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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*.

1. Introduction

Ovine footrot is a mulitfactorial 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; Konig 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 ($CuSO_4$ [5–10%] or $ZnSO_4$ [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 preparating 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 guanidinethiocyanate, 0.01 M Tris–HCl pH 7.5,