



## Longitudinal *Dichelobacter nodosus* status in 9 sheep flocks free from clinical footrot



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### ABSTRACT

Footrot is a widespread problem in Swiss sheep farming. The objectives of this study were to determine whether flocks which were clinically free from footrot carry virulent strains of *Dichelobacter nodosus*, and to describe the infection dynamics for flocks and individual sheep. To this purpose, a new PCR-diagnostic tool was used, which is able to distinguish benign from virulent *D. nodosus*. Nine farms were examined three times at intervals of 6 months. Cotton swabs were used to collect samples from the interdigital skin to analyze for the presence of virulent and benign strains of *D. nodosus*. Additionally, epidemiological data of the farms were collected with the aid of a standardized questionnaire. On four farms, benign strains were diagnosed at each visit; in one farm, benign strains were detected once only. Two flocks revealed sheep infected with virulent *D. nodosus* throughout the study but without clinical evidence of footrot. In two flocks, the virulent strains of *D. nodosus* were introduced into the flock during the study period. In one farm, clinical symptoms of virulent footrot were evident only two weeks after the positive finding by PCR. Only individual sheep with previously negative status, but none with previously benign status became infected with virulent strains during the study. The newly developed competitive RT PCR proved to be more sensitive than clinical diagnosis for detecting footrot infection in herds, as it unequivocally classified the four flocks as infected with virulent *D. nodosus*, even though they did not show clinical signs at the times of sampling. This early detection may be crucial to the success of any control program. Both new infections with virulent strains could be explained by contact with sheep from herds with virulent *D. nodosus* as evaluated from the questionnaires. These results show that the within-herd eradication of footrot becomes possible using the competitive PCR assay to specifically diagnose virulent *D. nodosus*.

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### 1. Introduction

Ovine footrot is a multifactorial disease, inducing interdigital lesions and lameness, and it accounts for impaired animal welfare in many countries worldwide (Pryor, 1954; Zhou and Hickford, 2000; Egerton et al., 2002; König et al., 2011; Vatn et al., 2012). The disease is caused by an infection of the interdigital skin with the gram-negative anaerobic bacterium *Dichelobacter nodosus* (*D. nodosus*), representing the necessary etiological agent (Egerton et al., 1969; Roberts and Egerton, 1969; Wani and Samanta, 2006; Kennan et al., 2011; Raadsma and Egerton, 2013). Footrot brings about losses in meat, wool, and milk production, and it increases

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labor and management efforts relating to treatment and eradication (Stewart et al., 1984; Wani and Samanta, 2006; Green and George, 2008; Nieuwhof et al., 2008; Stauble et al., 2014b). In the alpine area, footrot is of considerable importance, as foraging involves walking considerable distances which might be impaired when locomotor activity is affected (Stauble et al., 2014b).

The clinical manifestation of the disease ranges from mild interdigital dermatitis (benign footrot) over intermediate disease states (intermediate footrot) to complete separation of the horn shoe from the underlying tissue (virulent footrot) (Egerton and Parsonson, 1969; Egerton et al., 1969; Depiazzi et al., 1998; Abbott and Egerton, 2003). The degree to which clinical signs evolve depends on environmental conditions, differences in susceptibility of the host, and the virulence factors (proteases) released by the infecting *D. nodosus* strain (Graham and Egerton, 1968; Billington et al., 1996; Depiazzi et al., 1998; Zhou and Hickford, 2000). The degrees of virulence of various *D. nodosus* strains are mainly due to differences in the expression of the subtilisin-like extracellular proteases AprV2/B2, AprV5/B5 and BprV/B (Kennan et al., 2010; Stauble et al., 2014b). Among them, the expression of the two proteases known as virulent AprV2 and benign AprB2 was found to fully correlate with the clinical status of the studied individual sheep originating from various flocks in Switzerland, France, Germany and Norway (Stauble et al., 2014b). Further evidence was provided by the full genome sequence analysis of 103 strains of *D. nodosus* isolated from sheep all over the world, showing a bimodal population revealing the key single amino-acid differences between AprV2 and AprB2 (Kennan et al., 2014). In virulent strains, the AprV2 gene encodes the thermostable protease that is responsible for tissue damage typical for footrot (Kennan et al., 2010; Stauble et al., 2014a). Against this background, a competitive RT-PCR was recently developed to detect the genes encoding the virulent AprV2 (in this paper referred to as virulent strains) and the benign AprB2 proteases (in this paper referred to as benign strains) of *D. nodosus* simultaneously (Stauble et al., 2014a).

In Switzerland, a representative inquiry among sheep farmers who were members of the national sheep breeding association revealed that 57% of the respondents “had encountered problems with footrot in their sheep flocks in the past” (Greber and Steiner, 2013). Nevertheless, the control of footrot is still voluntary in most areas of Switzerland, except for two cantons in which within-herd control of virulent footrot based on findings at clinical examination is mandatory by law. The current control program consists of inspecting sheep’s feet, separating clinically affected from clinically healthy sheep, repeated footbathing in a disinfecting solution (CuSO<sub>4</sub> [5–10%] or ZnSO<sub>4</sub> [10–20%] or Formaldehyde [4–5%]) of affected individuals until clinical recovery and culling of non-responders (<http://bgk.caprovis.ch/cms09/showsingle.asp?lang=1&urlid=9>). Although repeatedly reported in the literature (Egerton et al., 1968; Egerton and Roberts, 1971; Jordan et al., 1996; Duncan et al., 2012; Kaler et al., 2012; Dhungyel et al., 2013; Strobel et al., 2014), neither vaccination nor antimicrobial treatment are accepted single measures of this control program. The polyvalent vaccine Footvax® (MSD, Luzern, Switzerland), administered prior to alpine pasturing in respective subpopulations of two non-sanitized flocks was only partially effective by reducing the lesion severity score and the new infection rate in vaccinated as compared to sham treated sheep (Hardi-Landerer et al., 2012). However, new outbreaks of virulent footrot in sanitized flocks occurred regularly, mainly during seasonal mixing of flocks of different origin on communal alpine pastures in the summer (Fringeli, 2010).

Currently, the political, societal and economical concern about effectively and sustainably controlling footrot without using antimicrobials is steadily increasing. The strategy of Swiss footrot control is under review, the consideration being the eradication

of virulent *D. nodosus* expressing the virulent protease AprV2 from sheep flocks nationwide, since (i) it is preferable for control programs to rely on the attributes of *D. nodosus* rather than the clinical signs (Allworth, 2014) and (ii) a rapid, practical and objective diagnostic tool for detecting virulent strains of *D. nodosus* is now available (Stauble et al., 2014a).

While preparing for this potentially upcoming novel footrot control program, the current descriptive longitudinal study was initiated, aiming to elucidate unexpected transmission or outbreaks of footrot caused by strains expressing the AprV2 protease. It was hypothesized that (i) the new PCR was suitable for detecting cases of virulent footrot in Swiss sheep flocks, (ii) new outbreaks caused by virulent strains of *D. nodosus* in formerly negative flocks would occur only following contact with individuals originating from non-sanitized flocks, and (iii) mutation from the benign to the virulent type of *D. nodosus* (mutation from AprB2 to AprV2) was unlikely to occur.

## 2. Materials and methods

### 2.1. Selection of flocks and sheep

Nine flocks located in 7 different Swiss cantons were used for this study according to convenient selection. Inclusion criteria for participation were that farmers were willing to volunteer for the whole study period, and that flocks were officially free of clinical footrot as documented by the footrot eradication program of the Swiss Consulting and Health Service for Small Ruminants (BGK) (<http://bgk.caprovis.ch/cms09/showsingle.asp?lang=1&urlid=9>). Flocks were sampled 3 times at 6 monthly intervals, starting in fall 2013. A representative number of sheep from each flock were selected so that laboratory analyses would correctly detect the presence of sheep carrying virulent *D. nodosus* within the herd with an accuracy of ≥95%. This number was calculated for each flock separately using specialized software (FreeCalc®, V2) assuming a prevalence of virulent strains in an affected herd of 20%, a specificity of the PCR for AprV2 of 98% and a sensitivity of 90% (Greber and Steiner, 2015; Locher, 2015). At first sampling (fall 2013), at least 8% of the total number of sheep from a respective flock were added to this number to correct for sheep potentially leaving the herd during the study. Individual sheep were selected according to the likelihood that they would remain in the respective flock during the whole study period, and if still present, the same sheep were sampled throughout the whole study. If the number of the selected sheep had dropped below the threshold, additional sheep were selected to meet the calculated minimal representative number. The study was performed with the permission of the ethical committees of the respective cantons.

### 2.2. Lesion scoring, collection of specimens and laboratory analyses

Feet were rated as either clinically healthy (score 0), showing mild signs of interdigital inflammation (score 1) or showing various degrees of clinical signs of footrot (scores 2–5) (<http://bgk.caprovis.ch/cms09/showsingle.asp?lang=1&urlid=9>), according to a scoring system adapted from (Egerton and Roberts, 1971). Specimens were taken immediately before routine claw-trimming by use of cotton swabs (2 mm 15 cm, Paul Hartmann AG, Heidenheim, Germany) from the interdigital space and, if present, from the outer rim of a lesion. For each of the four feet of an individual sheep, one clean quarter of one and the same swab was used so that each swab represented a 4 feet pooled sample of one sheep. After sampling, swabs were immediately soaked for at least one minute in 1 ml SV-lysis buffer (4 M guanidinetiocyanate, 0.01 M Tris-HCl pH 7.5,

**Table 1**  
Sampling schedule and number of sampled sheep of 9 flocks (A–I) free of clinical footrot.

Flock	1	2	3
A	7	14	23
B	14	25	25
C	8	14	27
D	11	19	17
E	5	4	8
F	4	14	14
G	9	17	21
H	6	8	4
I	8	12	28
Total	72	127	167

1 = number of sheep sampled once during the study; 2 = number of sheep sampled two times; 3 = number of sheep sampled three times.

1% b-mercapto-ethanol) to transfer the DNA and discarded thereafter (Stauble et al., 2014b). DNA-containing lysis buffer wells were kept at 4 °C until further processing at 1–6 weeks after collection. Analysis was performed by competitive real time PCR as described in detail by (Stauble et al., 2014a). This allowed the detection of the genes encoding the virulent thermostable protease AprV2 and/or the benign thermosensitive protease AprB2.

2.3. Animal status, flock status and collection of historic data

According to the result of the laboratory analyses, a footrot status was assigned to each sheep and to each flock at the time of the given sampling session. A sheep was rated *negative*, if the PCR was negative for both genes (AprV2 and AprB2); *virulent* if the PCR was positive for AprV2 only; *benign* if the PCR was positive for AprB2 only, and *mixed* if the PCR was positive for both AprV2 and AprB2. A flock was rated *Negative*, if all tested sheep of the respective flock were rated negative; *Virulent* if at least 1 of the tested sheep was either rated virulent or *mixed*, and *Benign*, if at least 1 sheep was rated *benign* and none *virulent* or *mixed*. At all three sampling sessions, historic data focusing on prophylactic measures, treatments, housing, pasturing, animal traffic and introduction of new sheep into the flock (originating from an outside farm during the preceding period) were collected, using a structured questionnaire. Decisions about treatments and other management measures concerning the flock were completely left to the discretion of the individual farmers.

3. Results

3.1. Animals sampled during the study

Throughout the study, 9 flocks (A–I) participated in this study; 366 sheep were sampled at least once, of which 167 (45.6%) were sampled 3 times. Detailed information concerning the sampling schedule of each flock separately is given in Table 1.

3.2. Flock status

Detailed information concerning the flock status and the results of the sampled sheep (for each sampling session separately) are given in Table 2. The status of 4 flocks (A–D) was *Benign* throughout the study. The status of flock E was *Benign* at first sampling and converted to *Negative* thereafter. The initial status of flock F was *Negative* twice and became *Virulent* during the period preceding the third sampling session. During that summer, some sheep of this flock were kept on a communal alpine pasture together with sheep originating from non-sanitized flocks. At the time of third sampling, clinical signs of virulent footrot were not

**Table 2**  
Footrot status of 9 flocks at 3 different sampling sessions and results of the competitive RT-PCR for AprV2/B2 of 366 sheep sampled at least once.

Flock	2013				spring 2014				2014				Flock status in fall 2014						
	# of sheep tested in fall	b (%)	v (%)	m (%)	n (%)	Flock status in fall 2013	# of sheep tested in spring 2014	b (%)	v (%)	m (%)	n (%)	Flock status in spring 2014		# of sheep tested in fall 2014	b (%)	v (%)	m (%)	n (%)	
A	37	34	91.9	3	8.1	B	38	6	15.8	32	84.2	B	29	29	100			B	
B	51	41	80.4	10	19.6	B	58	7	12.1	51	87.9	B	30	30	100			B	
C	43	42	97.7	1	2.3	B	45	12	26.7	33	73.3	B	29	29	100			B	
D	35	35	100			B	37	1	2.7	36	97.3	B	28	24	85.7	4	14.3	B	
E	17	3	17.6	14	82.4	B	10			10	100	N	10					N	
F	27			27	100	N	27			27	100	N	20	7	35	13	65	V	
G	37	9	24.3	4	10.8	V	38			38	100	N	31	24	77.4	3	9.7	4	12.9
H	15			8	53.3	V	11			9	81.8	V	8	3	37.5	2	25	3	37.5
I	43	1	2.3	15	34.9	V	43			32	74.4	V	30	6	20	4	13.3	20	66.7

b = sheep positive for AprB2; v = sheep positive for AprV2; m = sheep positive for AprB2/V2; n = sheep negative for AprB2/V2; B = flock rated benign; N = flock rated negative; V = flock rated virulent.

present in this flock, but occurred 2 weeks after PCR detected virulent *D. nodosus*. Flock G, although being clinically unsuspecting, started with a Virulent status, and was sanitized successfully before the second sampling according to the guidelines of the current Swiss footrot control program (<http://bgk.caprovis.ch/cms09/showsingle.asp?lang=1&urlid=9>) to reach the *Negative* status. An outbreak with a virulent strain occurred before the third sampling. Historical information from this flock revealed that some ewes and rams had participated in an exhibition that was not restricted to sheep originating from sanitized herds between the second and third sampling session. Quarantine- and prophylactic measures were not adequately adopted as given by the current footrot control program (1 instead of 4 weeks of isolation after the exhibition and footbathing once instead of weekly in a disinfecting solution during the quarantine period). Clinical signs of virulent footrot were not evident during the whole study period in this flock. Footbathing in a disinfecting solution was routinely performed once monthly by this farmer on a voluntary basis. Two flocks (H and I) were assigned the *Virulent* status throughout the study period. Sheep from both flocks were temporarily mixed during common alpine pasturing and for breeding purposes, the trading of sheep between the two flocks was a routine procedure. Clinical signs of virulent footrot were only present in the months before the first sampling session in flock H. Footbathing of both flocks in a disinfecting solution was performed voluntarily by the farmer on a routine basis in summer and fall throughout the study.

### 3.3. Changes of individual animal status

Selected results of the 167 sheep that were sampled 3 times throughout the study are described here. At the sampling in spring 2014, the number of sheep with a *negative* status had increased to 137 compared to 54 at first sampling in fall 2013: 76 of 88 (86%) of the *benign* sheep switched to *negative*, while this occurred in only 9 of 15 (60%) of the *virulent* sheep. The status of 70 of 76 (92%) sheep that were *benign* at first sampling and *negative* at second sampling switched back to the status *benign* at third sampling in fall 2014. In 26 out of 167 sheep (15.5%), the status switched to *virulent* during the study; the previous status having been *negative* in all these sheep. Thus, no sheep switched from the *benign* to the *virulent* status. The switch to the status *mixed* occurred in 4 sheep only. The original status of these was *negative* in 3 sheep and *virulent* in 1 sheep. Only 22 (6%) out of the total of 366 sheep sampled showed at least once the *mixed* status throughout the study.

## 4. Discussion

Six (4 *Benign* and 2 *Virulent*) out of the 9 flocks investigated in this study did not change the footrot status throughout the study period and one switched from *Benign* to *Negative*. The switch from *Negative* to *Virulent* was evident in 2 flocks, in both of which this could plausibly be explained by contact with sheep from non-sanitized flocks (common pasturing or common exhibition, respectively). At the individual sheep level, a switch from *negative* to *virulent* but not from *benign* to *virulent* was observed. The individual sheep status *mixed* occurred very rarely.

The results of this study have to be interpreted with some caution, as only 9 flocks were included in the study and only a representative selection rather than all the sheep from each herd were clinically examined and sampled. The latter is likely not be a major drawback, as the number of sheep per flock to be sampled was calculated in advance so that the chance of a false flock level status allocation was lower than 5%.

All 4 flocks that were classified as *Benign* throughout the study were sanitized within 5–20 years prior to this study. The finding

that sanitation of flocks with virulent footrot is usually followed by colonization of these flocks with benign strains agrees with information from the literature (Egerton and Parsonson, 1969).

New infection of individual sheep with virulent strains was observed in this study only after contact with non-sanitized flocks. Furthermore, only previously *negative* sheep acquired the virulent status, but not individuals colonized with benign strains 6 months earlier. The first finding agrees entirely with the literature (Allworth, 2014) and is most relevant for the potential success of any eradication program, as unexplained outbreaks of virulent footrot were not observed. From the second finding, it may be hypothesized that the mutation of *D. nodosus* strains found in Swiss sheep from the benign to the virulent status does not occur.

In Switzerland, sheep are usually kept indoors on dry straw bedding during the cold winter season. *D. nodosus* is less likely to colonize the sheep's feet if the environmental conditions are dry (Abbott and Lewis, 2005). Sheep's local defense mechanisms may be impaired at very low temperatures. Furthermore, footbathing of flocks at irregular intervals was quite common in these flocks. This may explain why in the *Benign* flocks of this study, it was very common that the status of sheep switched twice during the study period from *benign* to *negative* in winter and from *negative* to *benign* in summer. This is supported by the common finding that under current Swiss conditions, new outbreaks of footrot are rare during the winter season.

As shown in this study, sheep may be colonized with virulent strains without expressing any clear clinical signs of footrot (score >2). This finding was made possible by combining the clinical data with the laboratory results generated with the new PCR tool. This is in agreement with previous findings showing that highest numbers of virulent *D. nodosus* are found early during infection when clinical symptoms are still absent or only moderate (Stauble et al., 2014b). It also is in line with the literature (Moore et al., 2005; Vatn et al., 2012). It underscores the importance of using tools for control programs, which allow for the detection of specific virulence characteristics (Allworth, 2014) instead of relying exclusively on clinical attributes. Ample experience with the missing long-term success of the footrot control program partly implemented in Switzerland for many years, supports this finding (Fringeli, 2010). Frequent footbathing of the whole flock in a disinfecting solution, as voluntarily practiced by some of the farmers participating in the current study may further mask the presence of virulent strains potentially present in clinically sanitized flocks.

## 5. Conclusions

The results of this prospective longitudinal study are in agreement with all 3 initial hypotheses. Firstly, it did not unveil any obvious findings that, under Swiss conditions, might hamper the success of an ovine footrot control program focusing on the eradication of AprV2 positive *D. nodosus* from ovine feet. Secondly, the results of this study explained, why the currently implemented Swiss footrot control program was not successful in the long term. And finally, the study highlighted the fact that the newly developed competitive PCR for detecting the AprV2/B2 genes of *D. nodosus* may prove to be a very useful tool in a revised nationwide footrot control program in Switzerland.

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