term environmental reservoirs are less likely. The endemic nature of FR in the UK is likely due to the moisture content of soil that facilitates survival of *D. nodosus* and transmission of disease. It also may increase host susceptibility to phenotypically display disease. Further work on the duration of colonization of healthy feet in the absence of disease might assist in considering whether elimination of *D. nodosus* from flocks is feasible, and if not, what alternative control strategies would be most effective.

6.5 Future work

Future work should focus on the healthy feet of sheep as the primary site of persistence in the absence of disease. Analysis of further foot swabs from study 2 is in progress as part of a Master's degree. This will allow for the inclusion of additional sheep in the statistical model (Chapter 3) and provide more detailed information on the effect of climate on *D. nodosus* persistence on feet. It would be interesting to conduct further research on *D. nodosus* persistence in semi-arid climates. A longitudinal study, sampling a larger number of sheep may elucidate for how long *D. nodosus* is able to persist on lesion-free feet.

In this study, we isolated *D. nodosus* strains that were dominant during an outbreak, as identified by MLVA. This enabled a more detailed investigation of these strains. It may be possible to investigate characteristics of *D. nodosus* that are more likely to be associated with increased transmission and persistence. It would also be interesting to investigate faecal shedding in a flock with high FR prevalence. Since *D. nodosus* is able to adhere to soil and grass samples it may also be informative to sample the pasture in a flock with high prevalence. Environmental reservoirs, such as soil could become important in flocks where disease prevalence is high. Future work could also involve the investigation of alternative reservoirs of *D. nodosus*.

Regarding the MLVA, future work could involve the individual optimization of primers to improve the detection limit. More importantly the appearance of multiple peaks when testing cultured isolates should be further investigated. This could lead to a reduction of the threshold level and may enable to identify less dominant strains and hence provide a complete community profile. Any future research into the persistence of *D. nodosus* should also consider investigating the viability and pathogenicity of *D. nodosus* detected in different potential reservoirs, by conducting suitable culturing experiments. An investigation into the virulence status of *D. nodosus* in samples obtained from studies 1 and 2 is already in progress as part of different PhD project.

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Appendices

APPENDIX 1

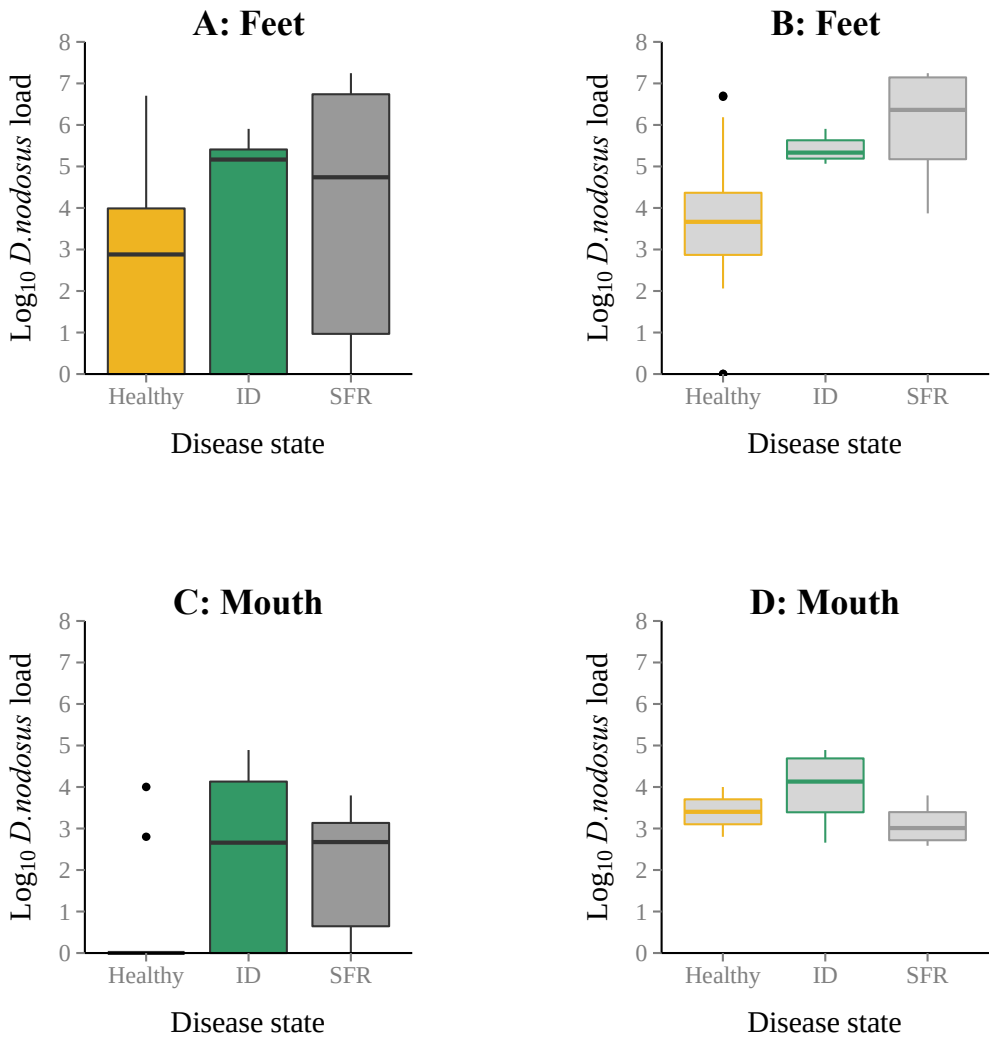
Study 1: Mean, minimum and maximum *D. nodosus rpoD* genome copies detected by sample type.

Sample type	Minimum load detected/sample	Maximum load detected/sample	Median load detected (All samples)	Median load detected (Positive samples)
Feet	2.06	7.24	2.60 (n=152)	3.95 (n=97)
Mouth	2.58	4.89	0.92 (n=38)	3.50 (n=13)
Soil	2.49	4.84	1.35 (n=88)	3.31 (n=36)
Grass	2.39	4.15	1.27 (n=24)	3.06 (n=10)
Faeces	2.51	4.45	0.93 (n=40)	3.09 (n=10)

D. nodosus load is presented as $\log_{10}$ *rpoD* genome copies present swab/sample $^{-1}$.

APPENDIX 2

Study 1: Figure and table showing the Log_{10} *D. nodosus* loads on feet and the gingival cavity according to disease state.



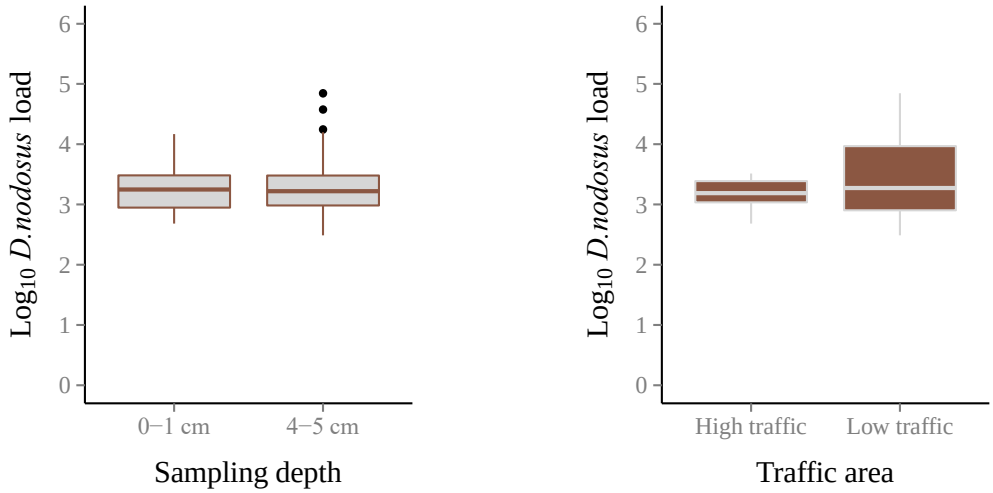
D. nodosus load in clinical swabs from feet and the oral cavity, collected, fortnightly over the study period (Week 1-7) according to disease state of the foot. **A, C**: *D. nodosus* load on foot swabs **(A) (n=152)** and oral cavity swabs **C (n=38)**, including negative samples **C, D**: *D. nodosus* load including only positive samples on foot swabs **(B) (n=97**) and in the oral cavity **(D) (n=10).** Bacterial loads are presented as Log_{10} +1/swab. Healthy feet: **n=137**, ID feet: **n=9**, FR feet: **n=6**.

Sample source	Healthy Feet (n=137)		Feet with ID (n=9)		Feet with SFR (n=6)	
	Median load**	**Number/ % positive**	**Median load**	**Number/ % positive**	**Median load**	**Number/ % positive**
All Feet (n=152)	2.88	90/65.7	5.17	4/44.4	4.74	4/66.7
Positive feet (n=97)	3.67		5.33		6.36	
Sample source	**Healthy sheep (n=25) ***		**Sheep with ID (n=7) ***		**Sheep with SFR (n=6) ***	
All Mouth (n=38)	0	2/8	2.66	4/57	2.67	4/66
Positive samples (n=10)	3.40		4.13		3.01	

*The number of times a sheep was classed as healthy or having ID or SFR lesions.

APPENDIX 3

Study 1: Quantification and detection frequency of *D. nodosus* in soil (0-1 cm, 4-5 cm, HTA's, LTA's)



Log_{10} *D. nodosus* load of positive soil samples g^{-1} taken at 0-1cm (n=16) and 4-5 cm (n=20) and between samples collected from high (n=17) and low (n=19) traffic areas.

APPENDIX 4

Study 1: Quantification and detection frequency of *D. nodosus* in ewes and lambs.

Comparison of $\log_{10}$ *D. nodosus* load and detection frequency between ewes and lambs

	Ewe feet positive samples (n=20)	**Lamb feet positive samples (n=20) ***	**P (Load all samples)**	**P (Load positive samples)**	**P Detection frequency**
Week 1	20 (100%)	20 (100%)	<0.001	<0.001	NS
Week 3	17 (85%)	10 (50%)	<0.1	NS	<0.05
Week 5	12 (60%)	6 (30%)	NS	NS	<0.1
Week 7	12 (60%)	3 (25%)	<0.1	NS	<0.1

*Only 12 feet in week 7 (data for 2 lambs not available), NS=not significant

APPENDIX 5

Study 1: Variables that were excluded from the binomial mixed effects regression model.

Variable	Reason for exclusion
D. nodosus is present in soil (1= present)	*D. nodosus* was present in soil every week
Sheep was treated with antibiotic injection (1=treated)	Only 1 sheep was treated on one occasion

Study 2: Variables that were excluded from the binomial mixed effects regression model

Variable	Reason for exclusion
D. nodosus is present in grass (1= present) *D. nodosus* load in grass	No *D. nodosus* positive grass samples from weeks 1-20
D. nodosus is present in the mouth (1= present) *D. nodosus* load in the gingival cavity	Only 1 *D. nodosus* positive mouth sample in study
D. nodosus is present in faeces (1= present) *D. nodosus* load in faeces	Only 1 *D. nodosus* positive mouth sample in study
Number of diseased feet	Only 1 foot was diseased most weeks
Sheep was treated with antibiotic injection (1=treated)	Only 1 sheep was treated with an antibiotic injection

APPENDIX 6: Correlations and associations of all model variables (study 1)

A: Correlations of all continuous variables

Continuous Predictor variables	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1 *D. nodosus* load on feet	1.00											
2 Number *D. nodosus* positive foot samples	**0.38**	1.00										
3 *D. nodosus* load in the mouth	0.04	**0.19**	1.00									
4 Number *D. nodosus* positive mouth samples	**0.18**	**0.51**	**0.24**	1.00								
5 *D. nodosus* load in soil	**0.32**	**0.81**	**0.20**	**0.70**	1.00							
6 Number of *D. nodosus* positive soil samples	**0.38**	**1.00**	**0.19**	**0.54**	**0.80**	1.00						
7 *D. nodosus* load on grass	**0.26**	**0.68**	0.03	**-0.20**	0.15	**0.67**	1.00					
8 Number of *D. nodosus* positive grass samples	**0.29**	**0.71**	0.01	**-0.24**	**0.34**	**0.69**	**0.93**	1.00				
9 *D. nodosus* load in faeces	**0.17**	**0.33**	0.01	-0.02	**0.64**	**0.29**	0.02	**0.38**	1.00			
10 Number of positive faecal samples (week)	**0.27**	**0.60**	0.03	-0.04	**0.68**	**0.56**	**0.41**	**0.71**	**0.92**	1.00		
11 *F. necrophorum* load on feet	0.15	**0.49**	**0.19**	**0.39**	**0.42**	**0.50**	**0.26**	**0.24**	0.06	**0.18**	1.00	
12 *F. necrophorum* load in the mouth	0.03	-0.06	0.00	0.15	0.11	-0.05	-0.16	-0.12	0.06	0.00	-0.06	1.00
13 Total rain (mm) 7 days prior sampling	-0.15	**-0.38**	0.07	**0.54**	0.15	-0.37	**-0.93**	**-0.87**	0.02	**-0.33**	-0.08	-0.14
14 Total rain (mm) 14 days prior sampling	**0.30**	**0.79**	0.09	0.07	**0.30**	**0.77**	**0.96**	**0.85**	-0.05	**0.34**	**0.40**	-0.17
15 Total rain (mm) 28 days prior sampling	**0.32**	**0.76**	0.04	-0.13	**0.54**	**0.71**	**0.71**	**0.95**	**0.63**	**0.89**	**0.56**	-0.08
16 Mean temperature (°C), 7 days prior sampling *	**-0.36**	**-0.95**	-0.16	**-0.38**	**-0.60**	**-0.94**	**-0.83**	**-0.77**	-0.11	**-0.45**	**-0.49**	-0.06
17 Mean temperature (°C), 14 days prior sampling *	**-0.35**	**-0.86**	-0.11	**-0.24**	**-0.84**	**-0.83**	**-0.53**	**-0.75**	**-0.78**	**-0.94**	**-0.47**	-0.07
18 Mean temperature (°C), 28 days prior sampling s*	**-0.39**	**-0.99**	-0.16	**-0.40**	**-0.78**	**-0.98**	**-0.73**	**-0.80**	**-0.40**	**-0.68**	**-0.56**	-0.06
19 Min. temperature (°C), 7 days prior sampling *	0.10	-0.15	0.08	-0.02	-0.15	-0.14	-0.09	-0.14	-0.16	**-0.18**	**-0.22**	**0.39**
20 Min. temperature (°C), 14 days prior sampling *	0.09	-0.15	0.09	-0.02	-0.14	-0.10	-0.09	-0.15	-0.16	**-0.18**	**-0.20**	**0.37**
21 Max. temperature (°C), 28 days prior sampling *	0.14	-0.12	0.05	-0.02	-0.11	-0.07	-0.07	-0.11	-0.14	-0.15	**-0.26**	**0.43**
22 Max. temperature (°C), 7 days prior sampling *	0.12	-0.13	0.06	-0.04	-0.12	-0.6	-0.06	-0.11	**-0.17**	**-0.18**	**-0.23**	**0.29**
23 Max. temperature (°C), 14 days prior sampling *	0.07	-0.15	0.10	-0.02	-0.14	-0.14	-0.11	-0.15	-0.15	**-0.18**	**-0.18**	**0.36**
24 Max. temperature (°C), 28 days prior sampling *	0.15	-0.12	0.04	-0.03	-0.11	-0.11	-0.05	-0.10	-0.16	-0.16	**-0.26**	**0.39**

Table continued	13	14	15	16	17	18	19	20	21	22	23	24
9 *D. nodosus* load in faeces												
10 Number *D. nodosus* positive faecal samples												
11 *F. necrophorum* load on feet												
12 *F. necrophorum* load in the mouth												
13 Total rain (mm) 7 days prior sampling*	1.00											
14 Total rain (mm) 14 days prior sampling*	**0.96**	1.00										
15 Total rain (mm) 28 days prior sampling*	**0.82**	**0.72**	1.00									
16 Mean temperature (°C), 7 days prior sampling*	**-0.99**	**-0.94**	**-0.73**	1.00								
17 Mean temperature (°C), 14 days prior sampling *	**-0.75**	**-0.54**	**-0.90**	**0.70**	1.00							
18 Mean temperature (°C), 28 days prior sampling *	**-0.95**	**-0.83**	**-0.84**	**0.95**	**0.88**	1.00						
19 Min. temperature (°C), 7 days prior sampling *	-0.13	-0.09	**-0.17**	0.12	**0.19**	0.15	1.00					
20 Min. temperature (°C), 14 days prior sampling *	-0.13	-0.10	**-0.17**	0.12	**0.19**	0.16	**0.99**	1.00				
21 Max. temperature (°C), 28 days prior sampling *	-0.10	-0.06	-0.14	0.09	**0.16**	0.12	**0.92**	**0.88**	1.00			
22 Max. temperature (°C), 7 days prior sampling *	-0.10	-0.05	-0.15	0.09	**0.18**	0.14	**0.89**	**0.86**	**0.81**	1.00		
23 Max. temperature (°C), 14 days prior sampling *	-0.14	-0.10	**0.18**	0.13	**0.18**	0.16	**0.97**	**0.99**	**0.82**	**0.82**	1.00	
24 Max. temperature (°C), 28 days prior sampling *	-0.09	-0.05	-0.14	0.08	0.16	0.12	**0.90**	**0.85**	**0.97**	**0.80**	**0.77**	1.00

R^2 values are shown. P-values with significance are shown in **bold, ***Rolling mean was used for temperature and rainfall variables.

B: Associations of continuous variables with categorical variables (Study 1)

	Foot had FR lesions (1=present)	**Sheep had FR (1=disease)**	***D.nodosus* present on feet (1=present)**	***D. nodosus* present in mouth (1=present)**	***D. nodosus* present on grass (1=present)**	***D. nodosus* present in faeces (1=present)**	***F. necrophorum* present in feet (1=present)**	***F. necrophorum* present in mouth (1=present)**	**Lamb or ewe (1=ewe))**	**Foot sprayed with antibiotic spray (1=yes)**
D. nodosus load on feet	0.05	0.85	**<0.01 (+)**	0.79	0.06	**<0.01 (+)**	**0.02 (+)**	0.86	**0.01 (+)**	**<0.01 (+)**
Number of *D. nodosus* positive feet	0.12	**0.02 (-)**	**<0.01 (+)**	0.41	**<0.01 (+)**	**<0.01 (+)**	**0.03 (+)**	**0.01 (+)**	0.36	0.63
D. nodosus load in mouth	**<0.01 (+)**	**<0.01 (+)**	0.82	**<0.01 (+)**	0.97	0.58	**0.04 (+)**	**0.02 (+)**	0.91	0.71
Number of *D. nodosus* positive mouth samples	0.32	**0.02 (+)**	**<0.01 (+)**	**<0.01 (+)**	**0.01 (-)**	**0.01 (+)**	**0.04 (+)**	0.66	0.76	0.44
D. nodosus load in soil	0.85	0.34	**<0.01 (+)**	**0.03 (+)**	**0.01 (-)**	**<0.01 (+)**	**<0.01 (+)**	0.66	0.36	0.16
Number of *D. nodosus* positive soil samples	0.12	**0.02 (-)**	**<0.01 (+)**	0.41	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	**0.01 (+)**	0.36	0.63
D. nodosus load on grass	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (+)**	0.13	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	0.76	**0.02 (-)**
Number of *D. nodosus* positive grass samples	**<0.01 (-)**	**0.01 (-)**	**<0.01 (+)**	0.13	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	0.76	**0.02 (-)**
D. nodosus load in faeces	0.43	0.11	0.81	0.05	**<0.01 (+)**	**<0.01 (+)**	**0.04 (-)**	**<0.01 (+)**	0.36	0.07

Table continued

Number of *D. nodosus* positive faecal samples	**0.04 (-)**	**<0.01 (-)**	**<0.01 (+)**	0.21	**<0.01 (+)**	**<0.01 (+)**	**0.02 (+)**	0.01	0.36	0.79
F. necrophorum load on feet	0.82	0.10	0.63	0.88	<**0.01 (+)**	<**0.01 (+)**	<**0.01 (+)**	0.21	**0.01 (-)**	0.56
F. necrophorum load in the mouth	0.99	**0.03 (+)**	0.17	<**0.01 (+)**	0.35	0.87	0.83	<**0.01 (+)**	<**0.01(+)**	0.88
Total rain (mm) 7 days prior sampling*	**0.03 (-)**	**0.01 (-)**	**<0.01 (+)**	0.13	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	0.76	**0.02 (-)**
Total rain (mm) 14 days prior sampling*	**0.03 (-)**	**<0.01 (-)**	**<0.01 (+)**	0.58	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	0.76	**<0.01 (-)**
Total rain (mm) 28 days prior sampling*	**0.04 (-)**	**<0.01 (-)**	**<0.01 (+)**	0.21	**<0.01 (+)**	**<0.01 (+)**	**0.02 (+)**	**<0.01 (+)**	0.36	0.79
Mean temp. (°C), 7 days prior sampling*	**0.03 (+)**	**<0.01 (+)**	**<0.01 (-)**	0.58	**<0.01 (-)**	**0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.76	**0.01 (-)**
Mean temp. (°C), 14 days prior sampling *	0.12	**0.02 (+)**	**<0.01 (-)**	0.41	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.36	0.63
Mean temp. (°C), 28 days prior sampling *	0.12	**0.02 (+)**	**<0.01 (-)**	0.41	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.36	0.63
Min. temp. (°C), 7 days prior sampling *	**0.03 (+)**	**<0.01 (+)**	**<0.01 (-)**	0.58	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.76	**<0.01 (+)**
Min. temp. (°C), 14 days prior sampling *	**0.03 (+)**	**<0.01 (+)**	**<0.01 (-)**	0.58	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.76	**<0.01 (+)**
Min. temp. (°C), 28 days prior sampling *	0.12	**0.02 (+)**	**<0.01 (+)**	0.41	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.36	0.63
Max. temp. (°C), 7 days prior sampling *	**0.03 (+)**	**<0.01 (+)**	**<0.01 (-)**	0.58	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.76	**<0.01 (+)**
Max. temp. (°C), 14 days prior sampling *	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (-)**	0.13	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.76	**0.02 (+)**
Max. temp. (°C), 28 days prior sampling *	0.12	**0.02 (+)**	**<0.01 (-)**	0.41	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	**<0.01 (-)**	0.36	0.63

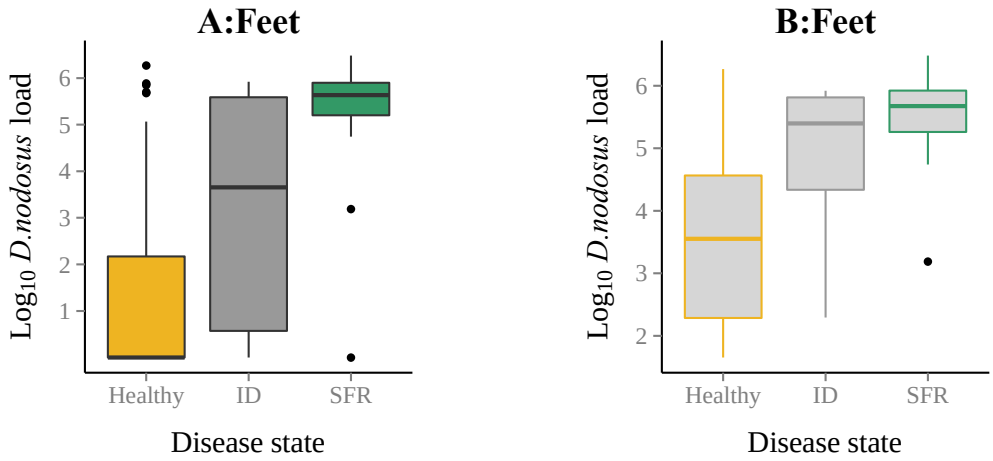
P-values with significance are shown in **bold, ***Rolling mean was used for temperature and rainfall variables. ^The direction of the effect in indicated in parentheses after the significant p-value: The plus symbol (+) indicates that the median is significantly higher in binomial 1 variable, the minus symbol (-) indicates that the median is significantly higher in binomial 0 variable.

C: Relationship between categorical model variables

Predictor variables	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1. Foot had FR lesion (1=present)	1.00										
2. Sheep had FR (1=diseased)	**<0.01**	1.00									
3. *D. nodosus* present on feet (1=present)	0.80	0.09	1.00								
4. *D. nodosus* present in mouth (1=present)	0.01	**<0.01**	0.67	1.00							
5. *D. nodosus* present grass (1=present)	0.12	**0.01**	**<0.01**	0.68	1.00						
6. *D. nodosus* present faeces (1=present)	0.50	0.7	**0.04**	1.00	**<0.01**	1.00					
7. *F. necrophorum* present on feet (1=present)	0.73	0.05	**0.04**	0.17	**<0.01**	0.10	1.00				
8. *F. necrophorum* present in mouth (1=present)	0.46	0.82	0.28	0.08	**<0.01**	0.71	**0.01**	1.00			
9. Age (lamb/ewe)	0.05	0.11	**0.01**	0.84	0.84	0.29	0.73	0.08	1.00		
10. Foot trimmed (1=trimmed)	0.07	0.89	0.89	0.61	1.00	0.67	0.67	0.67	1.00	1.00	
11. Antibiotic spray given (1=yes)	**<0.01**	0.14	0.23	0.93	**<0.01**	0.05	0.66	0.43	0.87	1.00	1.00
12. Body condition score	**<0.01**	**<0.01**	**0.03**	**0.02**	0.01	**0.03**	0.03	**0.03**	**<0.01**	0.93	0.54

APPENDIX 7

Study 2: *D. nodosus* loads on feet and the gingival cavity according to disease state



Log_{10} *D. nodosus* load on clinical swabs from the feet of 3 sheep that became diseased in study 2**. A, C**: *D. nodosus* load on foot swabs **(A) (n=148)** including negative samples **B**: *D. nodosus* load including only positive samples on foot swabs **(B) (n=60).**

APPENDIX 8

Study 2: detection of *D. nodosus* from weeks 1-3.

Log_{10} *D. nodosus rpoD* genome copies detected per swab/sample and number of samples where *D. nodosus* was detected/week

	Week 1		Week 2		Week 3	
Number of sheep with *D. nodosus* detected (n=21)		13		2		2
	Median load	**Number/ % positive**	**Median Load**	**Number/ % positive**	**Median load**	**Number/ % positive**
All feet (n=84)	0.00	27/32.14	0.00	3/3.57	0.00	4/4.76
Positive feet (n=34)	2.39		4.80		3.59	

APPENDIX 9: Correlations and associations between all model variables (Study 2)

A: Correlation of continuous variables (study 2).

Predictor variables	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1 *D. nodosus* load on feet	1.00														
2 Number of *D. nodosus* positive foot samples	0.06	1.00													
3 *F. necrophorum* load on feet	**0.51**	0.13	1.00												
4 Mean temp. (°C) 7 days prior sampling*	0.02	**-0.62**	0.02	1.00											
5 Mean temp. (°C) 14 days prior sampling*	0.02	**-0.69**	0.06	**0.98**	1.00										
6 Mean temp. (°C) 28 days prior sampling*	0.02	**-0.66**	0.02	**0.96**	**0.99**	1.00									
7 Min. temp. (°C) 7 days prior sampling*	-0.01	-0.14	0.03	**0.73**	**0.68**	**0.69**	1.00								
8 Min. temp. (°C) 14 days prior sampling*	0.01	**-0.62**	0.03	**0.91**	**0.93**	**0.93**	**0.77**	1.00							
9 Min. temp. (°C) 28 days prior sampling*	0.02	**-0.65**	0.06	**0.95**	**0.96**	**0.97**	**0.72**	**0.97**	1.00						
10 Max. temp. (°C) 7 days prior sampling*	0.06	**-0.71**	0.07	**0.93**	**0.95**	**0.94**	**0.50**	**0.85**	**0.90**	1.00					
11 Max. temp. (°C) 14 days prior sampling*	0.04	**-0.66**	0.04	**0.95**	**0.98**	**0.97**	**0.62**	**0.87**	**0.92**	**0.97**	1.00				
12 Max. temp. (°C) 28 days prior sampling*	0.03	**-0.64**	0.06	**0.93**	**0.96**	**0.97**	**0.67**	**0.90**	**0.92**	**0.93**	**0.97**	1.00			
13 Total rain (mm) 7 days prior sampling*	-0.04	0.02	-0.06	**-0.38**	**-0.36**	**-0.36**	-0.08	**-0.19**	**-0.29**	**-0.42**	**-0.40**	**-0.38**	1.00		
14 Total rain (mm) 14 days prior sampling*	0.01	0.01	-0.05	**-0.41**	**-0.43**	**-0.43**	-0.08	**-0.20**	**-0.32**	**-0.38**	**-0.49**	**-0.45**	**0.59**	1.00	
15 Total rain (mm) 28 days prior sampling*	0.08	-0.09	0.02	**-0.27**	**-0.23**	**-0.26**	-0.33	-0.16	-0.15	-0.16	**-0.28**	**-0.31**	**0.41**	**0.58**	1.00
16 Number of *D. nodosus* positive soil samples	-0.03	**0.36**	0.10	**-0.24**	**-0.29**	**-0.24**	**0.26**	0.07	0.08	**-0.39**	**-0.25**	**-0.22**	0.13	-0.15	**-0.20**
17 *D. nodosus* load in soil	0.09	**0.67**	0.16	0.06	0.06	0.14	**0.19**	**-0.26**	**-0.26**	0.10	0.13	0.15	0.18	**-0.28**	**-0.34**
18 Mean weekly soil moisture (%)	0.00	**0.44**	-0.07	**-0.72**	**-0.27**	**-0.71**	**-0.51**	**-0.51**	**-0.61**	**-0.80**	**-0.76**	**-0.73**	**0.61**	**0.69**	**0.68**
19 Mean weekly soil moisture 4-5 cm (%)	-0.06	-0.04	-0.15	**-0.39**	**-0.37**	**-0.40**	**-0.24**	-0.12	**-0.27**	**-0.39**	**-0.39**	**-0.38**	**0.51**	**0.53**	**0.23**
20 Mean weekly soil moisture 0-1 cm (%)	0.06	**0.61**	0.06	**-0.56**	**-0.57**	**-0.53**	**-0.43**	**-0.55**	**-0.52**	**-0.65**	**-0.60**	**-0.58**	**0.29**	**0.37**	**0.66**
21 Mean weekly soil moisture LTA (%)	0.02	**0.60**	-0.10	**-0.82**	**-0.80**	**-0.76**	**-0.64**	**-0.66**	**-0.70**	**-0.86**	**-0.83**	**-0.77**	**0.44**	**0.60**	**0.64**
22 Mean weekly soil moisture HTA (%)	-0.02	**0.31**	-0.01	**-0.62**	**-0.61**	**-0.62**	**-0.40**	**-0.39**	**-0.50**	**-0.70**	**-0.66**	**-0.65**	**0.64**	**0.68**	**0.64**
23 Mean weekly soil temperature (°C)	0.05	**-0.54**	0.05	**0.92**	**-0.93**	**0.91**	**0.65**	**0.88**	**0.88**	**0.93**	**0.95**	**0.91**	**-0.44**	**-0.44**	**-0.27**

Table continued								
Predictor variables	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
16 Number of *D. nodosus* positive soil samples	1.00							
17 *D. nodosus* load in soil	**0.63**	1.00						
18 Mean weekly soil moisture (%)	-0.12	-0.15	1.00					
19 Mean weekly soil moisture 4-5 cm (%)	**-0.42**	**-0.37**	**0.65**	1.00				
20 Mean weekly soil moisture 0-1 cm (%)	**0.26**	0.17	**0.66**	-0.14	1.00			
21 Mean weekly soil moisture LTA (%)	0.08	0.07	**0.89**	**0.33**	**0.84**	1.00		
22 Mean weekly soil moisture HTA (%)	**-0.22**	**-0.26**	**0.97**	**0.77**	**0.50**	**0.76**	1.00	
23 Mean weekly soil temperature (°C)	**0.18**	**-0.22**	**0.18**	**-0.70**	**-0.30**	**-0.62**	**-0.79**	1.00

R^2 value is shown, significant correlations are highlighted in **bold.** *****Temp.=temperature, max=maximum, min = minimum, rolling mean was used.

B

Correlation of continuous predictor variables with categorical variables of study 1

Continuous predictor variables \ Categorical predictor variables	**Foot had FR lesions (1=present)**	**Sheep had FR (1-diseased)**	***D.nodosus* present on feet (1=present)**	***D.nodosus* present in soil (1=present)**	***F. necrophorum* present on feet (1=present)**
D. nodosus load on feet	**<0.01 (+)**	0.44	**<0.01 (+)**	0.29	**<0.01 (+)**
F. necrophorum load on feet	**<0.01 (+)**	**<0.01 (+)**	**<0.01 (+)**	**0.04 (+)**	**<0.01 (+)**
Mean temp. (°C) 7 days prior sampling*	**0.04 (+)**	**<0.01 (+)**	0.99	0.51	0.71
Mean temp. (°C) 14 days prior sampling*	0.10	**<0.01 (+)**	0.59	0.38	0.51
Mean temp. (°C) 28 days prior sampling*	0.08	**<0.01 (+)**	0.89	**0.02 (+)**	0.86
Min. temp. (°C) 7 days prior sampling*	0.27	**<0.01 (+)**	0.53	**<0.01 (+)**	0.12
Min. temp. (°C) 14 days prior sampling*	0.07	**<0.01 (+)**	0.93	**0.01 (+)**	0.80
Min. temp. (°C) 28 days prior sampling*	0.09	**<0.01 (+)**	0.84	**0.01 (+)**	0.86
Max. temp. (°C) 7 days prior sampling*	0.05	**<0.01 (+)**	0.98	0.05	0.58
Max. temp. (°C) 14 days prior sampling*	0.17	**<0.01 (+)**	0.76	**0.01 (+)**	0.67
Max. temp. (°C) 28 days prior sampling*	0.11	**<0.01 (+)**	0.91	0.05	0.92
Total rain (mm) 7 days prior sampling*	0.60	0.17	0.85	0.66	0.86
Total rain (mm) 14 days prior sampling*	0.42	0.27	0.65	**<0.01 (-)**	0.93
Total rain (mm) 28 days prior sampling*	0.16	**<0.01 (+)**	0.70	**0.03 (-)**	0.70
Number of *D. nodosus* positive soil samples	0.62	0.30	0.42	**<0.01 (+)**	**<0.03 (+)**
D. nodosus count soil (week)	0.39	0.13	0.13	**<0.01 (+)**	0.07
Mean weekly soil moisture (%)	0.38	**<0.01 (-)**	0.85	0.01	0.99
Mean weekly soil moisture 4-5 cm (%)	0.37	**<0.01 (-)**	0.83	**<0.01 (-)**	0.82
Mean weekly soil moisture 0-1 cm (%)	0.60	0.52	0.31	0.25	0.28
Mean weekly soil moisture LTA (%)	0.70	0.14	0.57	0.19	0.35
Mean weekly soil moisture HTA (%)	0.54	**<0.01 (-)**	0.87	**<0.01 (-)**	0.79
Mean weekly soil temperature (°C)	0.10	**<0.01 (+)**	0.99	**0.02 (+)**	0.54

P-values with significance are shown in **bold, ***Rolling mean was used for temperature and rainfall variables. ^The direction of the effect in indicated in parentheses after the significant p-value: The plus symbol (+) indicates that the median is significantly higher in binomial 1 variable, the minus symbol (-) indicates that the median is significantly higher in binomial 0 variable.

C

Relationship between categorical variables using chi-square statistics

Predictor variables	**1**	**2**	**3**	**4**	**5**	**6**
1 Foot has FR lesions (1=present)	1.00					
2 Sheep has FR (1=diseased)	**<0.01**	1.00				
3 *D. nodosus* present on feet (1=present)	**<0.01**	0.72	1.00			
4 *D. nodosus* present in soil (1=present)	0.70	0.19	0.34	1.00		
5 ***F.*** *necrophorum* present on feet (1=present)	**<0.01**	0.12	**<0.01**	0.10	1.00	
6 Body condition score (Scale from 0-5)	0.39	**<0.01**	0.65	0.85	0.63	1.00