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Cross-infection of virulent *Dichelobacter nodosus* between sheep and co-grazing cattle

Maren Knappe-Poindecker [a,*], Marianne Gilhuus [b], Tim K. Jensen [c], Synnøve Vatn [d], Hannah J. Jørgensen [b], Terje Fjeldaas [a]

[a] Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, P.O. Box 8146 Dep, N-0033 Oslo, Norway
[b] Norwegian Veterinary Institute, P.O. Box 750 Sentrum, N-0106 Oslo, Norway
[c] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Copenhagen, Denmark
[d] Animalia-Norwegian Meat and Poultry Research Centre, P.O. Box 396 Økern, N-0513 Oslo, Norway

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ABSTRACT

Dichelobacter nodosus is the main aetiological agent of ovine footrot and the bacterium has also been associated with interdigital dermatitis is cattle. The aim of this study was to investigate possible cross-infection of virulent *D. nodosus* between sheep and co-grazing cattle. Five farms, where sheep previously diagnosed with virulent *D. nodosus* were co-grazing with cattle for different periods of time, were included. The study sample consisted of 200 cows and 50 sheep. All cows were examined for the presence of interdigital dermatitis, and ten ewes, preferably with symptoms of footrot, had the footrot scores recorded. On each farm, the same ten ewes and ten cows were chosen for bacterial analyses. Swabs were analysed for *D. nodosus* by PCR and culturing. *D. nodosus* isolates were virulence-tested and assigned to serogroups by *fimA* variant determination. Biopsies were evaluated histopathologically and analysed by fluorescent in situ hybridization for *D. nodosus*, *Treponema* spp. and *Fusobacterium necrophorum*. *D. nodosus* defined as virulent by the gelatin gel test were isolated from 16 sheep from four farms and from five cows from two of the same farms. All five cows had interdigital dermatitis. Two of the cows stayed infected for at least eight months. By pulsed-field gel electrophoresis (PFGE), the isolates from the five cows were found to be genetically indistinguishable or closely related to isolates from sheep from the same farm. This indicates that cross-infection between sheep and cows have occurred.

1. Introduction

Ovine footrot is a highly contagious and economically important disease (Whittington and Nicholls, 1995). *Dichelobacter nodosus*, a Gram negative anaerobic bacterium, is the primary aetiological agent, but development of disease depends upon the characteristics of the involved strain, the breed of sheep and environmental conditions (Beveridge, 1941; Egerton and Raadsma, 1991). *Fusobacterium necrophorum* has been considered a secondary pathogen in ovine footrot (Egerton et al., 1969; Bennett et al., 2009). Production of extracellular proteases by *D. nodosus* is assumed to be responsible for tissue damage (Thomas, 1964). In sheep, isolates that produce thermostable proteases are more likely to be virulent than isolates that produce thermolabile proteases (Depiazzi et al., 1991). Antigenetic classification of *D. nodosus* is based on its fimbrial antigens and there are 10 serogroups (A–I and M) (Claxton, 1989).

* Corresponding author. Tel.: +47 22 59 74 65; fax: +47 22 96 47 62.
E-mail address: maren.knappe-poindecker@nmbu.no
(M. Knappe-Poindecker).

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D. nodosus lacks strict host specificity, and transmission has been observed between sheep and other ungulates such as cattle and goats (Laing and Egerton, 1978; Ghimire et al., 1999). The bacteria is commonly isolated from cows with interdigital dermatitis (ID) as well as from cows without symptoms of foot disease, and all isolates from Norwegian cattle have previously been categorized as benign (Gilhuus et al., 2013; Knappe-Poindecker et al., 2013). *D. nodosus* has also recently been hypothesized to play a synergetic role together with *Treponema* spp. in the pathogenesis of digital dermatitis (DD) in some regions (Rasmussen et al., 2012).

In Norway, dairy cattle are housed during the winter without any contact with sheep. During the summer, all cows must be pastured at least 8 weeks, and co-grazing of sheep and cattle is commonly practised. This provides an opportunity for the bovine feet to be exposed to virulent *D. nodosus* if the sheep are infected with such isolates. In 2008, ovine footrot was diagnosed for the first time in 60 years in Norway (Meling and Ulvund, 2009). A study of co-grazing sheep and cattle during this outbreak indicated that cross-infection had occurred (Rogdo et al., 2012). If cross-infection occurs, it could have implications for the Norwegian footrot elimination programme.

The aim of this study was to investigate possible cross-infection of virulent *D. nodosus* between sheep and co-grazing cattle. Furthermore, the persistence of colonization in cattle and the impact of virulent *D. nodosus* on bovine claw health were studied.

2. Materials and methods

2.1. Study design and selection of farms

All Norwegian farms with dairy cattle co-grazing with sheep previously diagnosed with *D. nodosus* defined as virulent by the gelatin gel test (GG-test) were considered for this longitudinal study. The owners were interviewed about co-grazing routines, current claw health status of sheep and cattle, measures to improve the claw health and willingness to participate in the study. After this initial interview, only two farms were eligible, but during the following year, another three farms fulfilled the selection criteria.

2.2. Study sample

The study population consisted of five farms with 200 cows or heifers in the last two months of gestation and 725 ewes. The herd and flock figures are presented in Table 1. Farms 1 and 2 were included in the study in the autumn of 2010 and farms 3–5 were included in the autumn of 2011. Initially, ten ewes, preferably with symptoms of footrot, and ten cows or heifers on each farm were chosen for bacterial sampling. After an unsuccessful attempt to culture *D. nodosus* from cattle on farm 3, ten younger heifers were also sampled.

2.3. Clinical examination and recording of claw lesions

The clinical examination was performed and recorded by the first author. The ten sampled ewes had symptoms of footrot recorded in the autumn after Egerton and Roberts (1971) with modifications by Whittington (1995).

Cattle were examined for the presence of ID and samples were taken in the autumn at the end of the grazing season (between September and November), and in the spring before the start of the next grazing season (April or May). Between samplings, the cattle had no contact with sheep. All heifers in the last two months of gestation and all cows were examined in a stand-up trimming chute. Cows that were in labour ($n = 1$) and cows that were acutely ill ($n = 1$) were not examined. Only the hind feet were examined because they are more frequently affected by lesions than front feet (Sogstad et al., 2005).

2.4. Bacterial sampling and analysis

Samples for bacterial analyses were collected from the same 10 sheep that had foot health recorded. All cows on farms 1 and 2 had spent the same amount of time co-grazing with the sheep, and the first seven cows with ID and the first three cows with no symptoms of this disease were sampled. On farms 3–5, some cows had spent longer periods co-grazing with the sheep flock than others and the individuals sampled were chosen from these cows. There were fewer than seven cows with ID on farms 2 and 3, and in these herds, the number of samples from cows without symptoms was increased. If a sampled cow had left the herd during the winter, another cow was chosen for bacterial sampling as a replacement in the spring. Two culled cows were, however, not replaced.

Before sampling, the bovine skin was cleaned with tap water and dried off with paper towels. Ovine skin was only dried off with paper towels. Swabs and biopsies were taken from the plantar skin of the foot or the interdigital skin.

Table 1
Data from the five farms where cattle and sheep were co-grazing.

Farm	Sheep		Cows			Co-grazing		Number of weeks co-grazing
	Flock size[a]	Breed[b]	Number	Breed[c]	Housing	All cattle	Only heifers/dried-off cows	
1	400	NKS	50	NRF/HF	Tie stall	X		15
2	75	NKS	19	NRF	Tie stall	X		18
3	60	NKS/Texel	34[d]	NRF	Free stall		X	8
4	100	NKS	57	NRF	Free stall		X	16
5	90	NKS	40	NRF	Free stall		X	8

[a] Breeding sheep.
[b] NKS = Norwegian White Sheep.
[c] NRF = Norwegian Red, HF = Holstein/Friesian.
[d] Including 10 heifers.

Cows which tested positive for virulent *D. nodosus* isolates were followed up individually with therapy and retesting until they tested negative or were slaughtered. On farm 2, virulent *D. nodosus* was isolated from 4/10 cows, which is more than on the other farms where none or only one cow was infected with virulent strains. Because of the higher prevalence, all cows in this herd were examined in a trimming chute and had bacterial samples for PCR and culturing taken on 8 March 2012.

2.4.1. Sampling for D. nodosus—PCR, culturing, virulence-testing and serogrouping

Swabs for culturing were placed in a Transystem Amies agar gel medium with charcoal (Copan Innovation Ltd., Brescia, Italy), and swabs for real-time PCR were placed in tubes with sterile phosphate buffered saline (PBS) containing 0.02 M EDTA. Samples were sent by overnight courier to the Norwegian Veterinary Institute in Oslo for analysis.

DNA was extracted from the swabs in PBS with EDTA using a nucliSENS easyMAG extractor (bioMèrieux, Boxtel, The Netherlands) following the manufacturer's instructions. DNA from cultured isolates was obtained by diluting broth culture 1:5 in double distilled water followed by boiling for 1 min. Extracted DNA was stored at −20 °C. *D. nodosus* was detected using a real-time PCR as described previously (Frosth et al., 2012).

Culturing was performed on 4% hoof agar (HA) basically as described by Stewart and Claxton (1993), but with the addition of 1% 'Lab-Lemco' powder (Oxoid, Basingstoke, England) and 0.2% Tryptose (Oxoid) to the HA. When possible, at least two *D. nodosus* suspect colonies from each sample were subcultured onto 2% HA. An approximately 5 mm × 5 mm piece of agar with pure confluent bacterial growth was cut from the agar and transferred to HEPES-TAS broth (Stewart and Claxton, 1993). The broth was incubated anaerobically at 37 °C for 48–72 h. Purity of the broths was checked by phase contrast microscopy and the presence of *D. nodosus* was confirmed using real-time PCR as described above. The remaining broth cultures were used for virulence-testing by the GG-test, essentially performed as described by Palmer (1993). For the GG-test, 40 ml of a gel containing 1% Agarose NA (GE Healthcare, Uppsala, Sweden), 1% gelatin from porcine skin, Type A (Sigma-Aldrich, St. Louis, MO), 0.02 M Tris and 0.001 M $CaCl_2$ was prepared. To prevent growth of contaminant bacteria, 2.5 mM Sodium Azide (BHD Laboratory Supplies, Poole, England) was added. The gel was poured onto a 20 cm × 20 cm glass plate and when solid, 4 mm diffusion wells were made. Broth cultures were diluted 1:1 in a dilution buffer (pH 8.5) containing 0.1 M HEPES (Sigma-Aldrich, St. Louis, MO) before application to the gel. After incubation the gel was immersed in saturated Ammonium sulphate ($(NH_4)_2SO_4$) solution at 60 °C. The diameter of proteolysis around each well was measured and compared with a standard, which was calibrated according to Palmer (1993). Isolates were categorized as virulent or benign based on their ability to secrete thermostable proteases. Culture broths of virulent and benign control strains were included on each gel. Control strains of *D. nodosus* were AC 6465 ST 198 with thermostable proteases (virulent) and AC 6466 ST 305 with thermolabile proteases (benign).

In order to allocate the isolates to serogroups A–I, the variable region of the gene encoding the fimbrial subunit *fimA* was amplified by multiplex PCR (Dhungyel et al., 2002) with the previously described modifications (Gilhuus et al., 2013). DNA from Australian reference strains of *D. nodosus* representing serogroups A–I were included as positive controls. Distilled water was included as negative control.

2.4.2. Pulsed-field gel electrophoresis (PFGE)

Virulent *D. nodosus* isolates from cows were compared by PFGE with virulent isolates from sheep from the same farm. The isolates were analysed as described by Buller et al. (2010) with the following modifications: Isolates were cultured on 2% TAS (trypticase-arginine-serine) agar for 2–3 days and were suspended in 400 μl wash buffer. The agarose plugs were washed five times in Tris-EDTA (TE) buffer. Prior to restriction, plugs were washed twice with 0.6 ml TE at 25 °C for 20 min. DNA in each plug was restricted for 3 h at 30 °C in 100 μl volume containing 1× SuRE/Cut buffer A (Roche Diagnostics GmbH, Mannheim, Germany), 0.1 mg/ml Bovine Serum albumin (BSA) and 50 units of *Apa* I (Roche). An additional 10 units of *Apa* I was added halfway through the incubation period. Restriction fragments were separated in a CHEF-DR® III electrophoresis system (Bio-Rad, Hercules, CA) at 6 V/cm, with a pulse time of 0.2–25 s for 24 h. The gel was stained with ethidium bromide and visualized under UV light. DNA fragments larger than the smallest ladder fragment (48.5 kb) were included in the analysis and a unique electrophoretic banding pattern was defined as a pulsotype.

2.4.3. Sampling, histopathological evaluation and analyses of biopsies by fluorescent in situ hybridization (FISH)

Biopsies were taken on the border between healthy and diseased skin with a 3 mm biopsy punch (Miltex, Inc., USA) and immediately fixed in 10% neutral buffered formalin. Each biopsy punch was used only once. The biopsies were processed routinely for histopathology and FISH using oligonucleotide probes targeting 16S ribosomal RNA of *D. nodosus*, *F. necrophorum* and *Treponema spp*., as described by Rasmussen et al. (2012). The degree of epidermal damage was defined as score 0 (normal epidermis), score 1 (mild) as mild epithelial proliferation and hyperkeratosis, score 2 (moderate) as severe epithelial proliferation and hyperkeratosis (parakeratosis with increasing degeneration and mal-keratinization) and score 3 (extensive to diffuse) as severe epithelial proliferation with exudation, erosion or necrosis of the dermal papilla according to Rasmussen et al. (2012). If the dermis was included, the cellular, inflammatory response in the dermis was given score 0 (normal dermis), score 1 (mild) characterised by only a few lymphocytes and mononuclear cells, score 2 (moderate) with some lymphocyte or mononuclear cell infiltrations around small vessels, or score 3 (severe) with perivascular dermatitis. In each biopsy the presence of *D. nodosus, Treponema* spp. and *F. necrophorum* were scored 0 = no hybridization, or 1 = positive hybridization.

2.5. Treatments and measures

Cows which tested positive for virulent isolates were treated topically with activated copper and zinc chelate (Intra Hoof-fit®; Intracare b.v.) in the autumn of 2010 and with chlortetracycline (Cyclospray®; Eurovet) in the autumn of 2011 and retested and retreated until negative ($n = 4$) or slaughtered ($n = 1$). As a part of a herd treatment plan all cows on farm 1 were footbathed once in a stand-in footbath filled with 15% $ZnSO_4$ for 20 minutes later in the autumn of 2011. All cows on farm 2 were treated topically with chlortetracycline in the autumn of 2011 and with Intra Hoof-fit® in March 2012.

In the sheep flocks an elimination programme involving medical treatment or the slaughter of all sheep was performed shortly after sampling. There was no further follow-up for sheep which tested positive for virulent *D. nodosus*.

2.6. Statistical analysis

Data recorded at the farms were transferred to Stata (Stata SE/11, Stata Corp., College Station, TX, USA) for statistical analysis. The associations between ID, *D. nodosus* and *Treponema* spp. and between *D. nodosus* and *Treponema* spp. among the cows selected for bacterial sampling were analysed by Fisher's exact test. Two cows were diagnosed with DD and consequently excluded from the statistical analyses.

3. Results

3.1. Footrot in sheep

On farm 1, all ten sheep had severe symptoms of footrot, on farm 2 six had severe and four had mild symptoms, on farm 3 one had severe and nine had mild symptoms, on farm 4 three had mild and the other sheep had no symptoms, and on farm 5 two had severe and eight had mild symptoms.

3.2. Foot disorders in cattle

Interdigital dermatitis was diagnosed in 20.5% (41/200) of the cows in the autumn and 30.0% (56/187) of the cows in the spring. *D. nodosus* isolates characterised as virulent by the GG-test were only isolated from cows on farms 1 and 2. On these two farms ID was diagnosed in 17.4% (12/69) of the cows in the autumn and 18.5% (12/65) of the cows in the spring, versus 22.1% (29/131) and 36.1% (44/122) of the cows, respectively, on farms 3, 4 and 5, where no virulent isolates were detected. All the five cows, in which virulent *D. nodosus* was detected, had ID at the time of isolation.

3.3. Identification and characterization of D. nodosus

Results from PCR, culturing, virulence testing and FISH of *D. nodosus* on farm level are presented in Table 2. *D. nodosus* categorized as virulent by the GG-test was isolated from five cows, one on farm 1, hereafter referred to as cow 1a, and four cows on farm 2, hereafter referred to as cows 2a, 2b, 2c and 2d. At the time of sampling, all five cows had ID which was clinically impossible to distinguish from the lesions where only benign *D. nodosus* was isolated. In cow 1a, 2b, 2c and 2d, the ID healed and the cows eliminated the virulent isolates during the observation period.

On farm 2, cows 2a and 2b were positive for *D. nodosus* by PCR in the autumn of 2010. However, the samples were culture negative and the virulence could consequently not be tested. The two other cows, 2c and 2d, were not sampled in the autumn. Cow 2c was examined but not sampled. Cow 2d was neither examined nor sampled, because at that time she was too young to be included in the study. However, virulent *D. nodosus* was isolated from all these four cows the following spring. For further details on the follow-up testing of cows with virulent isolates, see Table 3.

Virulent *D. nodosus* was isolated from altogether 16 sheep from farms 1, 2, 3 and 5. For further information on

Table 2
Prevalence of *Dichelobacter nodosus* detected by PCR, culturing and fluorescent *in situ* hybridization (FISH) and characterised by virulence-testing and serogrouping in feet from sampled sheep (S) in the autumn (Au) and cattle (C) in the autumn and spring (Sp) on each farm.

Farm	Species	PCR		Culturing		FISH		Serogroups		Virulent isolates[a]		Benign isolates[a]	
		Au	Sp	Au	Sp	Au	Sp	Au	Sp	Au	Sp	Au	Sp
1	C	7/10	6/9	3/10	3/9	1/9	3/8	A	A	1 (1)	1 (2)	1 (2)	2 (4)
	S	10/10	–	8/10	–	6/10	–	A	–	8 (13)	–	0	–
2	C	8/10	9/10	1/10	4/10	2/10	5/10	A	A	0	4 (7)	1 (2)	0
	S	8/10	–	4/10	–	3/10	–	A	–	2 (3)	–	2 (3)	–
3	C	17/20[b]	9/9[b]	1/20	3/9	6/20	2/9	B, I	B, I	0	0	1 (2)	3 (3)
	S	9/10	–	5/10	–	0/9	–	A	–	5 (7)	–	0	–
4	C	10/10	10/10	8/10	3/10	5/10	3/10	A, B, I	B	0	0	7 (13)	3 (3)
	S	7/10	–	0/10	–	0/9	–	–	–	–[c]	–	–	–
5	C	10/10	10/10	5/10	5/10	3/9	4/10	A, C	B, C	0	0	4 (8)	5 (5)
	S	10/10	–	1/10	–	1/7	–	A	–	1 (1)	–	0	–

[a] Number of infected animals with number of isolates on each farm in parentheses.
[b] On farm 3, samples for bacterial analysis were collected from 20 cattle in the autumn and 9 cattle in the spring.
[c] On farm 4, the samples from sheep were culture negative and the virulence could consequently not be tested. Virulent *D. nodosus* has, however, previously been detected in this farm.

Table 3
Results from the PCR analysis, culturing (C), virulence testing (GG) and fluorescent *in situ* hybridization (FISH) for the entire observation period for the five cows with virulent *Dichelobacter nodosus*.

Cow	September/October 2010				January 2011[a]			May 2011				October 2011[a]			January 2012[a]			March 2012[a]		
	PCR	C	GG	FISH	PCR	C	GG	PCR	C	GG	FISH	PCR	C	GG	PCR	C	GG	PCR	C	GG
1A	+[b]	+	+	–	+	+	+	+	+	+	+	+	–[c]	na	–	–	na	mis[d]	mis	mis
2A	+	–	na[e]	–	mis	mis	mis	+	+	+	+	+	–	na	+	+	+	+	+	+
2B	+	–	na	–	mis	mis	mis	+	+	+	+	+	–	na	–	–	na	–	–	na
2C	mis	mis	mis	mis	mis	mis	mis	+	+	+	+	+	–	na	–	–	na	–	–	na
2D	mis	mis	mis	mis	mis	mis	mis	+	+	+	+	–	–	na	mis	mis	mis	–	–	na

[a] Biopsies were not taken and consequently FISH was not performed.
[b] Test positive.
[c] Test negative.
[d] Missing, samples not collected/symptoms not recorded.
[e] Not applicable.

identification and characterization of *D. nodosus* in the sheep, see Table 2. The results from the PFGE of *D. nodosus* isolates from cattle and sheep from farms 1 and 2 are presented in Fig. 1.

3.4. Histopathological evaluation and FISH regarding *Treponema spp.* and *F. necrophorum*

The results from the histopathological evaluation and FISH regarding identification of *Treponema* spp. and *F. necrophorum* on farm level are presented in Table 4. In the autumn of 2010, bacterial samples were collected from three of the five cows which coincidentally or later had virulent *D. nodosus* isolated, and *Treponema* spp. was not identified. The following spring all five cows were infected with *Treponema* spp.

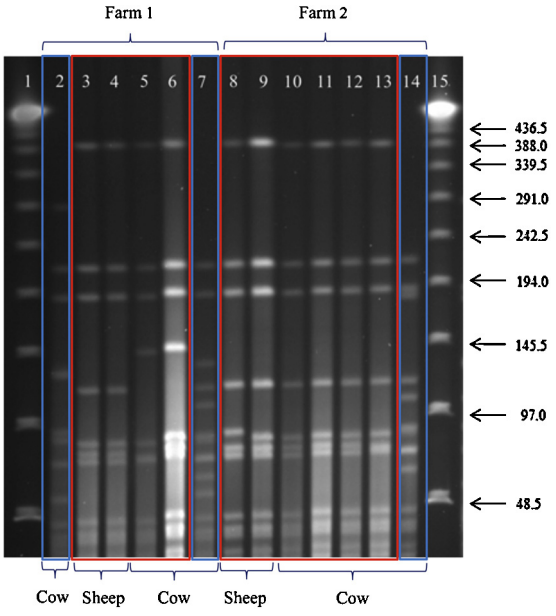

Fig. 1. Pulsed-field gel electrophoresis of *Apa* I-digested DNA from isolates of *Dichelobacter nodosus* from farms 1 and 2. Lanes labelled 1 and 15 represent lambda ladder size standards. Lanes outlined in blue are isolates defined as benign by the gelatin gel test and lanes outlined in red are isolates defined as virulent. Lanes 5 and 6 are isolates from cow 1a on farm 1 sampled in autumn and spring, respectively. Lanes 10, 11, 12 and 13 are isolates from cow 2c, 2b, 2d and 2a, on farm 2. Numbers to the right indicates molecular sizes (kilobases).

3.5. Associations between ID, *D. nodosus* and *Treponema spp.* in cattle

There was associations between ID and *D. nodosus* ($p = 0.01$, $n = 106$), between ID and *Treponema* spp. ($p < 0.001$, $n = 105$), and between *D. nodosus* and *Treponema* spp. ($p = 0.02$, $n = 105$).

4. Discussion

This study indicates that virulent *D. nodosus* was transferred from sheep with severe footrot to cattle when both were grazing on the same pasture. The infection persisted on the bovine feet for at least ten months, and one cow remained infected during an entire housing season. Consequently, cattle should be considered a possible reservoir for virulent *D. nodosus* after contact to sheep with severe footrot.

All 24 virulent isolates from sheep in the present study belonged to serogroup A, which is in agreement with previous research (Gilhuus et al., 2013). Virulent *D. nodosus* isolates belonging to serogroup A were also identified in cattle on the same farms, and the pulsotypes differed by 0–2 bands by PFGE. According to Tenover et al. (1995), isolates with identical pulsotypes are considered genetically indistinguishable, while isolates with 2–3 band differences are considered closely related. These results indicate that cross-infection occurred on two of the farms in this study. In sheep, direct contact on pasture is a risk factor for footrot, and cross-infections of *D. nodosus* between sheep and other species such as goats have been observed (Ghimire et al., 1999; Grøneng et al., 2013). Beveridge (1941) successfully infected a calf with footrot from sheep under experimental conditions, but concluded that cattle are not susceptible to natural infection with virulent isolates. Later studies have failed in transmission of virulent *D. nodosus* from sheep to co-grazing cattle (Laing and Egerton, 1978).

In addition to direct transmission, indirect transmission by means of such things as contaminated boots may be a potential route of transmission even though not identified as a risk factor (Grøneng et al., 2013). One of the cows in

Table 4
Results from the histopathological evaluation and fluorescent *in situ* hybridization (FISH) regarding *Treponema* spp and *F. necrophorum* in feet from sampled sheep (S) in the autumn (Au) and cattle (C) in the autumn and spring (Sp) on each farm.

Farm	Species	N	Dermis		Epidermis		Fluorescent *in situ* hybridization			
			Mean lesion score		Mean lesion score		*Treponema* spp.		*F. necrophorum*	
			Au	Sp	Au	Sp	Au	Sp	Au	Sp
1	C	9	1.0 ($n=7$)[a]	0.7 ($n=7$)	2.1	1.6 ($n=8$)	2/9	1/8	0/9	0/8
	S	10	1.6 ($n=8$)	–	2.3	–	4/10	–	2/10	–
2	C	10	0.9 ($n=9$)	1.1 ($n=9$)	1.3	1.4	1/10	5/10	0/10	0/10
	S	10	1.9 ($n=7$)	–	2.4	–	1/10	–	1/10	–
3	C	20/9[b]	0.3	0.7	0.6	0.4	2/20	3/9	0/20	0/9
	S	9	1.2	–	0.8	–	0/9	–	0/9	–
4	C	10	0.6	1.1	1.6	1.9	4/10	4/10	0/10	0/10
	S	9	1	–	0.1	–	0/9	–	0/9	–
5	C	10	0.6	0.3	1	1.3	4/9	4/10	0/9	0/10
	S	7	1.3 ($n=6$)	–	1.1	–	0/7	–	0/7	–
Total	C	59/49[b]	0.6	0.8 ($n=45$)	1.2	1.3 ($n=47$)	13/58	17/47	0/58	0/47
	S	45	1.4 ($n=39$)	–	1.4	–	5/45	–	3/45	–

[a] Insufficient amount of dermis present for appropriate scoring.
[b] On farm 3, samples for bacteriological analysis were collected from 20 cattle in the autumn and 9 cattle in the spring.

our study (cow 2c) was healthy and was not sampled in the autumn of 2011, but had ID and virulent *D. nodosus* was isolated the following spring. This cow may have been infected indirectly or was already infected when stabled but had not yet developed symptoms.

In our study, the presence of virulent *D. nodosus* in cattle was associated with ID. Recent studies have shown a strong association between benign *D. nodosus* and ID (Knappe-Poindecker et al., 2013) and in the present study it was impossible to distinguish between ID associated with virulent versus benign strains based on the symptoms and the histopathology. In the cows, the epidermal damage was mild and the inflammatory response of the dermis was minimal, indicating a superficial infection.

Farms 1 and 2, where virulent *D. nodosus* was isolated, had a 4.7% (autumn) and 17.6% (spring) lower prevalence of bovine ID than farms 3–5. Farms 1 and 2 had tie stall housing, as opposed to the three others which had free stalls, which are usually known to have a higher prevalence of ID because the more unhygienic environment favours infectious foot diseases (Sogstad et al., 2005). This environment has also probably contributed to the 14% increase in prevalence of ID on farms 3-5 during the housing season, as opposed to farms 1 and 2, where the prevalence was almost unchanged during the same period. Improvement of the environment, such as when grazing, is known to reduce the prevalence of ID (Holzhauer et al., 2012) and in four out of the five cows with virulent isolates in our study, the ID had healed after pasturing. At that time, these cows were negative by culture for *D. nodosus*, and at the next sampling also by PCR.

The longitudinal part of the study showed that even though virulent *D. nodosus* was isolated in only five out of 60 sampled cows, the infection may have endured for at least one housing season in all these five cows, and two cows remained infected for at least eight and ten months, respectively. This finding is in agreement with Wilkinson et al. (1970), who regularly observed *D. nodosus* in smears from a steer with ID over a nine month period. Cross-infection of both virulent and benign *D. nodosus* from cattle to sheep has been described previously (Egerton and Parsonson, 1966; Wilkinson et al., 1970), and if virulent *D. nodosus* is carried and transmitted by cattle, this must be considered during the design and implementation of footrot quarantine and elimination plans. The previous recommendation from the Norwegian Sheep Health Service, which deemed one season without co-grazing of sheep and cattle sufficient to eliminate bovine feet as a possible source of reinfection, must be reconsidered as the results from this study indicates that one grazing season without contact with sheep is insufficient to eliminate virulent isolates of *D. nodosus* from all cattle.

In one of the infected cows the bacterium was not eliminated despite several treatments using products that are documented to be effective against bovine DD (Manske et al., 2002; Holzhauer et al., 2011). However, for practical reasons, the chlortetracycline was used only once instead of the recommended three days in a row, which probably reduced the effect of the antibiotic.

Rasmussen et al. (2012) hypothesized that *D. nodosus* break down the epidermal barriers in cattle, making the skin more susceptible to secondary invaders such as *Treponema* spp. in DD. Our results, with an association between *D. nodosus* and *Treponema* spp., and because at least three of the five cows with virulent *D. nodosus* became infected with *Treponema* spp. between the autumn and spring samplings, are in accordance with this hypothesis. Previous studies of ovine footrot have observed that an initial infection with *D. nodosus* is often followed by an infection with treponemes (Beveridge, 1941; Thomas, 1962), and an association between ID and *D. nodosus* and a strong association between ID and *Treponema* spp. has also been found in cattle herds with no contact with sheep (Knappe-Poindecker et al., 2013). The presence of *Treponema* spp. in all five cows with virulent *D. nodosus* at spring samplings may be in better conformity with this hypothesized synergy than be a

consequence of the virulence of the present *D. nodosus* isolate.

In our study, *F. necrophorum* was absent in bovine skin samples analysed by FISH, which is in accordance with a previous study where a lower prevalence of *F. necrophorum* in Norway than in Denmark was found (Rasmussen et al., 2012). In cattle, the bacterium is associated with interdigital phlegmon and has been identified in DD lesions (Nagaraja et al., 2005; Rasmussen et al., 2012). In sheep, *F. necrophorum* is, together with the *D. nodosus*, associated with footrot (Bennett et al., 2009), and the bacterium has been reported necessary to induce footrot in experimental trials (Egerton et al., 1969). The results from our study, where *F. necrophorum* was detected by FISH and only identified in three sheep, do however suggest that this bacterium may not be involved in the pathogenesis. These results are in agreement with Witcomb et al. (2011), who revealed no such evidence.

The symptoms of ovine footrot varied from severe on farms 1 and 2 to very mild on farm 4, and the severity of symptoms on farms 1 and 2 could increase the risk of transmission of virulent *D. nodosus*. The epidermal damage in sheep also varied among the flocks and the damage was considerably greater on farms 1 and 2. Even though the inflammatory response of the dermis was more uniform, sheep from farms 1 and 2 scored higher (1.0–1.9) than sheep from the other three farms (0.3–1.3). One previous study of other co-grazing ungulates suggested that the degree of contact with the infected sheep is important (Belloy et al., 2007), and there was a closer contact between sheep and cattle on farms 1 and 2. On these two farms, all dairy cows and heifers co-grazed with sheep for an average of 16.5 weeks, as opposed to the other farms which only let heifers and dry cows co-graze for an average of 10.7 weeks. This suggests that the number of cattle co-grazing and the length of the grazing season may be important with respect to the occurrence of cross-infections.

A higher number of participating farms would have been favourable, but this was not possible as many farms had already started measures to eliminate virulent *D. nodosus* in sheep flocks and thus were no longer eligible (Vatn et al., 2012).

5. Conclusion

Our study indicates that cross-infection of virulent *D. nodosus* from sheep to co-grazing cattle occurs, and that the virulent isolates can persist on bovine feet for at least ten months. Grazing of cattle on pastures where sheep with ovine footrot are kept should be avoided. Virulent *D. nodosus* was isolated from one cow directly after the grazing season, which shows that one grazing season without contact with sheep is insufficient to eliminate virulent *D. nodosus* from bovine feet. Cattle must be considered a source of virulent *D. nodosus* in elimination programmes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.02.044.

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