

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Characterisation of *Dichelobacter nodosus* and detection of *Fusobacterium necrophorum* and *Treponema* spp. in sheep with different clinical manifestations of footrot



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ARTICLE INFO

Keywords:

Dichelobacter nodosus
Ovine footrot
Fusobacterium necrophorum
Virulence
Serogroup
Treponema spp.

ABSTRACT

The aim of this study was to determine the proportion of *Dichelobacter nodosus*, *Fusobacterium necrophorum* and *Treponema* spp. in sheep with different clinical manifestations of footrot compared to healthy sheep both at flock and individual level. The second aim was to characterise *D. nodosus* with respect to virulence, presence of *intA* gene and the serogroups.

Swab samples ($n = 1000$) from footrot-affected ($n = 10$) and healthy flocks ($n = 10$) were analysed for the presence of *D. nodosus*, *F. necrophorum* and *Treponema* spp. by real-time PCR and culturing (*D. nodosus* only). *Dichelobacter nodosus* isolates ($n = 78$) and positive swabs ($n = 474$) were analysed by real-time PCR for the *aprV2/B2* and the *intA* genes and by PCR for the *fimA* gene (isolates only).

D. nodosus was more commonly found in flocks affected with footrot than in clinically healthy flocks. A significant association was found between feet with severe footrot lesions and the *aprV2* gene and between feet with moderate or no lesions and the *aprB2* gene, respectively. *F. necrophorum* was more commonly found in flocks with footrot lesions than in flocks without lesions. No significant association was found between sheep flocks affected with footrot and findings of *Treponema* spp. or the *intA* gene. Benign *D. nodosus* of six different serogroups was detected in twelve flocks and virulent *D. nodosus* of serogroup G in one.

In conclusion, *D. nodosus* and *F. necrophorum* were more commonly found in feet with footrot than in healthy feet. The majority of *D. nodosus* detected was benign, while virulent *D. nodosus* was only detected in a single flock.

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1. Introduction

Ovine footrot is a contagious disease of which *Dichelobacter nodosus*, a Gram-negative anaerobic bacterium, is the causative agent (Beveridge, 1941). Footrot begins as an inflammation of the interdigital skin of sheep

<http://dx.doi.org/10.1016/j.vetmic.2015.02.034>

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and may progress to severe separation of the claw capsule from the underlying tissues. Footrot severity is dependent on the virulence of the infecting *D. nodosus* strain as well as other factors such as farm management (Wassink et al., 2003), sheep breed (Emery et al., 1984), environmental/climatic conditions (Graham and Egerton, 1968; Stewart et al., 1984), and the presence of co-infecting bacterial genera. *Fusobacterium necrophorum*, another anaerobic bacterium, has been suggested as a secondary pathogen in the disease development and could possibly increase its severity (Beveridge, 1941; Witcomb et al., 2014). Two subspecies of *F. necrophorum* are recognised: *necrophorum* (*F. n. necrophorum*) and *funduliforme* (*F. n. funduliforme*) (Shinjo et al., 1991). There are also speculations that *Treponema* spp. is involved in footrot development (Beveridge, 1941). *Treponema* spp. have been found associated with other claw diseases such as contagious ovine digital dermatitis (CODD) and bovine digital dermatitis (BDD) (Naylor et al., 1998; Walker et al., 1995).

D. nodosus strains have recently been shown to exist globally in two distinct forms: virulent and benign (Kennan et al., 2014). Using the protease genes *aprV2* and *aprB2*, Stäuble et al. (2014a) have developed a real-time PCR assay to distinguish the virulent and benign strains of *D. nodosus*. Furthermore, whole genome shotgun sequencing of a large number of *D. nodosus* isolates from different countries has revealed that some of the isolates contain a SNP in the region of the 3' end of the forward primer in Stäuble's assay (Kennan et al., 2014). Our study presents the development of an additional real-time PCR assay to target *aprV2* and *aprB2* and we compare the two assays with respect to analytical sensitivity and amplification efficiency.

Previously, the *intA* gene was suggested to be associated with virulence (Cheetham et al., 2006) but more recent studies have been unable to confirm this (Dhungyel et al., 2013; Kennan et al., 2014). Serogrouping of *D. nodosus* isolates could provide epidemiological information on strain diversity and is a prerequisite if specific vaccination is to be carried out (Dhungyel et al., 2014). Ten serogroups (A-I and M) of *D. nodosus* are recognised today (Claxton, 1989; Ghimire et al., 1998). The serogroup-specific PCR assay developed by Dhungyel et al. (2002) targeting the *fimA* gene can discriminate nine of them (A-I).

Ovine footrot has been reported in many countries (Bennett and Hickford, 2011) and the first Swedish case was diagnosed in 2004 (Olofsson et al., 2005). The disease has received considerable attention especially after an outbreak of footrot in Norway in 2008 (Meling and Ulvund, 2009). This outbreak was caused by a virulent *D. nodosus* strain of serogroup A that had been introduced in recent time (Gilhuus et al., 2013, 2014). In Sweden, diagnosis is mainly based on clinical examination of the feet of sheep according to a scoring system developed by Stewart and Claxton (1993); a lesion scoring ≥ 2 is used as definition of disease. Diagnosis is sometimes complemented by detection of *D. nodosus* by real-time PCR and positive findings must be reported to the Swedish Board of Agriculture (Jönköping, Sweden). The sheep industry in Sweden consists of about 10,000 flocks with an average size of 32 ewes; the most common breed is the Gotland Pelt sheep

(Swedish Board of Agriculture, 2013). In 2009, a prevalence study of footrot in slaughter lambs was conducted in Sweden which found 5.8% of individual sheep to be afflicted (König et al., 2011). This same study also showed that footrot was present throughout the country. Still, there is limited knowledge of how footrot manifests itself in Sweden and which strains of *D. nodosus* that are present. Hence the aim of this study was to determine the proportion of *D. nodosus*, *F. necrophorum* and *Treponema* spp. findings in sheep with different clinical manifestations of footrot compared to healthy sheep both at flock and individual level. A second aim was to characterise *D. nodosus* with respect to virulence, presence of *intA* gene and the serogroups present in the country.

2. Materials and methods

2.1. Sheep flocks and sampling procedure

The 20 sheep flocks in this study were selected by the Swedish Animal Health Service. The selection was based on the clinical status of the flocks using the predefined categories 1–4 (Table 1), and were selected from different geographical locations in Sweden (Fig. 1). Scoring and sampling took place during August to November in 2011 and in October 2012. This sampling period was chosen with regards to favourable environmental conditions for disease expression and transmission. In late summer the pastures are moist and the mean daily temperature is above 10 °C in large parts of Sweden. The clinical definition of footrot currently used in Sweden is a score ≥ 2 lesion. Thus categories 1 and 2 are regarded as clinically healthy and categories 3 and 4 as affected. Each foot of every sheep in the flocks was examined visually and scored according to the scoring system developed by Stewart and Claxton (1993) with scoring percentages in Table 2. The majority of the sheep flocks ($n = 14$, 70%) were scored and sampled by the same trained veterinarian. The other flocks were scored by veterinarians previously trained by the experienced veterinarian mentioned above. Participation by the sheep owners was voluntary and Gotland Pelt sheep was the most common breed.

In connection with the scoring, fifty swab samples were collected from each of the 20 sheep flocks ($n = 1000$ swabs in total). Sheep with lesions in the interdigital space (score ≥ 1) were prioritised in the sampling but clinically healthy sheep were also sampled in footrot-affected flocks if there were fewer than 50 sheep with lesions. Flocks where all feet scored 0 were sampled randomly. ESswabs (Copan Innovation Ltd, Brescia, Italy) were used to collect the

Table 1
Definition of flock clinical status.

Category	Criteria for clinical categorization of flocks
1	Flocks mainly with score 0 animals and no animal with a score >1
2	Flocks with many score 1 animals but no animals with a score >1
3	Flocks with a minimum of one animal with a score 2
4	Flocks with a minimum of one animal with a score 3

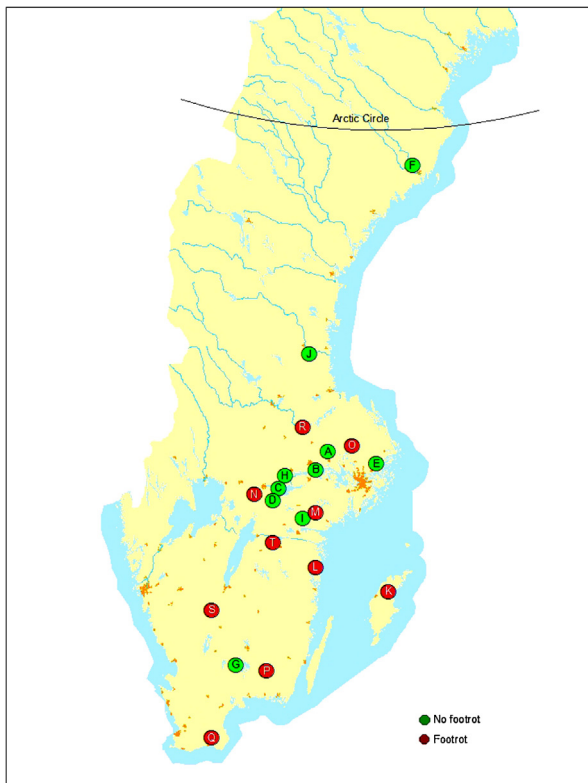


Fig. 1. Geographical distribution of 20 Swedish sheep flocks sampled for *D. nodosus*, *F. necrophorum* and *Treponema* spp.

samples from the interdigital skin and the same swab was used both for culturing and real-time PCR analysis. Samples were sent by regular mail to the National

Veterinary Institute (SVA) without special cooling and arrived in 1–3 days.

2.2. Culturing and DNA extraction

On the day of arrival at SVA, samples were cultured for *D. nodosus* on 4% hoof agar plates (SVA, Uppsala, Sweden) as described by Stewart and Claxton (1993). Plates were read four to six days after anaerobic incubation at 37 °C. Suspected colonies were subcultured and the duplex 16S rRNA/intA real-time PCR assay developed in this study was used for confirmation of *D. nodosus*. Confirmed isolates were then stored at –70 °C in serum broth with 15% glycerol (SVA, Uppsala, Sweden). For serogrouping and virulence determination, these frozen isolates were thawed and cultivated on fastidious anaerobe agar plates (Lab M Ltd, Bury, UK) with 10% defibrinated horse blood (Hätunalab AB, Bro, Sweden). The plates were incubated anaerobically at 37 °C for four days and all isolates were subcultured once on the same type of agar plates or until a pure isolate was obtained.

DNA was extracted directly from the swabs by using the EZ1 tissue kit (Qiagen, Hilden, Germany) on the EZ1 Advanced and from bacterial colonies by a simple boiling method as previously described (Frosth et al., 2012). The extracted DNA was stored at –70 °C until analysed.

2.3. Duplex real-time PCR assay for detection of the 16S rRNA and intA genes of *D. nodosus*

A duplex real-time PCR assay to detect both the 16S rRNA gene and the intA gene of *D. nodosus* was developed in this study; it also contained an internal amplification control. Primers and probe for the 16S rRNA gene were used as previously described (Frosth et al., 2012). For detection of the intA gene, primers and probe were

Table 2

Flock size, sheep breed and scoring percentages in each of the 20 Swedish sheep flocks (A–T).

Flock ID	Flock size (ewes)	Breed	Score 2 (%)	Score 3 (%)	Score 4 (%)	Score 2–5 (%)	Category
A	8	Klövsjö sheep	0	0	0	0	1
B	40	Gotland Pelt sheep	0	0	0	0	1
C	35	Gotland Pelt sheep	0	0	0	0	1
D	55	Finnsheep	0	0	0	0	1
E	35	Gotland Pelt sheep	0	0	0	0	1
F	32	Gotland Pelt sheep	0	0	0	0	2
G	150	Gotland Pelt sheep	0	0	0	0	2
H	150	Gotland Pelt sheep & crossbreds	0	0	0	0	2
I	73	Gotland Pelt sheep	0	0	0	0	2
J	119	Gotland Pelt sheep & crossbreds	0	0	0	0	2
K	110	Gotland Pelt sheep	0.7	0	0	0.7	3
L	164	Crossbreds	1.8	0	0	1.8	3
M	69	Gotland Pelt sheep	31	0	0	31	3
N	216	Gotland Pelt sheep & Leicester	17	0	0	17	3
O	18	Gotland Pelt sheep	16	0	0	16	3
P	116	Gotland Pelt sheep	25	3.4	0	28.4	4
Q	57	Gotland Pelt sheep & Leicester	48	4.6	0	52.6	4
R	70	Finnsheep & crossbreds	10	10	4	24	4
S	50	Gotland Pelt sheep & crossbreds	12	31.5	0	44	4
T	40	Gotland Pelt sheep	53	2.5	0	55.5	4

designed using the Primer Express Software v 3.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA) with the following sequences: 5'-CAAGCAGCTTATTTAGCCGAAGA-3' (*intAf*), 5'-TTGTGCGTGCTTTTCATTTTC-3' (*intAr*) and 5'-TTGCGCGGAATGGTACCAACTC-3' (*intAprobe* FAM-labelled) producing a 109-bp amplicon. The specificity of the assay was checked against GenBank sequences with the BLAST program package (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al., 1990). An internal positive amplification control (TaqMan Exogenous Internal Positive Control Reagents) from Thermo Fisher Scientific Inc. was included in the duplex-assay to distinguish true target negatives from PCR inhibition. The positive control reagents were used according to the manufacturer's instructions except that the internal positive control DNA was diluted 50 times before use.

The 16S rRNA/*intA* duplex real-time PCR was carried out in 15- μ l reaction mixtures containing 1 \times PerfeCTa qPCR ToughMix, Low ROX (Quanta BioSciences Inc., Gaithersburg, MD, USA), 0.1 mg/ml bovine serum albumin (BSA) (Sigma–Aldrich, St Louis, MO, USA), 400 nM of each primer, 100 nM of each TaqMan probe, 1 \times TaqMan Exogenous Internal Positive Control Reagents (Thermo Fisher Scientific Inc.) and 2 μ l of template DNA. The same PCR-amplification conditions as in Frosth et al. (2012) were used here and in the *aprV2/B2* assay below. The *D. nodosus* CCUG 27824T (Culture Collection, University of Göteborg, Sweden) strain was used as a positive control for both the 16S rRNA and the *intA* genes while *D. nodosus* AN484/05 (SVA strain collection) was used as a negative control for the *intA* gene.

Inclusivity and exclusivity (specificity) testing was performed on 55 bacterial and fungal strains as previously described (Frosth et al., 2012). The duplex real-time PCR developed in this study did not detect any false negative or false positive results.

2.4. Real-time PCR assay for detection and discrimination of the *D. nodosus aprV2/B2* genes

The primers and probes for the TaqMan-based real-time PCR assay targeting the *aprV2* and *aprB2* genes of *D. nodosus* were designed using the Primer Express Software v 3.0 (Thermo Fisher Scientific Inc.) with the following sequences: 5'-GAAGCGACTGGTTTGATAACTG-3' (*aprV2/B2F*), 5'-GAGCTGTCGCTCTTTCTTTGC-3' (*aprV2/B2R*), 5'-ATGCGGTGGTTATCCT-3' (*aprV2probe* FAM-MGBNFQ) and 5'-ATGCGGTGGTCGCTCCT-3' (*aprB2probe* VIC-MGBNFQ). The specificity of the primers and probes was checked against sequences in GenBank with the BLAST program. The real-time PCR assay produced a 71-bp amplicon and was carried out in 15- μ l reaction mixtures containing 1 \times TaqMan Gene Expression Master Mix (Thermo Fisher Scientific Inc.), 0.1 mg/ml BSA (Sigma–Aldrich), 400 nM of each primer, 100 nM of each probe and 2 μ l of template DNA. The PCR-program comprised of an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of 95 °C for 3 s and 60 °C for 30 s. The *D. nodosus* CCUG 27824T strain and AN484/05 were used as positive controls for *aprV2* and *aprB2*, respectively.

Exclusivity testing of the developed assay was performed on 45 bacterial and fungal strains as previously described (Frosth et al., 2012), in addition to 50 *D. nodosus* negative swab samples from five clinically healthy sheep flocks. The assay did not detect any false positives in the exclusivity testing either for strains or swabs.

A comparison of the detection limit and amplification efficiency of our *aprV2/B2* assay was made with that of Ståuble et al. (2014a). Primers, probes and TaqMan Genotyping Master Mix were ordered from Thermo Fisher Scientific Inc. and standard mode was used in the 7500 Fast Real-Time PCR System instrument to enable the PCR-program as described for their assay. The same detection limit (4 fg DNA) was achieved for both our and their assay, when run in parallel. The amplification efficiency was approximately 94% for both genes in our assay and 88% and 89%, respectively for *aprV2* and *aprB2* in theirs.

2.5. Real-time PCR analysis for detection of *F. n. necrophorum* and *F. n. funduliforme*

All 1000 swab samples were analysed for *F. n. necrophorum* and *F. n. funduliforme* by a real-time PCR assay targeting the *gyrB* gene, as described by Jensen et al. (2007) with some modifications. The *gyrB* assay was carried out in a single reaction—the *ssp. necrophorum* specific probe was labelled with FAM and the *ssp. funduliforme* with Cy5. The master mix used was 1 \times TaqMan Universal PCR Master Mix (Thermo Fisher Scientific Inc.) and BSA (Sigma–Aldrich) was added at a final concentration of 0.1 mg/ml. Template volume was 2.5 μ l. Each of the type strains of *F. n. necrophorum* CCUG 9994T and *F. n. funduliforme* CCUG 42162T (Culture Collection, University of Göteborg) were used as positive controls in all runs.

2.6. Real-time PCR analysis for detection of *Treponema* spp.

All 1000 swab samples were analysed for *Treponema* spp. by a real-time PCR assay developed in this study to target the 16S rRNA-tRNA^{leu} region of ISR2. Primers were those described by Stamm et al. (2002). The assay was carried out in 30- μ l reaction mixtures containing 1 \times SsoFast EvaGreen Supermix with Low ROX (Bio-Rad Laboratories Inc., Hercules, CA, USA), 0.1 mg/ml BSA (Sigma–Aldrich), 375 nM of each primer and 2 μ l of template DNA. The PCR-program comprised of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. After completion of the PCR-program, a continuous melt curve analysis (60–95 °C) was performed. DNA from *Treponema pedis* strain TA4, supplied by Dr. O. Svartström (Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences), was used as a positive control in the PCR runs.

All real-time PCR amplifications for *D. nodosus*, *F. necrophorum* and *Treponema* spp. were carried out in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.) and analysed with the 7500 Software v 2.0.6. Each real-time PCR run also included non-template controls in the form of DNase/RNase-free sterile

Table 3
Amplification efficiencies and detection limits for each PCR-target.

PCR-target	Standard curve slope	R ²	Amplification efficiency (%)	Limit of detection (fg DNA)	Genomic DNA per PCR-reaction (copies)
16S rRNA*	-3.461	0.997	95	4	3
<i>intA</i> *	-3.438	0.995	95	4	3
<i>aprV2</i> *	-3.485	0.998	94	4	3
<i>aprB2</i> *	-3.487	0.999	94	4	3
<i>F.n. funduliforme</i>	-3.734	0.998	85	10	4
<i>F.n. necrophorum</i>	-3.830	0.994	82	12	5
<i>Treponema</i> spp.	-3.540	0.996	92	32	10

* Assays developed within this study.

water (Sigma–Aldrich). Amplification efficiencies and detection limits for each PCR-target (Table 3), were determined using ten-fold serial dilutions of chromosomal DNA. This DNA was obtained from each of the positive controls respectively and real-time PCR was performed as described above. Each DNA dilution was run in six replicates in the real-time PCR analysis. Standard curves for amplification efficiency calculations were constructed by the use of the DNA dilutions positive for all six replicates. The DNA dilution with the lowest DNA amount positive for all six replicates was determined as the limit of detection.

2.7. *fimA* PCR analysis of *D. nodosus* isolates

All 78 *D. nodosus* isolates obtained by culturing in this study were analysed by the two multiplex PCR assays targeting the *fimA* gene as developed by [Dhungyel et al. \(2002\)](#), but with some modifications to determine serogroup affinity (A–I). The reverse primers were fluorescently labelled as follows: ADHI (FAM), BEG (HEX) and CF (NED). The two PCR assays, ABCDE and FGHI, were carried out in 25- μ l reaction mixtures containing 1x HotStarTaq Master Mix (Qiagen), 1.5 mM MgCl₂ (Thermo Fisher Scientific Inc.), 0.12 mg/ml BSA (Sigma–Aldrich), 1.2 μ M of forward primer, 400 nM of each of the labelled reverse primers and 2 μ l of template DNA. Each PCR run included DNA from the Australian *D. nodosus* prototypes (A–I) kindly provided by Dr. O. P. Dhungyel (Faculty of Veterinary Science, University of Sydney), as positive controls and DNase/RNase-free sterile water (Sigma–Aldrich) as non-template controls. Amplification was carried out in a 2720 Thermal Cycler (Thermo Fisher Scientific Inc.) with conditions as described by [Dhungyel et al. \(2002\)](#) except for an increased initial denaturation step of 15 min. The PCR products were analysed by capillary electrophoresis on an Applied Biosystems 3100 Genetic Analyzer (Thermo Fisher Scientific Inc.) and analysed with the Peak Scanner Software 2. The DNA ladder was Geneflo 625 ROX (EURx, Gdansk, Poland).

2.8. Statistical analyses

Descriptive statistics were used to present proportions of different bacterial findings for each flock. Associations between clinical status (categories 1–4) and bacterial

finding (yes/no) at the flock level were investigated using Fisher's exact-test. Univariable mixed-effect logistic regression models were used to investigate associations between footrot (score <2 vs. score \geq 2 and score \leq 2 vs. score \geq 3) and bacterial findings in feet. Mixed-effect models were used in order to take into account the random effect of sheep within flocks (that sheep within a flock are more similar than sheep between flocks) and an identity covariate structure was used. The clinical results (scoring and bacterial findings) from one randomly selected foot per individual (if more than one foot was sampled) were used in the mixed-effect models. All statistical analyses were performed using Stata Software (StataCorp., 2010; Stata Statistical Software: Release 13.1; College Station, TX, USA, StataCorp LP.). The distribution of sampled flocks was presented using ArcMap 10.2 (Esri Inc., CA, USA).

3. Results

3.1. Presence and characterisation of *D. nodosus* in flocks and sheep with different clinical manifestations of footrot by real-time PCR analysis of swab samples

The proportion of *D. nodosus* found in the 20 investigated flocks is shown in Fig. 2. *D. nodosus* was detected both in clinically healthy (categories 1–2) and footrot-affected flocks (categories 3–4), but was significantly more common

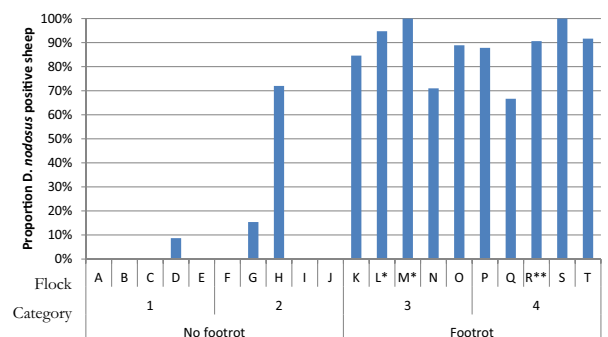


Fig. 2. Proportion *Dichelobacter nodosus* positive sheep in each of 20 investigated Swedish sheep flocks (A–T) with different clinical status according to categories 1–4. Flocks positive for the *intA*-gene are indicated with an asterisk and flocks positive for both the *intA* and the *aprV2*-gene are indicated with two asterisks.

Table 4

Distribution of bacterial findings, presence of the *intA* and *aprV2/B2*-genes in 20 Swedish sheep flocks with clinical status according to categories 1–4.

	Category				p-value
	1	2	3	4	
<i>D. nodosus</i>					
No	4	3	0	0	0.01
Yes	1	2	5	5	
<i>intA</i> in flocks with <i>D. nodosus</i>					
No	1	2	3	4	1.00
Yes	0	0	2	1	
<i>aprB2</i> in flocks with <i>D. nodosus</i>					
No	0	0	0	1	1.00
Yes	1	2	5	4	
<i>aprV2</i> in flocks with <i>D. nodosus</i>					
No	1	2	5	4	1.00
Yes	0	0	0	1	
<i>F. necrophorum</i>					
No	4	0	0	0	0.004
Yes	1	5	5	5	
<i>F. n. necrophorum</i>					
No	5	4	3	3	0.68
Yes	0	1	2	2	
<i>F. n. funduliforme</i>					
No	4	1	1	1	0.20
Yes	1	4	4	4	
<i>Treponema</i> spp.					
No	0	1	1	0	1.00
Yes	5	4	4	5	

in the affected flocks ($p = 0.01$) (Table 4). The *intA* gene was detected in three flocks in total, all of which were affected with footrot (categories 3–4), but the finding was not statistically significant. The *aprV2* gene was only detected in a single flock affected with footrot (category 4) while the *aprB2* gene was detected in twelve flocks—nine affected with footrot and three unaffected. There were no statistical differences in findings of the *aprV2* and *aprB2* genes for flocks with or without footrot.

Statistical analyses were also performed at the individual sheep level. However, not all swab samples could be included in the statistical analyses because some animals and even some feet had been sampled more than once. This left a total of 588 feet that were randomly selected for inclusion in the statistical analyses at individual level. Of these 588 feet, 506 were classified as not having footrot (score <2) and 82 as having footrot (score ≥ 2). Thirty of the 82 feet with footrot were considered to have severe footrot lesions (score ≥ 3). In total, *D. nodosus* was found in 236 feet (of 588, 40%); 77 of these feet were classified as having footrot and 159 as not. The results from the mixed-effect model showed that the odds of having footrot were 13 times higher in a foot with *D. nodosus* ($p = 0.002$). When comparing feet with severe footrot lesions (score ≥ 3) to feet with moderate footrot lesions (score 2) or feet without footrot (score <2), the odds of severe footrot was 19 times higher if there was *D. nodosus* ($p = 0.04$). There was no significant association between findings of *aprV2* or *aprB2* and feet with or without footrot. However, a comparison of feet with severe footrot lesions to feet with moderate or no lesions showed that the presence of *aprV2* significantly

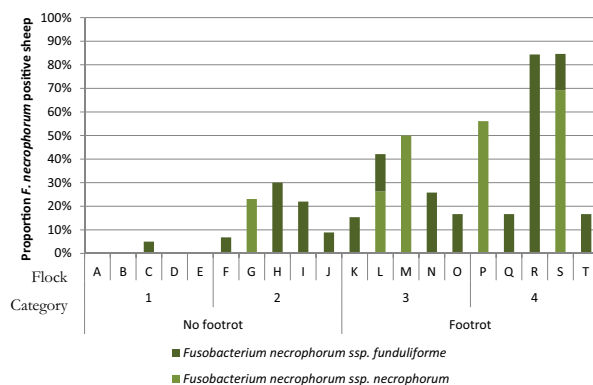


Fig. 3. Proportion *Fusobacterium necrophorum* positive sheep in each of 20 investigated Swedish sheep flocks (A–T) with different clinical status according to categories 1–4.

increased the odds of having severe footrot lesions ($p = 0.03$) and the presence of *aprB2* significantly lowered the odds of having severe footrot lesions ($p = 0.03$). The *intA* gene of *D. nodosus* was detected in 69 feet (from three sheep flocks) of which 44 (64%) were classified as not having footrot and 25 (36%) as having it. No statistical association was seen for the *intA* gene and feet with/without footrot or when severe footrot lesions were compared to feet with moderate or no lesions.

3.2. Presence of *F. necrophorum* and *Treponema* spp. in flocks and sheep with different clinical manifestations of footrot by real-time PCR analysis of swab samples

F. necrophorum ssp. *necrophorum* was detected in 5 of 20 investigated sheep flocks and *F. necrophorum* ssp. *funduliforme* was detected in 13 of 20 flocks. Two flocks, both affected by footrot, had both subspecies of *F. necrophorum* present (Fig. 3). *Treponema* spp. was detected in 18 of the 20 sheep flocks (Fig. 4). There was no significant association between findings of *F. n. necrophorum*, *F. n. funduliforme* or *Treponema* spp. and sheep flocks from the different categories but *F. necrophorum* was more commonly found in category 2–4 flocks than in category 1 flocks ($p = 0.004$) (Table 4).

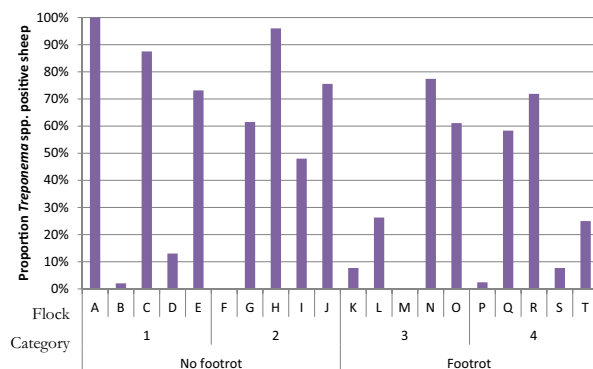


Fig. 4. Proportion *Treponema* spp. positive sheep in each of 20 investigated Swedish sheep flocks (A–T) with different clinical status according to categories 1–4.

In total, 134 of 579 investigated feet (nine feet had missing data for *Fusobacterium* and *Treponema* spp.) were *F. necrophorum* positive (47 *F. n. necrophorum* positive, 82 *F. n. funduliforme* positive and 5 that were positive for both subspecies). The results from the mixed-effect model showed that the odds of having footrot were 5.5 times higher in a foot with *F. necrophorum* ($p < 0.001$). The comparison of feet with severe footrot lesions to feet with moderate or no lesions found that the presence of *F. necrophorum* increased the odds of severe footrot lesions 4.9 times ($p < 0.01$). The *F. n. necrophorum* and *F. n. funduliforme* findings were also examined separately. The odds of footrot was 9.5 times higher in feet with *F. n. necrophorum* ($p < 0.001$). For feet with *F. n. funduliforme*, the odds of footrot were 2.5 times higher ($p = 0.047$). When feet with severe footrot lesions were compared to feet with moderate or no lesions there was a tendency that severe footrot lesions occurred if *F. n. necrophorum* ($p = 0.08$) or *F. n. funduliforme* ($p = 0.09$) were present. *Treponema* spp. was found in 273 (47%) of 579 feet but there was no statistically significant association.

3.3. Characterisation of *D. nodosus* isolates

Distribution of *D. nodosus* isolates from 11 flocks with respect to *intA*, virulence and serogroup are shown in Table 5. Twelve isolates contained the *aprV2* gene (virulent) while the remaining isolates ($n = 66$) contained the *aprB2* gene (benign). All virulent *D. nodosus* were isolated from one and the same flock (R) and they were also positive for the *intA* gene. The benign *D. nodosus* isolates were from ten different flocks and only a single isolate was *intA* positive (flock L). The most predominant serogroup was serogroup A which comprised 39 *D. nodosus* isolates from 6 different flocks. The virulent isolates belonged to serogroup G while the benign isolates belonged to six different serogroups: A, B, C, E, G and H. Three *D. nodosus* isolates from two different flocks could not be amplified by the serogroup-specific PCR assays of Dhungyel et al. (2002) but they were allocated to serogroup G on the basis of

whole genome shotgun sequencing as reported in Kennan et al. (2014).

4. Discussion

Footrot is a major animal welfare problem and reduces productivity (Green and George, 2008; Stewart et al., 1984). Knowledge of how footrot manifests itself in sheep and which strains of *D. nodosus* that are present in countries where footrot occurs are important for treatment, control and elimination of the disease.

In the present study, *D. nodosus* was found in flocks affected with footrot (score ≥ 2) and in clinically healthy flocks (score < 2) which is consistent with previous reports (Moore et al., 2005; Vatn et al., 2012). However, only three of the ten clinically healthy flocks were *D. nodosus* positive in our study of which two (G and H) had been diagnosed with footrot the year prior but undergone treatment.

Recently, whole genome sequencing of over 100 *D. nodosus* isolates divided them in two distinct groups, those carrying *aprV2* (virulent) or those with *aprB2* (benign). This distinction shows good correlation to severity of clinical manifestation (Kennan et al., 2014). Although the majority of *D. nodosus* was benign (*aprB2* positive) in our study, a significant association was seen between findings of *aprV2* and severe footrot lesions (score ≥ 3). Virulent *D. nodosus* (*aprV2* positive) was only detected in a single flock. This flock was also the only one that had score 4 lesions. In contrast to our findings, Moore et al. (2005) found almost only virulent strains regardless of disease severity (interdigital dermatitis or severe footrot). Likewise, Stäuble et al. (2014b) found only virulent strains in footrot affected flocks (score 1–5) and predominantly benign strains (score 0) in the unaffected ones. Our *aprV2/B2* real-time PCR assay had the same analytical sensitivity as the similar assay by Stäuble et al. (2014a). The advantages of our assay were that there were no known SNPs in the primers and probe design (Kennan et al., 2014) and the qPCR FAST format significantly reduced analysis time.

Table 5

Distribution of *Dichelobacter nodosus* isolates from 11 flocks with respect to *intA*, virulence as defined by *aprV2/B2* and *fimA* (Serogroup A–I).

Flock	No. isolates	<i>intA</i> pos	<i>aprB2</i> pos	<i>aprV2</i> pos	<i>fimA</i>									
					A	B	C	D	E	F	G	H	I	
G	2	0	2	0	2	0	0	0	0	0	0	0	0	0
H	3	0	3	0	0	0	2	0	0	0	1	0	0	0
K	1	0	1	0	1	0	0	0	0	0	0	0	0	0
L	8	1	8	0	6	0	1	0	0	0	0	1	0	0
M	10	0	10	0	10	0	0	0	0	0	0	0	0	0
N	1	0	1	0	0	1	0	0	0	0	0	0	0	0
O	10	0	10	0	10	0	0	0	0	0	0	0	0	0
Q	10	0	10	0	10	0	0	0	0	0	0	0	0	0
R	12	12	0	12	0	0	0	0	0	0	12	0	0	0
S	14	0	14	0	0	0	0	0	14	0	0	0	0	0
T	7	0	7	0	0	0	0	0	0	0	7	0	0	0
Total	78	13	66	12	39	1	3	0	14	0	20	1	0	0

Serogrouping of the *D. nodosus* isolates revealed that there are several different serogroups present in Sweden. This suggests that footrot has been present before the first diagnosed case in 2004. Serogroup A was the most predominant, which is consistent with a study in Norway by Gilhuus et al. (2013). However, all of our *D. nodosus* isolates of serogroup A were benign unlike those in Norway which were mainly virulent (Gilhuus et al., 2013). The only virulent *D. nodosus* isolates in our study belonged to serogroup G. They were also *intA* positive which further distinguishes them from the Norwegian outbreak strain which was *intA* negative and serogroup A (Gilhuus et al., 2013).

It has recently been suggested that *F. necrophorum* is a secondary pathogen in footrot development that possibly enhances disease severity (Witcomb et al., 2014). To our knowledge, there have been no footrot studies differentiating the two subspecies of *F. necrophorum* (*F. n. necrophorum* and *F. n. funduliforme*). In our study, *F. necrophorum* was more commonly present in category 2–4 flocks (score 1–5) than in category 1 flocks (score 0). There was a significant association with *F. necrophorum* at species level but only a tendency was seen at subspecies level for feet with different footrot scores, i.e. severe footrot lesions (score ≥ 3) as compared to moderate lesions (score 2) and healthy feet (score < 2). Whether there are differences in virulence between the two subspecies of *F. necrophorum* in regards to the development of ovine footrot would merit further studies.

No significant association between *Treponema* spp. and footrot was found in our study. However, *Treponema* spp. were detected in all but two flocks and often in high proportion of the animals. Further studies on the *Treponema* species and their possible role in footrot are warranted.

In our study, new tools were developed to rapidly detect the presence of virulent *D. nodosus* i.e., real-time PCRs for the *aprV2/aprB2* and *intA* genes. The only virulent *D. nodosus* (*aprV2* positive) detected in this study was also *intA* positive, and thus the combination of *aprV2* and *intA* real-time PCRs can be used for screening and tracing the spread of this particular strain to other flocks. The importance of rapid detection and virulence determination methods for *D. nodosus* has been highlighted by the recent outbreak of footrot in Norway (Gilhuus, 2014). The tools developed in this study are aimed to support the Swedish Animal Health Service's efforts to stop the spread of virulent footrot and to eliminate the disease at flock level. Thus further research on why benign *D. nodosus* seems to cause severe footrot lesions (score ≥ 3) in Swedish sheep flocks is necessary.

In conclusion, this is the first study on the presence of *D. nodosus*, *F. necrophorum* and *Treponema* spp. in Swedish sheep flocks with different clinical manifestations of footrot compared to healthy sheep flocks. This study also report initial data on *D. nodosus* isolates from Sweden regarding virulence, serogroup and presence of the *intA* gene. *Dichelobacter nodosus* and *F. necrophorum* were more commonly found in flocks and feet with footrot lesions than without lesions. *Treponema* spp. was common in both flocks and feet with and without footrot. The majority of

D. nodosus detected was benign and of six different serogroups, while virulent *D. nodosus* of serogroup G was only detected in a single flock. The *intA* gene was only found in *D. nodosus* from flocks with footrot but in both feet with and without footrot lesions.

Conflict of interest statement

None.

Acknowledgements

We thank Gull Holmström at the Department of Bacteriology, SVA for excellent technical assistance. Dr. O. P. Dhungyel is thanked for providing DNA from the Australian *D. nodosus* prototypes for serogroups A–I. Dr. Olov Svartström, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, is thanked for providing DNA from the *T. pedis* strain TA4. The Swedish Animal Health Service, the Swedish Board of Agriculture and the Swedish Farmers' Foundation for Agricultural Research are gratefully acknowledged for their financial contribution to the study.

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