Dichelobacter nodosus and Footrot in Swedish Sheep

Increased Knowledge and Improved Laboratory Diagnostics

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Abstract

Ovine footrot is a contagious bacterial disease primarily caused by *Dichelobacter nodosus*. Footrot affects the feet of sheep and is characterised by two major clinical presentations. The milder form consists of inflammation confined to the interdigital space (interdigital dermatitis or benign footrot) and the more severe form includes underrunning of the hoof horn (underrunning or virulent footrot). Disease severity is dependent on several factors including the virulence of the *D. nodosus* strain, environmental conditions, farming practices, host susceptibility, and co-infecting bacteria.

In Sweden, ovine footrot was first diagnosed in 2004, but fast and sensitive diagnostics for *D. nodosus* were not available. Likewise, knowledge was missing about the *D. nodosus* strains and co-infection with other lameness-associated bacteria. Hence the overall aim of this thesis project was to increase the knowledge about ovine footrot in Swedish sheep and to improve laboratory diagnostics for it.

In this thesis project, sensitive and specific real-time PCR methods to detect and discriminate between virulent and benign strains of *D. nodosus* were developed and used to characterise *D. nodosus* from Swedish sheep. The results showed that most of the Swedish *D. nodosus* are benign and that the virulent type is uncommon. *D. nodosus* isolates from seven other countries included in the study showed that the *D. nodosus* genome is highly conserved and that it exists as a globally distributed bimodal population. Furthermore, *D. nodosus* is mainly associated with the early stages of footrot whereas *Fusobacterium necrophorum* is associated with the later ones. This confirms the suggested role of *F. necrophorum* as an opportunistic pathogen rather than the primary pathogen. Although previously proposed, there was no evidence of *Treponema* spp. in disease development. Finally, a sample pooling method was developed to meet the demands for cost-efficiency in control programs. The method allows samples to be analysed in groups of five with no loss of sensitivity compared to individual samples. It has been implemented in the Swedish Footrot Control Program as a result of this thesis project.

Keywords: Dichelobacter nodosus, ovine footrot, diagnostics, virulence, serogroup, genome, real-time PCR, pooling of samples, *Fusobacterium necrophorum*, *Treponema*

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Dedication

To my family

"Har man tagit Fan i båten får man ro honom i land"
Svenskt ordspråk

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Frosth, S.**, Slettemeås, J.S., Jørgensen, H.J., Angen, Ø., and Aspán, A (2012). Development and comparison of a real-time PCR assay for detection of *Dichelobacter nodosus* with culturing and conventional PCR: harmonisation between three laboratories. *Acta Vet. Scand.* 54(6).

II Kennan, R.M., Gilhuus, M., **Frosth, S.**, Seemann, T., Dhungyel, O.P., Whittington, R.J., Boyce, J.D., Powell, D.R., Aspán, A., Jørgensen, H.J., Bulach, D.M., and Rood, J.I (2014). Genomic evidence for a globally distributed, bimodal population in the ovine footrot pathogen *Dichelobacter nodosus*. *mBio* 5(5):e01821-14.

III **Frosth, S.**, König, U., Nyman, A.K., Pringle, M., and Aspán, A (2015). Characterisation of *Dichelobacter nodosus* and detection of *Fusobacterium necrophorum* and *Treponema* spp. in sheep with different clinical manifestations of footrot. *Vet. Microbiol.* 179(1-2), 82-90.

IV Maboni, G., **Frosth, S**., Aspán, A., and Tötemeyer, S (2016). Ovine footrot: new insights into bacterial colonisation. *Vet. Rec.* 179(9).

V **Frosth, S**., König, U., Nyman, A.K., and Aspán, A (2016). Sample pooling for real-time PCR detection and virulence determination of the footrot pathogen *Dichelobacter nodosus. In manuscript.*

Paper IV is reproduced with the permission from BMJ & British Veterinary Association.

The contribution of Sara Frosth to the papers included in this thesis was as follows:

I Sara Frosth designed the study together with Anna Aspán and performed parts of the laboratory work. Sara Frosth analysed the results together with the other authors and wrote the first draft of the manuscript.

II Sara Frosth provided theoretical input and background data of *D. nodosus* isolates. Sara Frosth performed the experimental work on the Swedish *D. nodosus* isolates (culturing, DNA-extraction and next-generation sequencing) and contributed to writing the manuscript.

III Sara Frosth conceived the study together with Anna Aspán. Sara Frosth developed and/or improved the PCR assays used. Sara Frosth performed all PCR analyses and parts of the *D. nodosus* culturing work. Sara Frosth analysed the results together with the other authors and wrote the first draft of the manuscript.

IV Sara Frosth performed all the real-time PCR analyses for the detection of *F. necrophorum* and *Treponema* spp. and for virulence determination of *D. nodosus* which she had developed in paper III. Sara Frosth analysed the results together with the other authors and contributed to writing the manuscript.

V Sara Frosth planned the study with input from the other authors. Sara Frosth performed all the laboratory work and analysed the results. Sara Frosth wrote the first draft of the manuscript.

Abbreviations

aprB2	Acidic protease 2 gene from benign strains
AprB2	Acidic protease 2 from benign strains
aprV2	Acidic protease 2 gene from virulent strains
AprV2	Acidic protease 2 from virulent strains
BDD	Bovine digital dermatitis
bp	Base pair
BRIG	BLAST Ring Image Generator
CODD	Contagious ovine digital dermatitis
Contig	Contiguous sequence
D. nodosus	*Dichelobacter nodosus*
DNA	Deoxyribonucleic acid
EVOC	Ex vivo organ culture
F. necrophorum	*Fusobacterium necrophorum*
FAA	Fastidious anaerobe agar
fimA	Fimbrial subunit gene
FimA	Fimbrial subunit protein
HA	Hoof agar
IAC	Internal amplification control
ID	Interdigital dermatitis
intA	Integrase A gene
LOD	Limit of detection
MALDI-TOF-MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MGB	Minor groove binder
MHC	Major histocompatibility complex
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SJV	Swedish Board of Agriculture

SNP	Single nucleotide polymorphism
spp.	Species (plural)
SVA	National Veterinary Institute
TAS	Trypticase, arginine and serine
tRNA	Transfer ribonucleic acid
UK	United Kingdom

1 Introduction

1.1 Ovine footrot

Ovine footrot is a contagious animal disease primarily caused by the bacterium *Dichelobacter nodosus* (Beveridge, 1941). Its severity can depend on the virulence of the *D. nodosus* strain (Stewart *et al.*, 1986), environmental conditions (Depiazzi *et al.*, 1998; Graham & Egerton, 1968), farming practices (Wassink *et al.*, 2003), susceptibility of the host (Emery *et al.*, 1984), and co-infecting bacteria (Witcomb *et al.*, 2014; Egerton *et al.*, 1969). In addition, some sort of initial damage to the interdigital skin is believed to be required for the disease to develop (Egerton *et al.*, 1969; Beveridge, 1941).

The disease occurs worldwide in sheep and constitutes a major animal welfare problem due to the painful nature of the lesions that often result in lameness. The economic impact of footrot on the sheep industry is substantial; besides production losses in terms of meat and wool (Marshall *et al.*, 1991), there are high costs associated with prevention and treatment (Green & George, 2008).

1.1.1 Clinical signs

Ovine footrot starts as a mild inflammation of the interdigital skin of the foot and which may advance to result in complete separation of the hoof capsule from the tissues underneath (Beveridge, 1941). Two major clinical presentations of footrot exist. One milder form consists of inflammation confined to the interdigital space and a more severe form includes underrunning of the hoof horn (Egerton *et al.*, 1969; Beveridge, 1941). However, the terminology for these two conditions varies between countries. In Australia, benign and virulent footrot are used whereas the terms interdigital dermatitis (ID) and virulent footrot or underrunning footrot are used in the United Kingdom (UK) for the same conditions. The term footrot has also been

used for the more severe form of the disease (underrunning of the horn) in the UK, when ID and footrot were considered to be two separate diseases (Green & George, 2008).

The typical clinical sign of footrot is lameness, but not all animals with footrot are lame. Usually more than one animal in the flock is affected. More than one foot on the same animal can be affected; it often grazes on its front knees or lies down more often than unaffected animals. Early clinical signs include a reddening of the interdigital skin which is often moist and may suffer from hair loss. Later, a white-greyish exudate is often visible in the interdigital space. The inflammation can sometimes heal spontaneously, but may also progress to include the skin-horn junction of the inside wall. Once spread to the soft horn, underrunning can occur to various extents beginning at the heel and sole. In the worst case, the hard horn of the outer wall is also underrun and may eventually loosen from the underlying tissues. Prolonged infection can result in deformation of the hoof (Stewart & Claxton, 1993).

Different footrot scoring systems have been developed to aid in disease assessment and for standardisation purposes. One widely used system is the one developed by Egerton and Roberts (1971). This system, has a scale from 0 (healthy) to 4 (most severe clinical signs).

1.1.2 Transmission

The major risk of footrot introduction into a flock comes from contact with sheep from neighbouring flocks (Grøneng *et al.*, 2014; Raadsma & Egerton, 2013). Introduction of new animals also poses a risk but this can be reduced by only introducing animals from flocks certified free of footrot or by quarantine of the newly introduced animals (Abbott & Lewis, 2005).

Transmission of *D. nodosus* from infected to susceptible sheep occurs mainly via the environment – soil, pasture or bedding. If the sheep are in the same place at the same time when transmission of *D. nodosus* occurs, this is usually termed direct transmission in the literature, whereas indirect transmission refers to transmission to sheep in the same place but on different occasions. Muzafar *et al.* (2015) showed that direct transmission can occur rapidly; newborn lambs were colonised by *D. nodosus* within hours after birth, most probably due to contaminated straw bedding. Only a short exposure time of susceptible sheep to contaminated grounds was enough for indirect transmission to occur in the field, as first reported by Whittington (1995). Transmission can also occur mechanically via equipment or other objects that are in contact with the animals, for example knives used for foot trimming, gloves or boots.

Transmission is influenced by environmental conditions such as moisture, temperature, and pasture (Depiazzi *et al.*, 1998; Graham & Egerton, 1968). Wet and relatively warm weather (mean temperature above 10°C) facilitates transmission, whereas hot, dry weather inhibits it (Depiazzi *et al.*, 1998; Graham & Egerton, 1968).

1.1.3 Reservoirs

The major reservoir of ovine footrot consists of sheep that are either subclinically or chronically infected with *D. nodosus*. Subclinical carriers can harbour the bacterium in the interdigital skin without showing any clinical signs of disease. Chronic carriers are sheep that have not fully recovered after treatment or that have been affected by footrot for a long time. In both cases, new horn can be formed over bacteria that have remained within the foot. These encapsulated bacteria can survive for several years (Beveridge, 1941).

D. nodosus has been isolated from other ruminants including goats (Claxton & O'Grady, 1986), cattle (Egerton & Parsonson, 1966), deer (Skerman, 1983) and moose (Frosth, unpublished). Goats are as equally susceptible to footrot as sheep. Initially, cattle were considered unlikely potential reservoirs (Beveridge, 1941) and were later thought only capable of harboring benign *D. nodosus* (Wilkinson *et al.*, 1970). More recent research, however, has shown that cattle can be infected with virulent *D. nodosus* and for a long time (Knappe-Poindecker *et al.*, 2014a). Virulent *D. nodosus* isolated from cattle has been shown to induce severe disease in lambs under experimental conditions (Knappe-Poindecker *et al.*, 2014b). In turn, both benign and virulent strains of *D. nodosus* isolated from sheep have been successfully transferred to cattle, which then developed ID (Knappe-Poindecker *et al.*, 2015).

Survival of *D. nodosus* in the environment, i.e. outside of its host and niche, is believed to be limited, but recent *in vitro* studies have shown that the bacterium can survive at most 40 days in soil (Muzafar *et al.*, 2016; Cederlöf *et al.*, 2013; Enlund, 2010). This is much longer than the previously reported survival time of days up to two weeks. It is also longer than the 14-day recommended rest time for fields that have been used by footrot-affected sheep (Whittington, 1995; Beveridge, 1941). However, whether these surviving bacteria are capable of causing an infection has not been investigated. Previous studies show that the bacterium was not infectious after more than one week outside the host (Beveridge, 1941).

1.1.4 Differential diagnosis

The main differential diagnosis for ovine footrot is contagious ovine digital dermatitis (CODD), previously known as severe virulent ovine footrot

(Harwood *et al.*, 1997). CODD begins as an inflammation at the coronary band (from which the hoof wall grows) and not in the interdigital skin. However, both diseases can lead to underrunning of the hoof wall (Duncan *et al.*, 2014). CODD has so far only been reported in the UK and its aetiology is unclear (Duncan *et al.*, 2014). *Treponema* species (spp.) may be associated with CODD, but other bacteria such as *D. nodosus* might also be (Duncan *et al.*, 2012; Moore *et al.*, 2005b; Naylor *et al.*, 1998).

Other differential diagnoses include white line lesions, interdigital hyperplasia, and granulomas. White line lesions are characterised by a weakening of the sole-wall junction of the hoof (white line) which can vary in extent from a small lesion to more severe cases where the white line is separated (Winter & Arsenos, 2009). Soil, stones and other debris can accumulate in the pocket generated by the separation and lead to infections and pus (Winter & Arsenos, 2009). The cause of white line lesions is unknown despite its frequent occurrence in sheep in the UK and other countries (Winter & Arsenos, 2009). Almost 10% of Swedish slaughter lambs had white line separation in a footrot prevalence study performed in 2009 (König *et al.*, 2011).

Interdigital hyperplasia is the outgrowth of skin folds in the interdigital space. When these continue to grow, they are pressed between the digits during walking and cause pain (Winter, 2004). The cause is unknown, but since it is believed to be hereditary, sheep with interdigital hyperplasia should not be bred (Winter, 2004). Just under 1% of the Swedish slaughter lambs had interdigital hyperplasia in the footrot prevalence study by König *et al.* (2011).

Granulomas consist of outgrowths of granulation tissue and are most commonly found at the toe of the hoof (Winter, 2004). Granulomas are often caused by over-trimming, but can also develop after sole injuries (Winter, 2004).

1.2 Footrot in Sweden

1.2.1 First diagnosed case

The first case of ovine footrot in Sweden was clinically diagnosed and bacteriologically confirmed in 2004 (Olofsson *et al.*, 2005). Suspicion of disease had, however, existed earlier and *D. nodosus* was detected already in 1981 in cattle (Plym Forsell & Andersson, 1981). The first sheep farm diagnosed with footrot was located in western Sweden and was newly established. Sheep had been introduced from five other Swedish farms and they were all Swedish born, but it could not be excluded that there had been imported animals earlier in the chain of infection. The sheep farm consisted of

some hundred Gotland Pelt sheep and had experienced problems with lameness (Olofsson *et al.*, 2005).

1.2.2 Diagnosis and scoring system

Diagnosis of footrot in Sweden is mostly based on clinical signs. Sampling and detection of *D. nodosus* is only used as a complement to the clinical diagnosis, due to the many reports that the bacterium is also found in healthy feet (Vatn *et al.*, 2012; Calvo-Bado *et al.*, 2011; Moore *et al.*, 2005a; Depiazzi *et al.*, 1998; Glynn, 1993). The scoring system for assessment of the sheep feet is the one described by Stewart & Claxton (1993), and it is a modification of the system described by Egerton & Roberts (1971). An overview of the scoring system is given in Table 1 and photos illustrating each score can be seen in Figures 1-6. Scores 1 and 2 are characterised by benign footrot or ID, whereas scores 3, 4 and 5 are characterised by virulent or underrunning footrot (Stewart & Claxton, 1993).

Table 1. *Footrot scoring system currently used in Sweden (Stewart & Claxton, 1993).*

Score	Definition
0	Healthy foot
1	Mild to moderate inflammation limited to the interdigital skin
2	Necrotising inflammation of the interdigital skin also involving the soft horn of the inside wall
3	Necrotising inflammation with underrunning of the soft horn of the heel and sole
4	Necrotising inflammation with underrunning extending to the outside wall of the sole
5	Necrotising inflammation of the deeper tissues (laminae) of the outside wall with underrunning of the hard horn

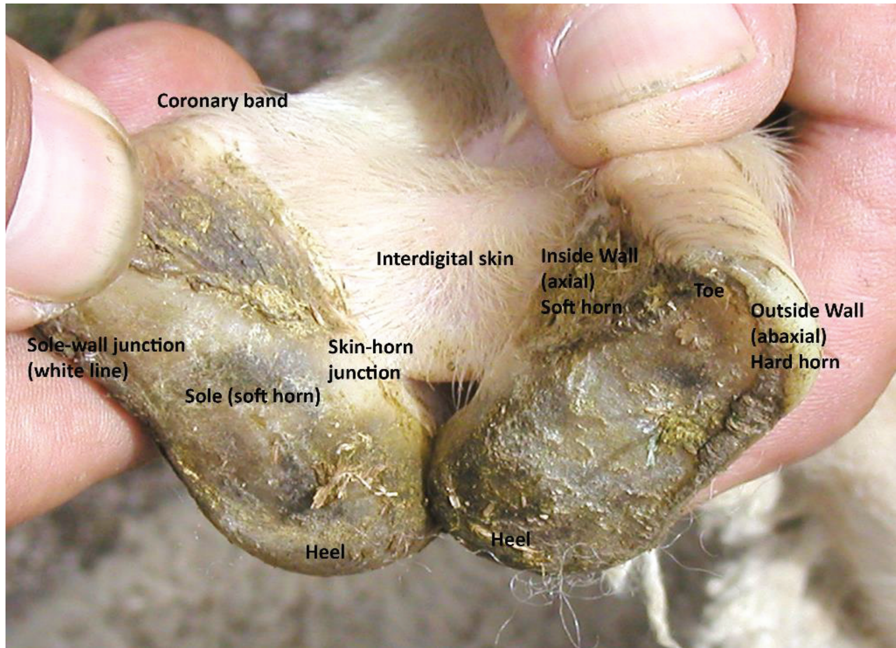

Figure 1. Score 0 = healthy. The different parts of the sheep's foot are labelled. Photo: Ulrika König.

Figure 2. Score 1 = mild inflammation of the interdigital skin including reddening and hair loss. Photo: Åsa Hilmersson.

Figure 3. Score 2 = necrotising inflammation of the interdigital skin involving the skin-horn junction. Photo: Ulrika König.

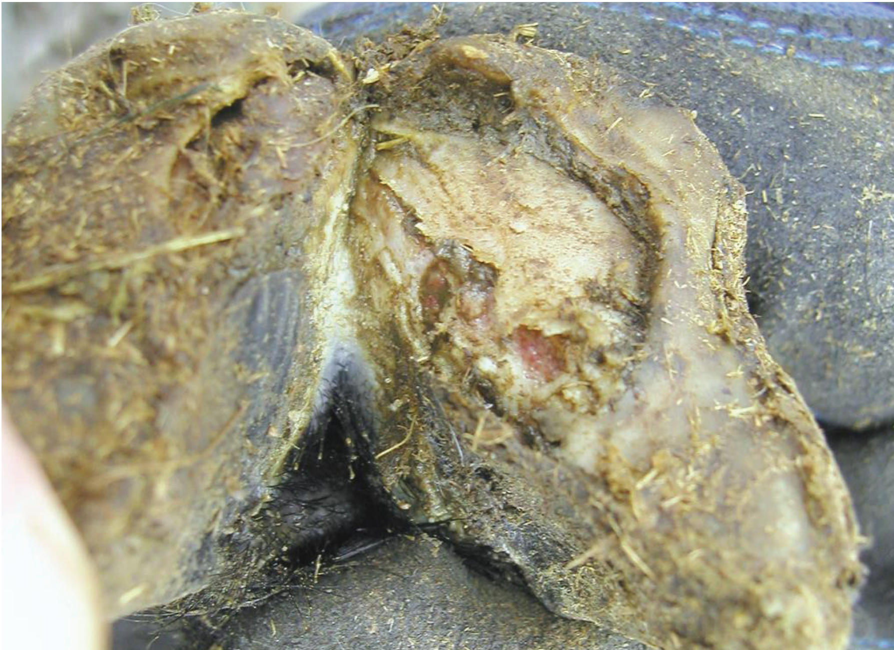

Figure 4. Score 3 = necrotising inflammation and underrunning of the soft horn. Photo: Ulrika König.

Figure 5. Score 4 = underrunning extending to the outside wall. Photo: Ulrika König.

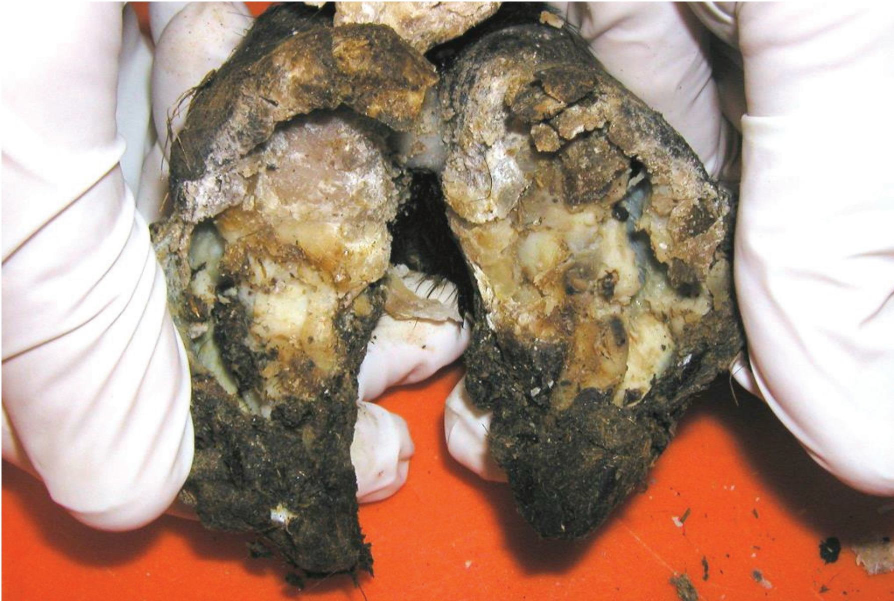

Figure 6. Score 5 = necrotising inflammation of the deeper tissues (laminae) including underrunning of the hard horn. Photo: Ulrika König.

In Sweden, footrot is diagnosed on flock level. Disease is defined as the presence of lesions scored ≥2 and the term footrot is used regardless of the extent of the lesions. Score 1 lesions are not regarded as footrot in Sweden due to the belief that this mild inflammation is caused by factors other than *D. nodosus*, for example wet pastures, manure or infection with *Fusobacterium necrophorum.* Virulence determination of *D. nodosus* isolates has not been available previously in Sweden and the few strains tested did not always correlate with the clinical signs. As a result of this thesis project, there is now a quick and cost-effective test to determine the virulence of *D. nodosus* isolates (papers III and V).

Why does virulence of the infecting *D. nodosus* strain not always correlate with clinical disease? Benign footrot or ID (scores 1 and 2) are thought to be caused by benign *D. nodosus* strains whereas virulent or underrunning footrot (scores 3-5) are thought to be caused by virulent strains (Stewart & Claxton, 1993). However, virulent *D. nodosus* has been found in benign footrot (Stäuble *et al.*, 2014b; Moore *et al.*, 2005a) and benign *D. nodosus* has been found in virulent footrot (Frosth *et al.*, 2015). In the study by Stäuble *et al.* (2014b), virulent *D. nodosus* in healthy animals was explained by early- or subclinical infection. Different percentages of score 4 lesions (>10%=virulent, 1-10%=intermediate, 1%<=benign footrot) have been used in Australia for classification of disease at the flock level (Stewart & Claxton, 1993). That a classification system is needed at flock level – in addition to the scoring system at an individual level – demonstrates the complexity of disease.

1.2.3 Prevalence

A prevalence study on footrot in Swedish slaughter lambs was conducted in 2009 by König *et al.* (2011). In that study, which was based on clinical examination of feet from 500 lambs, 5.8% had footrot as defined by a score ≥2 footrot lesion (König *et al.*, 2011). This figure might, however, be an underestimate because animals with more severe footrot lesions were not included. These animals are not routinely sent for slaughter (König *et al.*, 2011).

Footrot is a notifiable disease in Sweden (SJVFS 2013:23) which means that all index cases (the first identified case in a flock) are reported to the Swedish Board of Agriculture (SJV); Figure 7 shows the total number of reports from 2008 to 2015. These reports are, however, not a good estimate of the true prevalence because the notification requirement is based on laboratory diagnostics (detection of the bacterium) whereas diagnosis in practice is mainly based on clinical signs. The willingness to take samples has also probably decreased because of the reports that *D. nodosus* is found in healthy sheep as

well as affected ones. The Farm and Animal Health, an advisory company working with animal health issues in the sheep, pig and cattle industries, has recorded all new clinical cases of footrot among their clients since the first diagnosed case in 2004 (Figure 7). In 2015, Farm and Animal Health had 2347 sheep flock clients (Ulrika König, personal communication) which is 26% of the total number of sheep flocks in the country (Statistics Sweden, 2016).

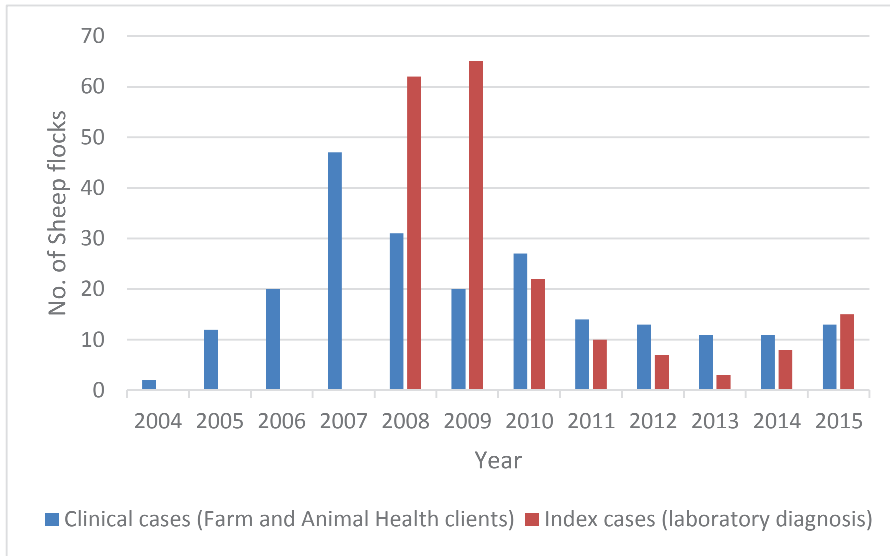

Figure 7. The number of clinical cases of footrot among clients of Farm and Animal Health and index cases based on laboratory diagnosis reported to the SJV between 2004 and 2015 (National Veterinary Institute, 2016; Swedish Board of Agriculture, 2009-2016). Note that the obligation to report index cases to SJV did not begin until 2008.

1.2.4 Control program

Farm and Animal Health has operated a voluntary Footrot Control Program (Klövkontrollen) since 2009 (Farm and Animal Health, 2016). The program is open to all sheep flocks whether they are clients of the Farm and Animal Health or not. In 2015, a total of 336 sheep flocks were enrolled in the program (National Veterinary Institute, 2016). The program is based on regular inspections of the feet of the sheep and is funded by the sheep farmers and the SJV. The purpose of the program is to detect and eradicate footrot from affected flocks and to enable trading of animals from footrot-free flocks. For example, certificates declaring freedom from footrot are required by several of the sale auctions for rams.

In brief, all the sheep feet of a flock except slaughter lambs are inspected between mid-August and mid-October every year, either by a veterinarian or the animal owner. If there are no clinical signs of footrot, the flock is declared free of footrot and obtains F-status (Figure 8).

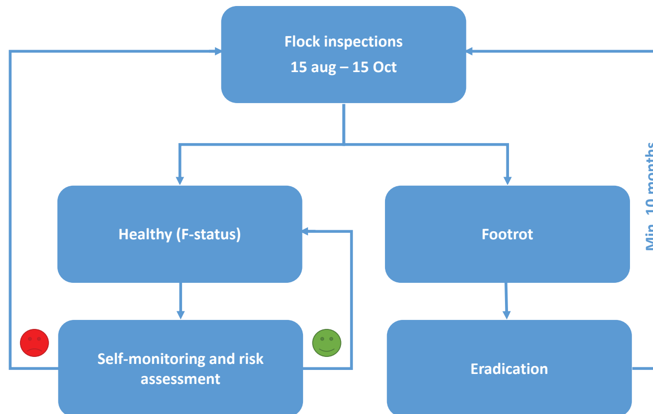

Figure 8. Schematic overview of the Swedish Footrot Control Program. Modified from Farm and Animal Health (2016).

Flocks entering the program for the first time are always inspected by a veterinarian, but flocks already enrolled may be inspected by the animal owner as part of a yearly self-monitoring. However, the animal owner must have been present at the first veterinary inspection to be trained in clinical examination and differential diagnosis of footrot. In addition, he or she must fill in a form describing routines, any contacts, observed lameness, and diagnoses of lameness during the year. Based on the information given by the animal owner, Farm and Animal Health performs a risk-assessment and F-status is issued if the flock passes the assessment. Otherwise a veterinarian will visit the farm and perform an additional inspection.

Since 2015, all newly enrolled flocks, as well as flocks suspected to have footrot are sampled at the first contact (five swab samples) for *D. nodosus* detection and virulence determination by the real-time polymerase chain reaction (PCR) analysis developed in this thesis project (papers I and III) (National Veterinary Institute, 2016). Flocks affected by footrot, but who wish to eradicate disease can obtain F-status, at the earliest, 10 months after treatment if no clinical signs of footrot are present at the repeated veterinary inspection.

1.2.5 Treatment and eradication of disease

The treatment and eradication of footrot currently used by Farm and Animal Health, is mainly based on footbathing with 10% (w/v) zinc sulphate although antibiotics are used in severe cases. Tetracycline for systemic treatment is the most commonly used antibiotic for footrot in Sweden. Antimicrobial susceptibility testing of Swedish *D. nodosus* isolates showed high susceptibility to several common antibiotics, including tetracycline, with the exception of one isolate (Frosth *et al.*, 2013). All isolates were susceptible to penicillin in the same study.

In short, for eradication of footrot, the feet of all sheep in the flock are inspected and chronically infected sheep are culled. The remaining sheep are subjected to a footbath with 10% (w/v) zinc sulphate solution for 15 to 30 minutes and transferred to a clean, hard surface for 30 to 60 minutes. The footbathing procedure is repeated twice after 1 to 7 days, followed by a repeated inspection and culling of affected sheep. A third inspection of the feet is performed about a year later. Preventive footbathing of the entire flock is not recommended in Sweden as it is believed that it could mask clinical signs and hence delay eradication measures. Footbathing is, however, always recommended when new animals are introduced; these should also be quarantined and have all their feet inspected before they are released into the rest of the flock.

1.2.6 Sheep population and farming practices

The sheep industry in Sweden is small compared to sheep rearing countries such as Australia and the UK. Even the neighbouring country Norway has over three times more sheep than Sweden. However, the sheep population, unlike those of pigs and cattle, is increasing in Sweden. In 2015, there were 594,753 sheep and lambs, which is an increase of 38% since the year 2000 (Statistics Sweden, 2016). There were 9,110 flocks in the same year with an average flock size of 32 ewes (Statistics Sweden, 2016). Most are located in the southern part of Sweden with the county Gotland having the densest sheep population (20.8 sheep per km^2) (Statistics Sweden, 2016). The most common breed in Sweden is the Gotland Pelt sheep (Figure 9). Movement of animals between different regions in the country is limited and sheep are often housed indoors during the winter (approximately between October to April).

Figure 9. Gotland Pelt sheep (foreground, grey with black head and legs) and Finnsheep (background, white) are two common sheep breeds in Sweden. Photo: Sara Frosth.

1.3 *Dichelobacter nodosus*

D. nodosus is the causative agent of ovine footrot. The bacterium was first described in 1941 although the disease was discovered already around 1810 (Beveridge, 1941). At the time of description, the bacterium was given the name *Fusiformis nodosus* (Beveridge, 1941), but it has been renamed twice since then, first to *Bacteroides nodosus* (Mraz, 1963) and then to the present *D. nodosus* (Dewhirst *et al.,* 1990). The type strain of *D. nodosus* is ATCC 25549^{T} (=CCUG 27824).

The genus name *Dichelobacter* is derived from the Greeks *dichelos* meaning cloven hoofed and *bacter* meaning rod (Dewhirst *et al.*, 1990), which is appropriate since it is a rod-shaped bacterium of ungulates. The species name, *nodosus*, meaning full of knots, reflects the shape of the bacterial cell which has rounded swollen ends (Dewhirst *et al.*, 1990). Besides the swollen ends, the cell has variable numbers of fimbriae or pili that cause the twitching motility of the cell and hence the spreading of the colonies on agar plates (Rood *et al.*, 2015).

The rod-shaped cells, are 3 to 6 µm long and 1.0 to 1.7 µm in diameter (Rood *et al.*, 2015). The rods are Gram-negative, and straight or slightly curved. They grow under anaerobic conditions but are not extremely sensitive

to oxygen (Rood *et al.*, 2015). Myers *et al.* (2007) showed that *D. nodosus* was still viable after 10 days in aerobic conditions.

1.3.1 Taxonomy

D. nodosus is the sole species within the genus of *Dichelobacter* and together with the *Cardiobacterium* and *Suttonella* genera, it constitutes the family of *Cardiobacteriaceae* (Dewhirst *et al.,* 1990). The *Cardiobacteriaceae* family in turn belongs to the class of *Gammaproteobacteria.* The *Cardiobacterium* genus, as well as the *Suttonella* genus, both contain two species each: *C. hominis* and *C. valvarum* and *S. indologenes* and *S. ornithocola,* respectively. All five currently recognised species of the family are associated with pathogenic disorders of animals or humans (Moore *et al.*, 2014).

1.3.2 Serogroups

Ten different serogroups of *D. nodosus,* designated A-I and M, are currently recognised (Ghimire *et al.*, 1998; Claxton, 1989). The serogroup classification is based on the fimbriae of the bacterium (Egerton, 1973). The serogroups are divided into two classes depending on sequence similarity and organisation of the fimbrial genes. Class I contains the majority of the *D. nodosus* serogroups – A, B, C, E, F, G, I and M – whereas class II only contains serogroups D and H (Ghimire *et al.*, 1998; Mattick *et al.*, 1991).

1.3.3 Virulence factors

Virulence of *D. nodosus* depends mainly on type IV fimbriae and expression of extracellular serine proteases (Kennan *et al.*, 2010; Kennan *et al.*, 2001). The genetic element *intA,* consisting of an integrase gene, was previously believed to be associated with virulence (Cheetham *et al.*, 2006), and hence the real-time PCR targets the *intA* gene in this thesis project (paper III). A correlation between the *intA* gene and virulence has, however, not been found in more recent studies, neither in the comparison of different phenotypic virulence tests by Dhungyel *et al.* (2013) nor in the genetic comparison of *D. nodosus* isolates for virulence in this thesis project (paper II).

Type IV fimbriae

D. nodosus has type IV fimbriae, enabling the bacterium to adhere and move into the damaged tissue of the host by twitching motility, which is essential for virulence (Han *et al.*, 2008). Other functions of the polar type IV fimbriae in *D. nodosus* are extracellular protease secretion and natural transformation (Kennan *et al.*, 2001). The type IV fimbriae of *D. nodosus* consist mainly of polymers of the major fimbrial subunit protein (FimA) and are highly

immunogenic. Other Gram-negative bacteria with type IV fimbriae include *Pseudomonas aeruginosa*, *Suttonella indologenes*, *Kingella kingae*, *Neisseria meningitidis*, *Moraxella bovis* and *Eikenella corrodens.*

Extracellular proteases

D. nodosus produces three closely related extracellular subtilisin-like serine proteases (subtilases) capable of tissue degradation, of which at least the acidic protease 2 from virulent strains (AprV2), is crucial for virulence (Kennan *et al.*, 2010). The AprV2 protease from virulent *D. nodosus* is thermostable and responsible for the elastase activity, two characteristics traditionally used for virulence determination (Kennan *et al.*, 2010). AprV2 differs from its benign counterpart, AprB2, by a single amino acid alteration (Tyr92Arg) (Riffkin *et al.*, 1995). At the gene level, the difference between the protease gene variants *aprV2* and *aprB2* consists of a two-base pair (bp) change from TA to CG at position 661/662 (accession No. L38395) (Riffkin *et al.*, 1995). The other two proteases are acidic protease 5 (AprV5 and AprB5) and basic protease (BprV and BprB) from virulent and benign strains, respectively.

1.3.4 Genome

The first *D. nodosus* genome to be sequenced was strain VCS1703A (GenBank accession number NC_009446.1). Whereas it is not the type strain, it has been used in several virulence studies (Myers *et al.*, 2007). The genome of *D. nodosus* is very small, about 1,400,000 bp. Nevertheless, one-fifth of the genome is believed to have originated from other organisms (Myers *et al.*, 2007), of which the largest region – probably acquired by lateral gene transfer – is an incorporated Mu-like bacteriophage (Myers *et al.*, 2007).

The biosynthetic ability of *D. nodosus* is surprisingly high given the size of the genome, although amino acid processing pathways are lacking (Myers *et al.*, 2007). Genes for amino acid transportation have, however, been identified and it is proposed that the extracellular proteases provide the cell with amino acids by degradation of host proteins (Myers *et al.*, 2007).

1.3.5 Immunology

D. nodosus infection of the epidermis causes an immune response, although it is not long-lasting (Egerton & Roberts, 1971). Understanding the immune response is important in many aspects, for example natural resistance and vaccine development, both of which can be used to control disease.

Natural resistance to footrot differs between sheep breeds (Emery *et al.*, 1984). British breeds are more resistant whereas merinos are more susceptible (Emery *et al.*, 1984). Footrot resistance is associated with variation in the

major histocompatibility complex (MHC) II (Escayg *et al.*, 1997). A commercially available test based on this has been developed and is used in breeding programs in New Zealand (Hickford, 2001).

In 1969, the first footrot vaccine was developed and used in research (Egerton, 1970). It was based on *D. nodosus* whole cells, but later purified fimbriae were shown to be equally effective (Egerton *et al.*, 1987; Every & Skerman, 1982). Several different vaccines against footrot have been developed since then, consisting of either whole cells or purified fimbriae (Dhungyel *et al.*, 2014). Nevertheless, there is only a single commercial vaccine available (Footvax, MSD Animal Health), and its protective effect is short-lived, possibly due to antigenic competition (Dhungyel *et al.*, 2014). Footvax contains many different serogroups (nine of ten, A-I), since immunity is serogroup-specific. An efficient vaccine against all *D. nodosus* serogroups would be ideal since multiple serogroups can be present within a flock (Claxton *et al.*, 1983). Furthermore, *D. nodosus* may seroconvert (Kennan *et al.*, 2003). Cross-protective antigens have been identified by reverse vaccinology (Myers *et al.*, 2007), and these may contribute to the development of an improved vaccine in the future.

1.4 Laboratory diagnostics of *Dichelobacter nodosus*

1.4.1 Detection

The presence of *D. nodosus* in footrot lesions can be demonstrated by different methods such as Gram-staining followed by microscopy, culturing, or PCR analysis. Microscopy and culturing results are preferably confirmed by an additional method, for example PCR or matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS).

Culturing

Culturing of *D. nodosus* is performed under anaerobic growth conditions with agar media including hoof agar (HA) (Stewart & Claxton, 1993; Thomas, 1958), trypticase, arginine and serine (TAS) agar (Skerman, 1975), and Eugon agar (Stewart & Claxton, 1993; Gradin & Schmitz, 1977). A high agar concentration (4% w/v Difco agar) is used which promotes growth of *D. nodosus* while inhibiting growth of contaminating bacteria (Thorley, 1976; Skerman, 1975). Plating shortly after sampling and careful drying of the plates prior to inoculation also helps to prevent contamination.

Agar plates are inoculated in a special way when using swabs. Swabs are used for the primary streak but a sterile toothpick or the back of a cotton swab is then used to make a grid pattern in the agar. Plates are incubated at 37 °C for

4-6 days and *D. nodosus* colonies grow out of the streaks made in the agar as flat semicircles (Figure 10).

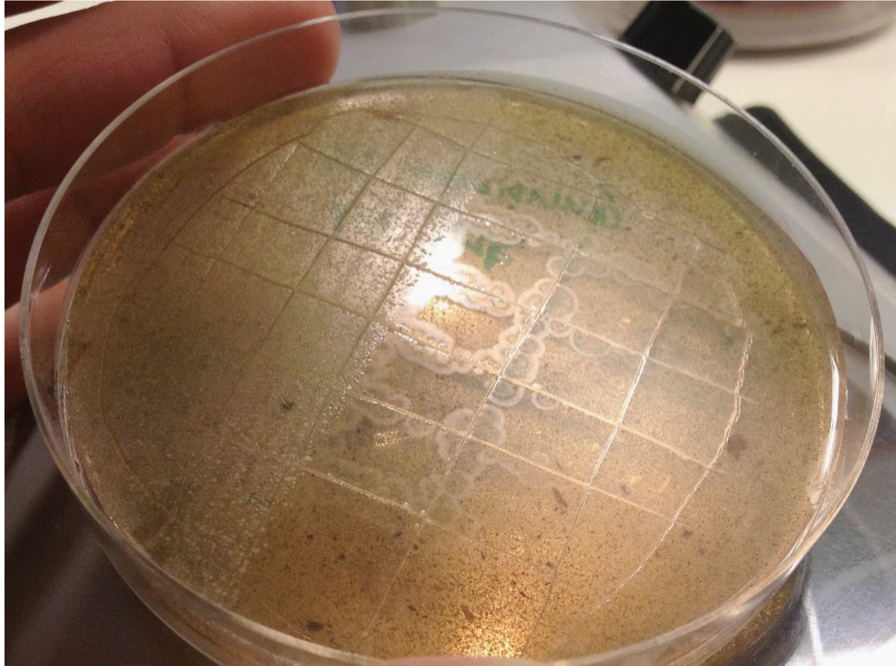

Figure 10. A clinical swab sample inoculated on 4% HA. Growth of *D. nodosus* can be seen around the streaks of the grid pattern in the middle of the plate. Photo: Sara Frosth.

A stereomicroscope is needed to examine the agar plates and to pick colonies for subculturing. Typical colonies of *D. nodosus* on HA can be recognised by their granulated outer zones, and distinct bands of seemingly no growth between the outer and the more central parts of the colonies. Colony appearance can, however, vary depending on the agar media used, agar concentration, and characteristics of the *D. nodosus* isolate, especially at lower agar concentrations (Skerman *et al.*, 1981; Thorley, 1976).

PCR

Detection of *D. nodosus* by PCR without the need for culturing was first described by La Fontaine *et al.* (1993). This method was later adjusted by Moore *et al*. (2005a) to be better suited for analysis of clinical swab samples.

In this thesis project (paper I), a real-time PCR was developed targeting the *16S* ribosomal ribonucleic acid *(rRNA)* gene. Two previous methods targeted this gene as well, but our method has higher sensitivity, specificity and shorter analysis time. Our PCR assay was further improved to include an internal

amplification control (IAC) and an additional target, *D. nodosus intA* gene, (paper III).

1.4.2 Virulence determination

Several methods for virulence determination of *D. nodosus* exist and all of them in some way examine the extracellular proteases that *D. nodosus* produces. The elastase (Stewart, 1979) and gelatin-gel tests (Palmer, 1993) are examples of virulence tests based on phenotypic expression of the proteases measuring elastase activity and thermostability, respectively.

More recently, gene-based tests in the form of real-time PCR assays have been developed, both in this thesis project (paper III) and by Stäuble *et al.* (2014a). Both of these real-time PCR assays identify and differentiate between *aprV2* and *aprB2,* using a common primer pair together with specific probes for each gene variant.

1.4.3 Serogrouping

Nine of ten *D. nodosus* serogroups A-I can be detected and discriminated by multiplex PCRs developed by Dhungyel *et al.* (2002). The PCRs target the major fimbrial subunit gene *(fimA)* of *D. nodosus* type IV fimbriae using a common forward primer and nine serogroup-specific reverse primers (Dhungyel *et al.*, 2002). Currently, there is no PCR that can readily detect the tenth serogroup M. The primers developed specifically for serogroup class I, however, can be used for amplification and subsequent sequencing for identification of that particular serogroup (Zhou & Hickford, 2001).

1.5 Other bacterial species implicated in footrot

Footrot is considered by many to be a polymicrobial disease even though the aetiology is attributed to *D. nodosus*. *Fusobacterium necrophorum* and *Treponema* spp. have been found in footrot lesions, (Egerton *et al.*, 1969; Roberts & Egerton, 1969; Beveridge, 1941) of which *F. necrophorum* is perhaps the most studied and debated. Indeed, many bacterial genera reside in the interdigital skin of the ovine foot; differences in variety and quantity as they relate to clinical condition have been reported by Calvo-Bado *et al.* (2011). For example, the genus *Peptostreptococcus* is associated with healthy feet, *Corynebacterium* with ID, and *Staphylococcus* with underrunning footrot.

1.5.1 *Fusobacterium necrophorum*

F. necrophorum is a Gram-negative, anaerobic bacterium that colonises the alimentary tract of animals and humans. It produces several toxins including a

potent leukotoxin (Tan *et al.*, 1996). Two subspecies of the bacterium are currently recognised – *necrophorum* and *funduliforme* (Shinjo et al., 1991). The *necrophorum* subspecies is more pathogenic than *funduliforme* due to its higher lipopolysaccharide content and higher leukotoxin production (Tan *et al.*, 1996). Its role in ovine footrot is debated. Some researchers believe that colonisation by *F. necrophorum* is a prerequisite for *D. nodosus* to infect the ovine interdigital skin (Egerton *et al.*, 1969; Roberts & Egerton, 1969) whereas others consider it to be an opportunistic pathogen (Witcomb *et al.*, 2014). Some sort of initial damage to the interdigital skin seems to be required (Egerton *et al.*, 1969; Beveridge, 1941) but it is unclear if that damage is caused by *F. necrophorum* or merely by wet grounds or mechanical trauma.

1.5.2 *Treponema* spp.

The genus *Treponema* consists of Gram-negative, anaerobic bacteria that are spiral-shaped and motile. Treponemes are very difficult to culture due to their fastidiousness. Treponemes colonise the intestinal tract and feet of animals and the oral cavity and intestinal- and genital tracts of humans. Both pathogenic and non-pathogenic members of the genera have been identified. The genus belongs to the family of *Spirochaetaceae.* Spirochaetes were found in footrot lesions already in 1941 (Beveridge, 1941). More recently, *Treponema* spp. were identified in a sheep with ID (Calvo-Bado *et al.*, 2011). *Treponema* spp. have also been found associated with CODD (Moore *et al.*, 2005b; Naylor *et al.*, 1998) and bovine digital dermatitis (BDD) (Walker *et al.*, 1995), both of which are diseases affecting the feet of animals.

2 Aims of the Thesis

The overall aim of this project was to increase the knowledge about ovine footrot in Swedish sheep and to improve laboratory diagnostics for it. More specifically, the aims were:

- To develop sensitive and specific real-time PCR methods for detection and virulence determination of *D. nodosus.*

- To investigate the genetic relationship between virulent and benign *D. nodosus* isolates and between *D. nodosus* isolates from different countries.

- To characterise *D. nodosus* from Swedish sheep.

- To study the bacterial colonisation in ovine footrot.

- To develop a pooling method that allows for quick and cost-efficient, yet sensitive real-time PCR analysis of *D. nodosus.*

3 Comments on Materials and Methods

This chapter presents general considerations regarding the materials and methods used in the thesis project. Detailed information regarding the same can be found in each paper.

3.1 Sampling

3.1.1 Sheep flocks

All sheep flocks described in papers III and V were enrolled in the Swedish Footrot Control Program. Only 3.7% of Swedish flocks are thus far enrolled in the program, but these are often involved in live animal trading and hence it is extra important that they are controlled. The study flocks were selected on the basis of convenience, then clinically examined and sampled in conjunction with routine inspections within the Footrot Control Program. The footrot scoring system was the one described in Stewart & Claxton (1993), which is a modification of Egerton & Roberts (1971) system. Score ≥2 lesions were considered as footrot. The majority of the sheep flocks (*n*=24, 64.8%) were scored and sampled by one and the same veterinarian who is highly experienced in footrot scoring. The remaining 13 flocks were scored and sampled by a small number of veterinarians trained in footrot scoring by the experienced veterinarian mentioned above. Besides clinical status of the sheep flocks, geographical location was also considered in paper III.

The UK sheep in paper IV were also selected on the basis of convenience. All feet were scored and sampled during six visits to an abattoir. Two persons scored and sampled all feet, one of which was in charge and present during all visits for standardisation purposes. The scoring system was modified from Parsonson *et al.* (1967). Feet were classified as healthy, ID or footrot according to the following criteria: absence of lesions in the interdigital skin=healthy, slight lesions with ≤5% of the skin affected=mild ID, lesions with >5% of the

skin affected=moderate/severe ID, and presence of underrunning lesions=footrot.

3.1.2 Sampling methods

Swabbing of feet was chosen as sampling method because it is non-invasive. Different types of swabs were used in paper I for culturing and real-time PCR, merely due to the fact that one and the same swab had not yet been evaluated for both methods. In paper III, the ESwab (Copan Innovation Ltd, Brescia, Italy) was used for both culturing and PCR analysis. Biopsy sampling (paper IV) was performed on slaughtered sheep to investigate bacterial colonisation in footrot. Biopsy material is advantageous since it allows for studies of bacteria that have penetrated the skin.

Figure 11. Sampling of the interdigital skin with an ESwab. Photo: Ulrika König.

3.1.3 Sampling site

The samples, both swabs and tissue biopsies, were taken from the interdigital skin of the sheep feet. However, it is also possible to sample the edge of footrot lesions, if present. The feet were generally not cleaned before sampling except for the biopsy sampling. The swab was gently pressed against the interdigital skin and twisted a half turn; this gave enough material for both culturing and PCR (Figure 11). Excessive swabbing often resulted in overgrowth of contaminating bacteria but had little effect on the PCR analysis.

3.1.4 Transport of samples

The time from sampling until culturing has been reported as important for successful culturing, hence plating already in the field has been suggested (Stewart & Claxton, 1993). Our swab samples were, however, plated in the laboratory to ensure consistency and sterility in the plating procedure. All swab samples were sent by regular mail to the National Veterinary Institute (SVA) where they arrived within one to three days after sampling.

3.1.5 Pooling of samples

Pooling of individual samples is used in some control programs to obtain a flock diagnosis at reduced work load and analysis costs (National Veterinary Institute, 2016). A common risk of pooling is reduced sensitivity due to dilution effects. Even if the samples are treated to achieve a higher bacterial concentration, the additional handling can reduce sensitivity. Hence we chose to pool samples in groups of five in paper V. This entailed only a minor deviation from the DNA extraction procedure developed for individual samples in paper I. More samples than five could probably be pooled, considering the favorable results presented in paper V, but that would need to be evaluated.

3.2 Culturing

Culturing – often considered the "gold standard" of methods – was performed to evaluate the developed *D. nodosus* real-time PCR (paper I) and to obtain clinical *D. nodosus* isolates for further characterisation (paper III). Culturing was performed on 4% (w/v Difco agar) HA as described previously (Stewart & Claxton, 1993; Thomas, 1958) since this method is well-established at the SVA (Båverud *et al.*, 2005). Low strength (2% w/v Difco agar) HA was, however, not used since it was perceived as more difficult to work with and gave inferior growth compared to 4% HA. Therefore, 4% HA was used both for culturing and subculturing. Moreover, it was an advantage to produce and store only one kind of HA-plate since the sustainability was relatively short (two weeks).

In short, HA-plates were inoculated using ESwabs as described in section 1.4.1, on the same day the samples arrived at the laboratory. The plates were pre-incubated for 10-30 min at 37 °C to assure dryness, then incubated in anaerobic jars for 4-6 days at 37 °C. Despite the pre-incubation, contaminating bacteria were still a problem, especially when the samples were delayed in the mail. This confirms that transport time can negatively affect culturing results. Three-day old samples could, however, be successfully cultured in some cases. Subculturing as early as possible, preferentially on day four was important, even if the colonies were still extremely tiny at this point. Colonies were picked with sterile toothpicks while using a stereomicroscope and inoculated on 4% HA, again in a grid pattern.

Prior to freezing and next-generation sequencing (NGS), *D. nodosus* was inoculated on Fastidious Anaerobe Agar (FAA) (Lab M Ltd, Bury, UK) plates containing 10% (v/v) defibrinated horse blood (Håtunalab AB, Bro, Sweden) to obtain single and distinct colonies (Figure 12). *D. nodosus* obtained from the FAA-plates was frozen and stored at -70 °C in serum broth with 15% glycerol. The FAA and HA-plates were produced at the SVA as well as the sheep hoof

Figure 12. Growth of *D. nodosus* on a FAA plate. Photo: Sara Frosth

powder required for the HA-plates. The sheep hoof powder was prepared as previously described (Stewart & Claxton, 1993; Thomas, 1958) with some modifications. In short, sheep legs were cut off as close to the hoof as possible in connection with autopsy and then boiled under pressure until the claw capsules fell off. The claw capsules were air dried at room temperature for about two to three weeks. A bench grinder was used to make powder of the dry claw capsules and a strainer (mesh size 1.4 mm) was used to remove to large particles. The powder was then autoclaved for 20 min at 121 °C.

3.2.1 Identification of isolates

D. nodosus colonies grown on HA were recognisable by their typical colony morphology, as described in section 1.4.1. All colonies were confirmed by the real-time PCR method developed in paper I and improved in paper III. In addition, MALDI-TOF-MS was used to identify some of the colonies; this proved to work well after the database had been updated with spectra from six additional *D. nodosus* isolates (Pringle, 2013). MALDI-TOF-MS is extremely quick and easy to perform, but since subtyping of *D. nodosus* is not possible with it, additional methods are required for further characterisation such as virulence testing.

3.3 DNA-extraction

Extraction of bacterial DNA can be performed by several different methods, depending upon the bacterium, sample matrix and downstream application. Cost per sample and level of automation are also important factors in choice of method, especially in large-scale diagnostic settings. The two main steps of bacterial DNA-extraction are breakage of the bacterial cell wall (cell lysis) to release the DNA and purification of the DNA from cell debris and proteins.

Since *D. nodosus* is a Gram-negative bacterium, the thin cell wall can be relatively easily disrupted by heat or chemicals. Boiling, which is quick and inexpensive, was used to extract DNA from *D. nodosus* colonies grown on agar plates for PCR analysis. A higher quality and purity of the DNA is,

however, required for NGS, so for this purpose two Qiagen (Hilden, Germany) kits were used: DNeasy Blood & Tissue and EZ1 Tissue. The first is a silica-based manual kit and the second a magnetic bead-based kit for the automated EZ1 Advanced system (Qiagen). Automated DNA-extraction was used to extract DNA directly from swab samples. Automated DNA-extraction is a very convenient and quick method (approximately 15 minutes on the EZ1 Advanced). A drawback with automated systems is, however, that they are expensive to buy and that the accompanying kits are also often more expensive than equivalent manual kits. Automated DNA-extraction could probably have been used to extract DNA from the biopsy samples as well, after some initial processing, but another manual silica-based kit, the QIAamp Cador (Qiagen), was used instead since an automated system was not available at the laboratory where they were processed.

3.4 Real-time PCR

Real-time PCR is a further development of conventional PCR in which target DNA is amplified and detected continuously during the analysis, i.e. in real-time, unlike conventional PCR that detects the DNA at the end of the analysis. Detection by fluorescence is done within the tube or assay plate, which significantly reduces the risk of contamination or “carry-over” compared to conventional PCR where the tubes need to be opened for analysis of the PCR-products on agarose gels. To further reduce the risk of contamination during PCR analysis, separate laboratories were used for the different parts of the PCR analysis (preparation of master mix, addition of target DNA, and running of the instrument).

Real-time PCR was chosen as analytic method and used in all but one of the papers because it is sensitive, specific, fast, and particularly suitable for bacteria that are difficult to culture like *D. nodosus* and *Treponema* spp. Conventional PCR assays were formatted for real-time PCR in order to reduce the work load and for easier data interpretation.

Real-time PCR assays for detection and virulence determination of *D. nodosus* were designed using the software Primer3 (Untergasser *et al.*, 2012) and Primer Express v 3.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The latter allows design of minor groove binder (MGB)-DNA probes. These have higher sequence specificity than standard probes (Kutyavin *et al.*, 2000) which is necessary for discriminating between the 2-bp difference in *aprV2* and *aprB2* of *D. nodosus* (Riffkin et al., 1995).

Since part of the specificity testing (inclusivity) for the virulence PCR *(aprV2/B2)* is not described in paper III, detailed materials and methods are

presented here. The same applies for a comparison that was made between the developed virulence PCR and the gelatin-gel test developed by Palmer *et al.* (1993). The results from both the inclusivity test and the comparison with gelatin-gel test are presented in section 4.1.2. Inclusivity testing was performed on 100 *D. nodosus* samples whereof 70 swabs and 30 isolates included the reference strains ATCC 25549^T, ATCC 27521 and ATCC 31545. All 100 samples were sequenced with the primers in Table 2 or by NGS (paper II) to determine the nucleotide composition in positions 661/662 of *aprV2/B2* (accession No. L38395).

Table 2. *Primers and their sequences used for PCR and DNA sequencing.*

Primer name	Sequence (5'-3')	Amplicon size (bp)
aprV2_B2 Sekv. F	TACCGCGAACAATGGCACTA	416
aprV2_B2 Sekv. R	GAATCGTAACCACCGCAACG	416
***aprV2/B2*F**	GAAGGCGACTGGTTTGATAACTG	113
aprV2_B2 Sekv. 3 R	GTTACCGCAGCGATTGTGC	113

Most *D. nodosus* isolates and swabs were successfully PCR-amplified and sequenced using the first primer pair in Table 2 (aprV2_B2 Sekv. F/R, *n*=74), but some of the swab samples (*n*=8) failed to yield a PCR product with these primers probably due to single nucleotide polymorphisms (SNP) in the primer regions. These eight samples were, however, successfully analysed with the second primer pair in Table 2, for which *aprV2/B2*F was also the forward primer of the real-time PCR assay. Master mix and PCR-conditions were the same as for the real-time PCR detecting *Treponema* spp. in paper III. All real-time PCR products were sent to Macrogen (Seoul, Korea) for sequencing using the same primers. In addition, 23 of the sequenced isolates, including ATCC 25549^T, were gelatin-gel tested by the Norwegian Veterinary Institute (*n*=14) or previously at the University of Bristol (*n*=9).

3.5 Next-generation sequencing

There are several different technologies and instruments available for next-generation sequencing (NGS) today, each with its advantages. Important factors to consider when choosing the most suitable technique for a specific project include read length, quality (error rate), time and costs.

The sequencing by synthesis (SBS) technology was used to sequence 103 *D. nodosus* isolates on the GAIIx and MiSeq instruments from Illumina. The maximum read lengths of these instruments are only about 300 bp (250 bp at the time of sequencing), but the sequence quality is high and the paired-end

option is advantageous in the subsequent sequence assembly. The sequence runs of especially the MiSeq, which is the smaller and newer of the two instruments, are also quick and inexpensive.

All genomes were reference assembled using the Short Read Mapping Package (SHRiMP) (Rumble *et al.*, 2009), and the publically available *D. nodosus* genome VCS1703A (GenBank accession number NC_009446.1) as well as *de novo* assembled using Velvet (Zerbino & Birney, 2008). In reference-guided assembly, reads are mapped to a reference; this is especially suitable for genomes with high sequence identity, which often results in fewer contiguous sequences (contigs) than a *de novo* assembly. A disadvantage is, however, that unique sequences not found in the reference genome are lost. No sequences are lost in a *de novo* assembly, where the genome is built without the help of a reference, but the assembly can be more fragmented (i.e., contain more contigs). Both SHRiMP and Velvet have been specifically developed for short read data.

The relationship between the sequenced genomes and the reference genome VCS1703A was visualised using BLAST Ring Image Generator (BRIG) (Alikhan *et al.*, 2011) and further analysed by Molecular Evolutionary Genetics Analysis v. 6 (MEGA6) (Tamura *et al.*, 2011) and SplitsTree (Huson & Bryant, 2006). All tools or softwares used for genome assembly and comparative analysis were freely available.

3.6 Statistical analysis

Fisher's exact test, which is a statistical significance test, was used to examine the association between clinical status or condition and bacterial findings in papers III and IV. This statistical test was chosen in paper III due to the small sample size (flock level), but it was also used in paper IV (individual feet) since it is equally valid for larger sample sizes. For the individual feet analysis in paper III, mixed-effects logistic regression models were used that considered clustering on flock level, i.e., sheep within a flock are more similar than sheep in different flocks.

The McNemar's test was used in paper I to determine whether there was a significant difference between the developed *D. nodosus* real-time PCR and culturing or conventional PCR. The McNemar's test was chosen since it is suitable for comparisons of paired proportions, from nominal data. To determine the degree of agreement between the methods used in paper I, Cohen's kappa was used.

4 Results and Discussion

4.1 Improved *Dichelobacter nodosus* diagnostics (papers I, III and V)

At the time of the first diagnosed footrot case in Sweden in 2004, there were no laboratory diagnostics available in the country for *D. nodosus.* Samples were sent to the UK for bacteriological culturing, confirmation and virulence testing (Olofsson *et al.*, 2005).

Although culturing of *D. nodosus* was rapidly established at the SVA (Båverud *et al.*, 2005), there was soon a need for a faster and more sensitive detection method due to the increasing number of samples. Hence a real-time PCR was developed that was specific for *D. nodosus*, and could be used directly on the sampling material without previous culturing (paper I). This real-time PCR was later improved to include an internal amplification control (IAC) and an additional target, the *D. nodosus intA* gene (paper III). IACs are commonly used in PCR-based methods to avoid false negative results.

The *intA* gene was included to provide information about virulence since an association between the *intA* gene and virulence had been found in a study by Cheetham *et al.* (2006). This association could, however, not be confirmed, neither by an Australian study (Dhungyel *et al.*, 2013) nor in paper II. Later, the *aprV2* gene was shown to be a key virulence factor (Kennan *et al.*, 2010) and therefore a real-time PCR assay targeting this gene was developed (paper III). To meet the demands for cost-efficiency often imposed on control programs, a sample pooling method was developed in paper V.

4.1.1 Detection

A real-time PCR assay targeting the *16S rRNA* gene was developed for detection of *D. nodosus* in paper I. Important factors when developing new real-time PCR assays include analytical specificity and sensitivity (Bustin *et*

al., 2009). The developed assay showed 100% analytical specificity meaning that it was able to detect all the *D. nodosus* isolates present and that it did not detect any of the non-target strains i.e., it did not yield any false positive results. High analytical specificity is especially important when analysing sampling material directly, as compared to analysing pure cultures. The analytical sensitivity of the developed assay was high; the limit of detection (LOD) was 4 fg *D. nodosus* genomic DNA which corresponds to approximately 3 genome equivalents.

Furthermore, comparisons with existing methods are important. The assay developed in paper I was compared with culturing, which is often considered the "gold standard", and with conventional PCR. The real-time PCR had higher diagnostic sensitivity than culturing. This was not unexpected since *D. nodosus* is a fastidious microbe (Table 3). However, it was somewhat surprising that the difference in diagnostic sensitivity was as great as 54.8% since a much lower difference (17.3%) was obtained in a previous study (König *et al.*, 2011). The proportion of *D. nodosus* positive samples for each method, and especially for culturing, was also markedly lower in this study than in König *et al.* (2011) (Table 3).

Table 3. *Comparison of diagnostic sensitivity of real-time PCR and culturing of D. nodosus in two studies.*

	No. of samples	Real-time PCR pos (%)	Culturing pos (%)	Difference (%)
Paper I	126	81.7	27.0	54.8
König *et al.* 2011	29	96.6	79.3	17.3

These differences could possibly be because scoring and sampling in the field is more difficult to perform than in a laboratory setting. Moreover, König *et al.*'s samples were analysed more rapidly after sampling (no transport time), which is more favourable, at least for culturing (Stewart & Claxton, 1993).

The difference in diagnostic sensitivity between the developed real-time PCR and conventional PCR was not as great as between real-time PCR and culturing, which was as expected. However, the advantages of real-time PCR over conventional PCR are numerous and important, for example increased specificity, reduced analysis time, and less risk of contamination. The real-time PCR detected 8% more positive samples than conventional PCR.

4.1.2 Virulence determination

In paper III, a real-time PCR assay targeting the *aprV2/B2* genes of *D. nodosus* was developed for virulence determination. The assay showed high analytical sensitivity (LOD = 4 fg *D. nodosus* genomic DNA corresponding to

approximately 3 genome equivalents) and high analytical specificity (100%). All 100 samples of the inclusivity testing which had had their *aprV2/B2* type determined by sequencing, were correctly identified as virulent or benign by the developed real-time PCR. The 100 tested samples consisted of both isolates and swabs; the distribution of virulent and benign *D. nodosus* are given by sample type in Table 4.

Table 4. *Distribution of virulent and benign D. nodosus by sample type included in the inclusivity testing.*

Sample type	Virulent *(aprV2)*	Benign *(aprB2)*	No. of samples
Isolate	9	21	30
Swab	7	63	70
Total	**16**	**84**	**100**

In addition, the real-time PCR was compared with the gelatin-gel test (Palmer, 1993) since the latter is a frequently used test for virulence determination of *D. nodosus.* Twenty-three of the isolates in Table 4 were gelatin-gel tested (17 benign and 6 virulent) and the results were consistent with the real-time PCR except in two cases. One of the isolates was assessed as intermediate in the gelatin-gel test, even when retested, but as benign *(aprB2)* by the real-time PCR. The other isolate came out as benign in the gelatin-gel test but as virulent *(aprV2)* in the real-time PCR. Since both isolates had been sequenced, it was evident that the real-time PCR identified these two isolates correctly regarding the *aprV2/B2* variant.

The *D. nodosus* real-time PCR assays for detection and virulence determination were developed to complement each other, but the virulence-PCR alone could be used for both purposes in the same way that the similar assay by Stäuble *et al.* (2014a) is being used. However, a disadvantage with this approach is that neither of these assays includes an IAC which means a potential risk of false negative results. Furthermore, *D. nodosus* with potential SNPs in the *aprV2/B2* genes can be missed. Lack of amplification do not necessarily mean that a sample is negative and consequently uninteresting, but on the contrary it can be very interesting and important for future diagnostics. For example, a *D. nodosus* isolate with a histidine in position 92 in AprV2/B2 instead of the tyrosine (virulent) or arginine (benign) was discovered in this thesis project when the two PCR assays were used in parallel (Frosth, unpublished). The developed assays can be analysed simultaneously on the same assay plate since they both use the same PCR-program.

In conclusion, sensitive and specific real-time PCR methods can detect and discriminate between virulent and benign strains of *D. nodosus.*

4.1.3 Pooling of samples

The real-time PCR methods for detection and virulence determination of *D. nodosus* in papers I and III are less time consuming and laborious than culturing followed by phenotypic virulence testing. However, analysis of large number of samples, which is common in control programs, can still be costly. Hence a method for pooling of samples was developed in paper V.

In paper V, swab samples taken within the Swedish Footrot Control Program were analysed by the real-time PCR assays for detection and virulence determination, individually and in pools of five. For each pool, one positive and four negative *D. nodosus* samples were included since that was the most difficult sample combination expected; if it worked this would ensure that the pooling method would work well for any possible sample combination. The pooled analysis showed that all pools containing a single *D. nodosus* positive sample (*n*=41) could readily be detected and virulence determined despite the positive sample being mixed with four negative samples. The maintenance of diagnostic sensitivity was probably due to the concentration step implemented prior to bacterial lysis and DNA-extraction. The concentration step consisted of centrifugation and it was performed on both the individual and the pooled samples. A common risk with pooling of samples is otherwise reduced sensitivity due to dilution effects. The developed method has been implemented in the Swedish Footrot Control Program (National Veterinary Institute, 2016) on the basis of the results of this study.

In conclusion, the pooling method for real-time PCR detection and virulence determination of *D. nodosus* has no loss of sensitivity compared to individual samples, and is faster and more cost-efficient.

4.2 Investigation of the *D. nodosus* genome (paper II)

Only a single *D. nodosus* isolate, the virulent VCS1703A, had been fully sequenced when this thesis project started (Myers *et al.*, 2007). Sequencing can generate important knowledge about the genetic relationship of bacterial populations. Hence, 103 *D. nodosus* isolates of different disease phenotypes and from different countries were sequenced in paper II.

In paper II, the results from Myers et al. (2007) were confirmed: *D. nodosus* has a relatively small genome (1.39 Mb) with no current genome reduction. The 103 genome sequences were 95% conserved (Figure 13). Analysis of the SNPs divided the 103 isolates into two separate clades (Figure 14), which correlated with the single amino acid difference (Tyr92Arg) between the protease variants AprV2 and AprB2 (Riffkin *et al.*, 1995). There was no genetic evidence of any intermediate variants of the bacterium although

D. nodosus strains of intermediate virulence have been reported (Stewart *et al.*, 1986). The division into two distinct clades also correlated with assigned disease phenotypes — clade I contained isolates of virulent disease phenotype and clade II benign disease phenotype. However, this was not the case for two of the Swedish isolates. They were grouped in clade II (AprB2), although they had been classified as having virulent phenotypes (score 3 lesions; paper III).

Sequence variability was primarily found in eight specific regions: bacteriophage, *vrl*, *vap1, vap2,* Omp1, *hsdS, pgr,* and RTX (Figure 13). These regions had been previously identified by Myers *et. al.* (2007). The largest of them was the bacteriophage that had been identified as an integrated Mu-like bacteriophage in VCS1703A (Myers *et al.*, 2007). More than a quarter of the sequenced isolates (*n*=29) contained this bacteriophage, or a closely related one. Only one of the 17 Swedish isolates contained the identical bacteriophage although almost all of the Swedish benign strains (*n*=12) contained a closely related variant.

The *vrl* region – which most probably is also of bacteriophage origin (Billington *et al.*, 1999) – could be identified in full or in part in 27 of the isolates, including 8 from Sweden. This region has previously been found to be associated with virulent isolates (Haring *et al.*, 1995; Katz *et al.*, 1991) although no direct functional association has been demonstrated.

The *vap* regions (*vap1* and *vap2)* are believed to have arisen by insertion of genetic material such as a bacteriophage or a plasmid into the *tRNA* gene(s) (Rood, 2002). Different integrase genes, including the *intA* gene, have been found adjacent to these *vap* regions (Cheetham *et al.*, 1999; Bloomfield *et al.*, 1997; Cheetham *et al.*, 1995). All of the 103 sequenced isolates contained genes within this region to some extent. Virulent isolates from Sweden and Australia exhibited the largest *vap* regions. No correlation was found between the *vap* regions and the clade division (clade I and clade II respectively).

In conclusion, *D. nodosus* isolates are genetically highly conserved and exist in a bimodal population structure that is globally distributed. Benign isolates from Sweden are more diverse than virulent ones. Furthermore, the results from this study validate the use of real-time PCR for virulence determination in paper III.

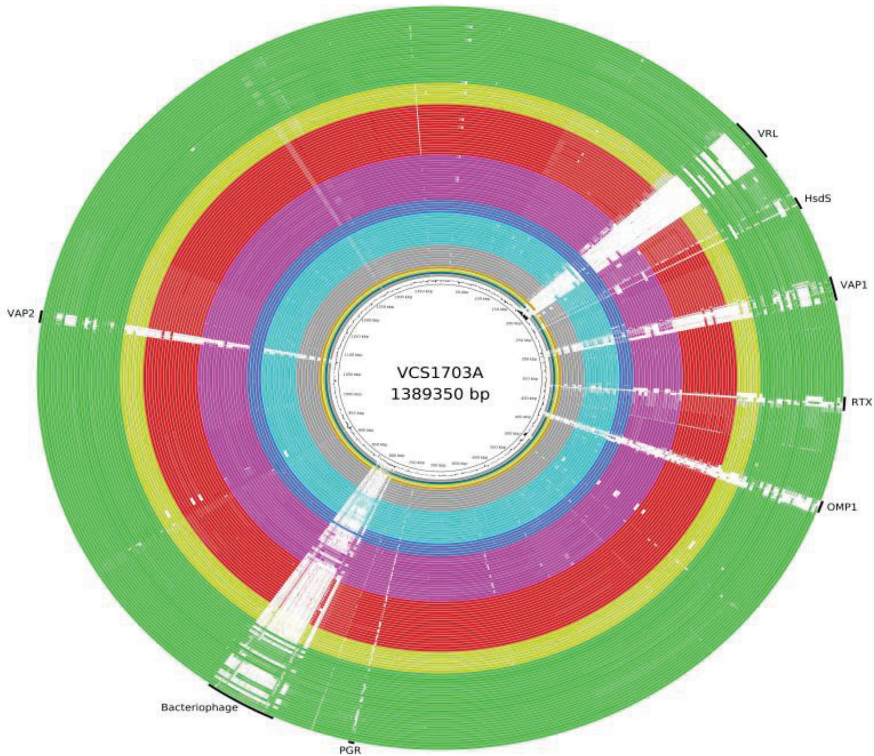

Figure 13. BRIG diagram showing the genetic relationship between *D. nodosus* strain VCS1703A and the 103 sequenced isolates. Each isolate is represented by a coloured ring which in turn indicates the country of origin and protease genotype as follows: yellow and green (Australia), pink and red (Norway), light blue and dark blue (Sweden), grey (Denmark). Isolates from UK, Nepal, Bhutan and India are the four innermost rings. Green, red, dark blue, and grey indicate virulent isolates (AprV2) and yellow, pink, and light blue indicate benign isolates (aprB2). Solid colours represent >95% and white regions <50% sequence identity with VCS1703A (Kennan *et al.*, 2014).

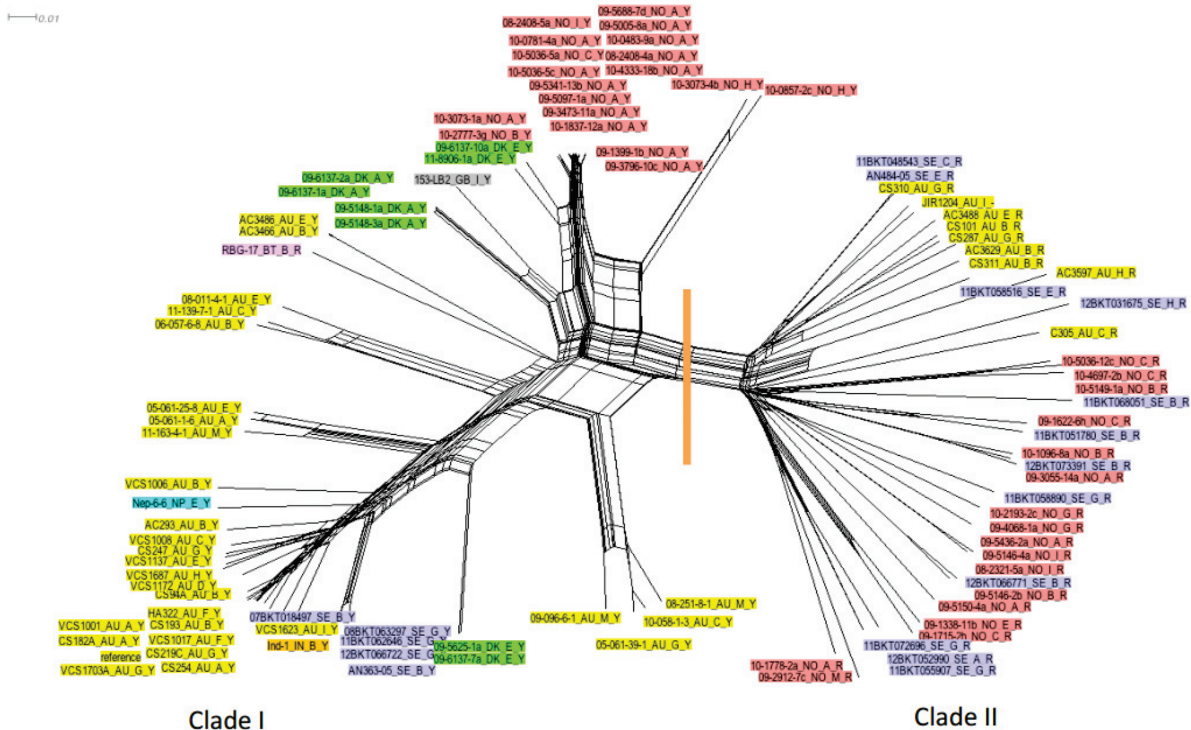

Figure 14. Network diagram showing the genetic relationship between 103 *D. nodosus* isolates. The vertical orange line indicates the clade division. The taxon colouring indicates geographical origin as follows: yellow (Australia), red (Norway), lilac (Sweden), green (Denmark), pink (Bhutan), blue (Nepal), orange (India), and grey (UK) (Kennan *et al.*, 2014).

4.3 Characterisation of *D. nodosus* from Swedish sheep (papers I, III and V)

Seventeen *D. nodosus* isolates from Swedish sheep were whole genome sequenced and analysed in paper II. Additional *D. nodosus* isolates and samples were characterised in this thesis project and are presented here:

4.3.1 Benign *D. nodosus* predominate in Swedish sheep

The prevalence of virulent and benign strains of *D. nodosus* in Swedish sheep has not yet been thoroughly investigated; nevertheless, available data indicate that benign *D. nodosus* predominate and that virulent *D. nodosus* are relatively uncommon.

In paper III, most of the *D. nodosus* were benign. Virulent *D. nodosus* was only found in 1 of the 20 investigated Swedish sheep flocks. All of the *D. nodosus* in papers I and V were benign. Although virulence testing was not established until late 2014 in routine diagnostics at the SVA (at which time the real-time PCR developed in paper III was implemented), stored samples were analysed retrospectively. In addition to diagnostic samples, these included samples from an eradication study including 19 sheep flocks with footrot (König & Björk Averpil, 2010) and samples from the footrot-prevalence study based on clinical signs (König *et al.*, 2011).

To date, virulent *D. nodosus* have only been found in nine Swedish sheep flocks since the first diagnosed case in 2004; the geographical distribution can be seen in Figure 15. Additional information regarding these virulent *D. nodosus* is given in Table 5. This is different from UK sheep where virulent *D. nodosus* are more prevalent than benign ones; see paper IV and Moore *et al.* (2005a).

Table 5. *Year and county of detection, serogroup, and presence of the intA gene for each of the nine virulent D. nodosus found in Sweden.*

Year	County	Serogroup	*intA* gene
2005	Västra Götaland	B	Positive
2007	Blekinge	B	Positive
2008	Skåne	G	Positive
2010	Skåne	A	Positive
2011	Dalarna	G	Positive
2014	Västra Götaland	n.a.	Positive
2015	Blekinge	n.a.	Positive
2016	Blekinge	n.a.	Positive
2016	Västra Götaland	n.a.	Positive

n.a.=not analysed

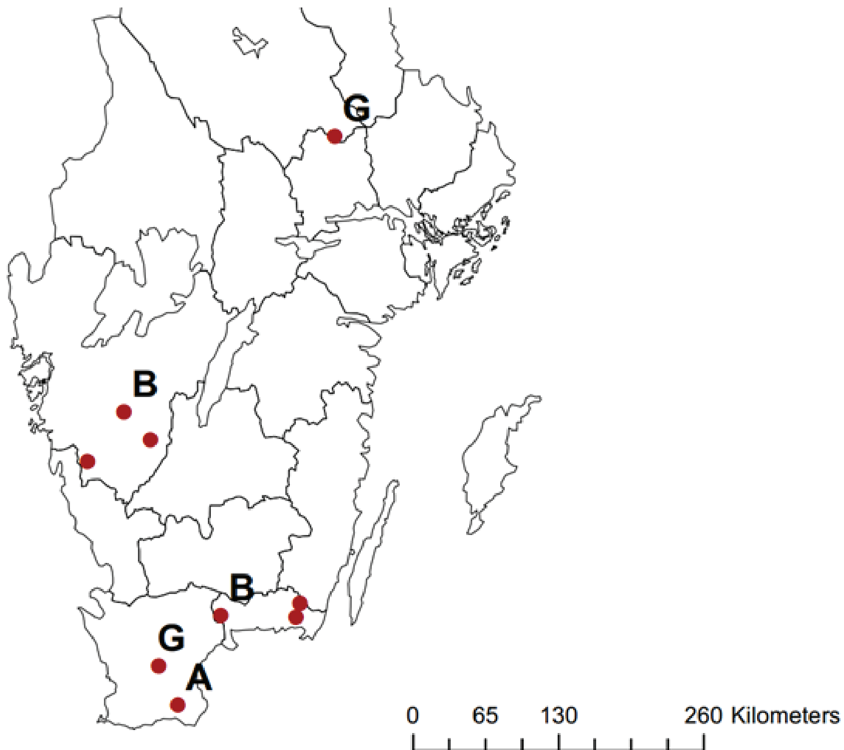

Figure 15. Map of southern part of Sweden showing the geographical distribution of Swedish sheep flocks with virulent *D. nodosus*. Letters indicate *D. nodosus* serogroups. Created in ArcMap 10.3.1 (Esri Inc., CA, USA).

4.3.2 Virulent *D. nodosus* contain the *intA* gene

The nine virulent *D. nodosus* detected in Swedish sheep flocks so far all contained the *intA* gene. This distinguishes them from the *D. nodosus* strain that recently caused an outbreak of ovine footrot in Norway (Gilhuus *et al.*, 2013). The *intA* gene is, however, also present in benign *D. nodosus* from Swedish sheep. In paper III, the *intA* gene was found in 17% (2/12) of the flocks and in 20% (41/208) of the samples with benign *D. nodosus* present. In paper V it was found in 13% (1/8) of the flocks and in 8% (5/61) of the samples. In paper I, 14% (14/103) of the samples with benign *D. nodosus* present contained the *intA* gene.

4.3.3 Most *D. nodosus* serogroups are represented in Sweden

Virulent *D. nodosus* of three different serogroups have so far been found in Sweden (Table 5). Considerably more benign *D. nodosus* have been found, but serogrouping is not routinely performed in the diagnostics at SVA. However,

serogrouping has been performed on benign *D. nodosus* isolates from paper III, from the eradication study (König & Björk Averpil, 2010), and from the prevalence study (König *et al.*, 2011), in this thesis project. Allocation into serogroups was done by multiplex PCR assays which can detect serogroups A-I (Dhungyel *et al.*, 2002). A few of the samples were not successfully amplified and hence were thought to belong to serogroup M, since that serogroup is not detected by the multiplex PCR. Moreover, serogroup M has been detected in Norway, although in only a few of the isolates (Gilhuus *et al.*, 2013). However, after whole genome sequencing of three of our isolates (paper II), it was clear that they did not belong to serogroup M, but had merely failed to amplify due to SNPs in the primer regions. The three isolates belonged to serogroup G. All serogroups except F were detected among the 151 Swedish benign *D. nodosus* isolates (Figure 16), which is similar to the findings from Norway (Gilhuus *et al.*, 2013).

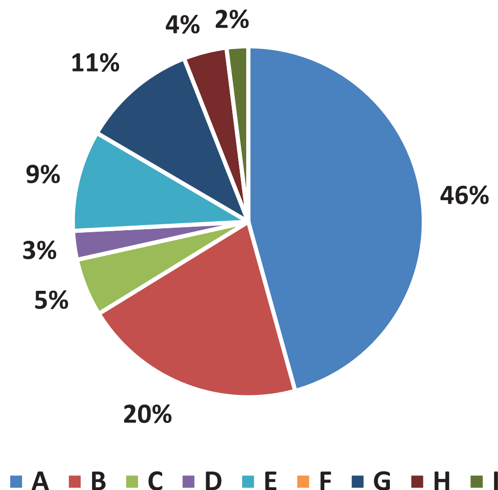

Figure 16. Distribution of *D. nodosus* serogroups in Swedish benign isolates (*n*=151).

This suggests that *D. nodosus* has probably been present for a long time in Sweden. It is possible that footrot may have been misdiagnosed before it attracted attention in 2004. It is also possible that, at least in cases with mild symptoms, it was previously unnoticed.

In conclusion, most *D. nodosus* in Swedish sheep are benign and the virulent type is uncommon. The few virulent *D. nodosus* so far detected have contained the *intA* gene. Most *D. nodosus* serogroups have been detected in Swedish sheep, indicating that *D. nodosus* has probably been present for a long time and is not a recent introduction.

4.4 Bacterial colonisation in ovine footrot (papers III and IV)

D. nodosus was recognised as the causative agent of ovine footrot by Beveridge in 1941. Later, Roberts and Egerton (1969) reported that *F. necrophorum* was needed before infection of *D. nodosus* could occur despite that Beveridge (1941) previously had found that *F. necrophorum* and spirochaetes merely enhanced disease severity. The role of bacterial colonisation in ovine footrot is unclear. Hence we investigated *D. nodosus, F. necrophorum* and *Treponema* spp. colonisation in sheep with different clinical manifestations of footrot (papers III and IV). In paper III, swab samples from Swedish sheep were investigated whereas biopsy samples from UK sheep were investigated in paper IV. All samples were analysed by the real-time PCR assays developed in papers I and III.

4.4.1 *Dichelobacter nodosus*

The investigation of Swedish sheep in paper III found that *D. nodosus* was more common in sheep with footrot lesions than in healthy sheep, both at flock and individual levels. The investigation of UK sheep (paper IV), which also included analysis of bacterial load, found that both the presence and load of *D. nodosus* was higher in sheep with interdigital dermatitis (ID) and underrunning footrot than in healthy sheep, in line with previous studies (Witcomb *et al.*, 2015; Witcomb *et al.*, 2014; Calvo-Bado *et al.*, 2011; Moore *et al.*, 2005a). The highest *D. nodosus* prevalence and load were found in samples with ID, which supports the theory that *D. nodosus* has an important role in early stages of the disease development (Witcomb *et al.*, 2015; Witcomb *et al.*, 2014).

In both studies, *D. nodosus* could be found in healthy feet as well as in feet with footrot lesions, which is consistent with previous reports (Vatn *et al.*, 2012; Calvo-Bado *et al.*, 2011; Moore *et al.*, 2005a; Depiazzi *et al.*, 1998; Glynn, 1993). However, the *D. nodosus* frequencies differed between the two studies. *D. nodosus* was found in 58% of healthy feet (46/79) in paper IV and in 31% of healthy feet (159/506) in paper III. It is possible that the difference could be even greater since the definition of healthy feet differed in the two papers; only feet where interdigital lesions were completely absent were classified as healthy in paper IV whereas feet with score 1 lesions (corresponding to mild ID) were classified as healthy in paper III in addition to feet with score 0 lesions (corresponding to complete absence of lesions). When score 0 feet were investigated in paper V, only 6% (9/152) of the Swedish sheep were positive for *D. nodosus*. Important to note, however, is that different sample types were used in these two papers; it remains to be investigated if these sample types give comparative results.

In conclusion, *D. nodosus* is mainly associated with the early stages of footrot, confirming that it is the primary pathogen in ovine footrot.

4.4.2 *Fusobacterium necrophorum*

F. necrophorum was present in 23% (134/579) of the swab samples from Swedish sheep (paper III) and in 15% (36/241) of the biopsies from UK sheep (paper IV). There was a significant association between *F. necrophorum* and feet with underrunning footrot (score ≥3) in both papers. The highest load of *F. necrophorum* was also found in feet with underrunning footrot, as shown in paper IV. This is similar to previous reports (Witcomb *et al.*, 2015; Witcomb *et al.*, 2014) and together these studies support *F. necrophorum* as an opportunistic or secondary pathogen rather than a prerequisite for *D. nodosus* infection of the interdigital skin (Roberts & Egerton, 1969).

Both papers also report data about the subspecies of *F. necrophorum*. The majority of *F. necrophorum* detected in UK sheep (paper IV) consisted of the *necrophorum* subspecies (97%, 35/36) whereas the *funduliforme* subspecies was more common in Swedish sheep (65%, 87/134) (paper III). The *necrophorum* subspecies has been reported as the more pathogenic of the two (Tan *et al.*, 1996), but this has not yet been studied in sheep with footrot. In conclusion, *F. necrophorum* is associated with later stages of footrot (underrunning or score ≥3), confirming the suggested role of *F. necrophorum* as an opportunistic rather than primary pathogen.

4.4.3 *Treponema* spp.

The results of paper III showed that *Treponema* spp. were commonly found in Swedish sheep flocks (90%, 18/20) and in a high proportion of the animals (47%, 273/579), regardless of clinical status. In paper V, *Treponema* spp. were found in all clinical conditions in sheep from the UK but at a very low frequency (8%, 20/421). Therefore, no significant association between *Treponema* spp. and ovine footrot could be found in either study, in spite of the fact that spirochaetes have been proposed to play a role in disease development (Calvo-Bado *et al.*, 2011; Beveridge, 1941).

The great difference between Swedish and UK sheep is intriguing but it remains to be investigated whether this difference is real or due to the different sample types used. The number of samples was also relatively limited. Swab samples do possibly contain more free-living and non-pathogenic *Treponema* spp. than biopsy samples. Low detection of *Treponema* spp. in biopsy samples from UK sheep has been previously reported (Calvo-Bado *et al.*, 2011).

In conclusion, involvement of *Treponema* spp. in ovine footrot is not evident, although previously proposed.

5 Conclusions

This project has increased the knowledge about ovine footrot in Swedish sheep and improved laboratory diagnostics for it. The specific conclusions are:

- Sensitive and specific real-time PCR methods can detect and discriminate between virulent and benign strains of *D. nodosus.*

- *D. nodosus* isolates are genetically highly conserved and exist in a bimodal population structure that is globally distributed.

- Most *D. nodosus* in Swedish sheep are benign and the virulent type is uncommon. Benign isolates are more diverse than virulent ones.

- *D. nodosus* is mainly associated with the early stages of footrot whereas *F. necrophorum* is associated with the later ones. Involvement of *Treponema* spp. in footrot is not evident.

- The pooling method for real-time PCR detection and virulence determination of *D. nodosus* has no loss of sensitivity compared to individual samples, and is faster and more cost-efficient.

6 Future Perspectives

"Knowledge brings more questions than answers" as Eduardo Giannetti wrote in Lies We Live By (2001) – a quotation that describes this research area very well. Footrot research has namely been conducted for over 200 years and still new questions arise.

Firstly, it would be very interesting to investigate why benign *D. nodosus* appears to give rise to virulent or underrunning footrot (score ≥3 lesions) in Swedish sheep even though those kind of lesions are believed to be exclusively caused by virulent strains. This question could be answered by – experimental challenge studies – using benign *D. nodosus* isolated from Swedish sheep with more severe footrot lesions. Such studies could at the same time include evaluation of new treatment strategies, for example with penicillin as an antimicrobial agent and footbath solutions without heavy metals. However, since challenge studies involve animal testing: they should be avoided as much as possible. Furthermore, they are not completely easy to perform (Knappe-Poindecker *et al.*, 2014b).

An option, or at least a first step, might be to study potential differences between virulent and benign *D. nodosus* in an *in vitro* model system. An *ex vivo* organ culture (EVOC) has been developed by Maboni *et al.* (2016) that permits studies on how different bacteria infect the ovine interdigital skin without using sheep experimentation. Moreover, the EVOC model permits studies of the early stages of host response, which is especially interesting since an intense host response is hypothesised to exacerbate disease severity. The host response in footrot is not well understood nor is the microbial community. A study investigating both is currently underway (Maboni *et al., in manuscript*), and preliminary data indicate that the microbial communities in the interdigital skin clearly differ in healthy and footrot affected feet, and upon different levels of inflammation.

The comparability of results from swabs and biopsies is also presently being studied (Maboni *et al., in manuscript*), and not only with respect to single species, but at microbial community level as well. Preliminary data from this study shows that the two sample types differ in bacterial prevalence and load.

Biopsy material from Swedish sheep with footrot would also be interesting to investigate with the aim of identifying and differentiating any treponemal species present. Investigation of biopsy material instead of swabs would probably increase the chance of detecting pathogenic species of *Treponema*, since if present, they must have been able to penetrate the interdigital skin.

Further, to obtain a better estimate of the distribution of *D. nodosus* and of virulent and benign strains in Sweden, prevalence studies are needed on randomly selected sheep. Such studies could be carried out at abattoirs and also used for surveillance purposes.

Finally, epidemiological studies combined with data on microbial communities and aspects on *D. nodosus* virulence would contribute to a more complete picture that may be needed for proper control of the disease.

7 Populärvetenskaplig Sammanfattning

Fotröta hos får är en smittsam sjukdom som orsakas av bakterien *Dichelobacter nodosus*. Sjukdomen förekommer i de flesta lammproducerande länder och utgör ett stort djurskyddsproblem. Dessutom medför sjukdomen betydande ekonomiska konsekvenser får fårnäringen; förutom direkta produktionsförluster är stora kostnader förknippade med behandling och förebyggande åtgärder.

En vanlig smittväg är genom inköp av djur till besättningen. Fotröta angriper klövarna och drabbade djur blir ofta halta. Sjukdomen kan uppträda med varierande svårighetsgrad från benign (lindrig) fotröta där den känsliga huden mellan klövarna inflammerats till virulent (allvarlig) fotröta där inflammationen även trängt in under själva sulan så att köttklöven blottas. I de värsta fallen kan klövväggen separeras från de underliggande vävnaderna. Vanlig behandling är fotbad i zinksulfatlösning (10%) eventuellt i kombination med antibiotika. Fotröta är en komplex sjukdom och dess svårighetsgrad beror på flera faktorer inklusive den infekterande *D. nodosus*-stammens virulens (förmåga att orsaka sjukdom), miljöförhållanden, klimat, fårras och samtidig infektion av andra bakteriearter.

I Sverige diagnosticerades fotröta för första gången år 2004 men snabb och känslig laboratoriediagnostik fanns inte att tillgå. Dessutom saknades kunskap om svenska *D. nodosus*-stammar och om samtidig infektion med andra bakteriearter som vanligtvis associeras med hälta var vanlig. Därför var det övergripande syftet med denna avhandling att öka kunskapen om fotröta hos svenska får samt att förbättra laboratoriediagnostiken av *D. nodosus*.

I denna avhandling utvecklades känsliga och specifika realtids-PCR metoder för att upptäcka och särskilja mellan virulenta- (aggressiva eller högvirulenta) och benigna (godartade eller lågvirulenta) stammar av *D. nodosus*. Dessa metoder användes sedan för att karaktärisera *D. nodosus* hos svenska får.

Resultaten visade att de flesta *D. nodosus* i Sverige är benigna medan den virulenta varianten är ovanlig. Flertalet av de tio serogrupper som finns beskrivna hos *D. nodosus* kunde påvisas hos svenska får i dessa studier vilket indikerar att bakterien förmodligen funnits i Sverige under en lång tid och inte nyligen introducerats. Genetisk analys av *D. nodosus* isolat från ytterligare sju länder förutom Sverige visade att *D. nodosus* genomet (den totala mängden arvsmassa) är välbevarat och att det existerar i två varianter (virulent respektive benign). Det fanns inga genetiska bevis för att det skulle existera några andra varianter än dessa två trots att *D. nodosus* med intermediär virulens rapporterats. Även om de flesta svenska *D. nodosus*-stammar är benigna förekommer allvarliga fall av fotröta vilket behöver studeras vidare.

Samtidig infektion med andra bakteriearter såsom *Fusobacterium necrophorum* och *Treponema* har rapporterats varav den första är kanske allra mest omdebatterad. *F. necrophorum* och dess roll i fotröta har delat forskarna i två läger; de som tror att *F. necrophorum* behövs för att *D. nodosus* infektion skall kunna ske och de som tror att *F. necrophorum* är mer av en opportunistisk patogen som passar på när *D. nodosus* redan orsakat skada. I dessa studier förknippades *D. nodosus* framför allt med de lindriga stadierna av fotröta medan *F. necrophorum* förknippades med de mer allvarliga vilket stärker hypotesen att det är *D. nodosus* som infekterar först medan *F. necrophorum* har en mer opportunistisk eller sekundär roll. Även om *Treponema* föreslagits vara inblandad i sjukdomsutvecklingen hittades inga bevis för det i denna studie. Det skulle dock vara intressant att undersöka vilka arter av *Treponema* man kan hitta hos får om det är samma som orsakar sjukdom hos gris och nöt eller om det är några som är specifika för just får.

Slutligen utvecklades en poolningsmetod för att möta kraven på kostnadseffektivitet som ofta ställs på kontrollprogram. Metoden gör det möjligt att analysera prover i grupper om fem utan minskad känslighet jämfört med individuella prover. Poolningsmetoden utvärderades tillsammans med realtids-PCR metoderna för detektion och virulensbestämning av *D. nodosus* och är sedan 2014 implementerad i det svenska kontrollprogrammet för fotröta (Klövkontrollen), som ett direkt resultat av dessa studier.

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