Prevalence of *Dichelobacter nodosus* in western Austrian sheep flocks: Comparison of bacterial cultures, clinical foot rot and lameness with PCR and analysis of sample pooling for PCR diagnosis

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Prävalenz von *Dichelobacter nodosus* in Schafherden in Westösterreich: Vergleich von Bakterienkulturen, klinischer Klauenfäule und Lahmheit mit PCR und Analyse des Probenpoolings zur PCR-Diagnose

Moderhinke ist eine hoch ansteckende und multifaktorielle Klauenkrankheit, die durch *Dichelobacter nodosus* (*D. nodosus*) verursacht wird und die Hauptursache für Lahmheit bei Schafen ist. Ziel dieser Querschnittsstudie war es, die Prävalenz von *D. nodosus* in Westösterreich auf Tier- und auf Betriebsebene zu ermitteln.

Klinische und bakteriologische Untersuchungen von interdigitalen Klauenabstrichen wurden mit den Resultaten der Real-time-PCR zum Nachweis von mit *D. nodosus* infizierten Tieren ausgewertet. Zusätzlich wurden gepoolte Vier-Fuss-Abstriche zur Erkennung von Moderhinke analysiert. Im Rahmen der Studie wurden insgesamt 3156 Schafe aus 124 Betrieben auf Lahmheit und klinische Anzeichen von Klauenfäule untersucht.

Die gefundene Herdenprävalenz von *D. nodosus* betrug 30,65 %. Die Bakterienkultur zeigte im Vergleich zur PCR eine Sensitivität von 75,0 % bzw. eine Spezifität von 100,0 % ($p < 0{,}001$). Die klinischen Fussfäule-Scores (Ckorr = 0,87; $p < 0{,}001$) und Lahmheits-Scores (Ckorr = 0,71; $p < 0{,}001$) korrelierten stark mit dem Nachweis von *D. nodosus* durch die PCR. Das Ergebnis zeigte, dass die klinische Untersuchung zur Identifizierung von mit *D. nodosus* infizierten Tieren in Herden genutzt werden kann, zur Bestätigung der Diagnose jedoch eine PCR eingesetzt werden muss. *D. nodosus* konnte mit risikobasierten Pools aus fünf Proben ge-

Abstract

Ovine foot rot is a highly contagious and multifactorial claw disease, caused by *Dichelobacter nodosus (D. nodosus)* and is the main cause of lameness in sheep. The aim of this cross-sectional study was to determine the prevalence of *D. nodosus* in western Austria both at animal and farm levels. Real-time PCR was evaluated in comparison with clinical and bacteriological investigations from interdigital foot swabs to detect *D. nodosus*-infected animals. In addition, the use of pooled four-foot swabs to detect foot rot was determined. In course of the study a total of 3156 sheep from 124 farms were examined for lameness and clinical signs of foot rot.

The found flock prevalence of *D. nodosus* was 30,65 % with bacterial culture showing a sensitivity of 75,0 % and a specificity of 100,0 % ($p < 0{,}001$) respectively, compared with PCR. Furthermore, clinical foot rot scores (Ckorr = 0,87; $p < 0{,}001$) and lameness scores (Ckorr = 0,71; $p < 0{,}001$) highly correlated with the detection of *D. nodosus* by PCR. The result showed that the clinical examination can be used to identify animals infected with *D. nodosus* in flocks, but PCR must be used to confirm the diagnosis. *D. nodosus* could be detected equally well with risk-based pools-of-five samples as with undiluted samples ($p < 0{,}001$), suggesting that a pool-of-five samples might be a suitable and cost-effective method for detecting *D. nodosus* in sheep flocks.

This study provides an overview of foot rot in Tyrolean sheep flocks and outlines the possibilities and limitations of the various diagnostic tools for *D. nodosus*. Further studies to investigate possible influencing factors, including alpine pasturing, management factors and biosecurity predispos-

nauso gut nachgewiesen werden wie mit unverdünnten Proben (p < 0,001), was darauf hindeutet, dass ein Pool aus fünf Proben eine geeignete und kostengünstige Methode zum Nachweis von *D. nodosus* in Schafherden sein könnte.

Diese Studie gibt einen Überblick über die Klauenfäule in Tiroler Schafherden und zeigt die Möglichkeiten und Grenzen der verschiedenen Diagnoseverfahren für *D. nodosus* auf. Weitere Studien zur Untersuchung möglicher Einflussfaktoren, einschliesslich Alpweiden, Bewirtschaftungsfaktoren und Biosicherheit, die die Klauenfäule prädisponieren, sind für die Gestaltung wirksamer zukünftiger Bekämpfungsprogramme in Alpenregionen erforderlich.

Schlüsselwörter: *Dichelobacter nodosus*, Klauenfäule, Herdenprävalenz, PCR, Schafe

ing to foot rot are necessary for the design of effective future control programs in alpine regions.

Keywords: *Dichelobacter nodosus*, foot rot, intra-flock prevalence, PCR, Sheep

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Introduction

Ovine foot rot is a highly contagious and multifactorial claw disease, which is the main cause of lameness in sheep flocks. The causative agent is the Gram-negative, anaerobe bacterium *Dichelobacter nodosus (D. nodosus).*[6,8,19,26] It is widespread worldwide and, despite improved husbandry conditions, continues to cause high economic losses, especially in countries with intensive sheep farming and suitable climatic conditions. These countries include, but are not limited to, Australia, the United Kingdom (UK), Bhutan, Iran, India, Greece, New Zealand, Switzerland, Norway and Sweden.[26] Clinical foot rot is very painful and poses a relevant animal welfare challenge.[8] Some affected sheep show supporting leg lameness, limb unloading and they sometimes walk or support their weight on their carpal joints during feeding. The latter is commonly seen in sheep on pastures, although not affected animals exhibit lameness.[19,26] The effects of the disease on the profitability of sheep farming are considerable.[26] This is particularly evident in alpine regions, since substantial distances must be covered by sheep to obtain sufficient feed.[22] Together with contagious ovine digital dermatitis (CODD), ovine interdigital dermatitis (OID), white line disease and claw joint abscesses, foot rot is the most common cause of lameness in sheep.[8] Infection of the interdigital space (IDS) and the expression of clinical signs depend on the virulence of the *D. nodosus* strains and additional favorable factors. The latter include inappropriate feeding, injuries to the IDS, poor claw hygiene, wet bedding, physical status and/or the presence of other bacteria, such as *Fusobacterium necrophorum* (*F. necrophorum*).[8,26] *F. necrophorum* is not a primary cause of foot rot, but can assist *D. nodosus* to infect the IDS and increases the severity of the disease.[25] The incubation period of foot rot is two to three weeks.[1,8,26] Its clinical manifestation ranges from mild interdigital dermatitis to underrunning of the claw horn and loss of the horn capsule accompanied by a pungent, putrid odor.[8,26]

D. nodosus is divided into serogroups A to I and M, based on the sequence variation in the type 4 fimbrial gene fimA.[14] The serogroups do not permit any conclusions to be drawn about virulence and pathogenicity.[26] However, by detecting the gene sequences of the extracellular acidic serine protease alleles *AprB2* and *AprV2*, it is possible to differentiate between benign and virulent strains.[23,26] In this respect *AprB2* is produced by benign and *AprV2* by virulent strains.[22] The *AprV2* and *AprB2* alleles differ by a two bp substitution, TA/TG, at position 661/662. This single nucleotide polymorphism (SNP) leads to an amino acid change (Tyr92Arg) in the entire structure of the serine proteases *AprB2* (thermolabile) and *AprV2* (thermostable), which affects the elastase activity in the entire protein.[13,14,21,22] At position 92, *AprV2* encodes a tyrosine residue and *AprB2* encodes an arginine residue.[13] Benign strains can induce inflammation of the IDS, which is mainly associated with redness and hair loss. Virulent strains are considered to be the main triggers of foot rot and they can lead to severe clinical courses within one or two weeks after infection.[1,19,26] The disease begins with inflammation of the IDS, but the result commonly leads to severe lameness.[14,26] The protease (*AprV2*) causes the claw horn to be further infiltrated by *D. nodosus*, starting from the IDS via the sole to the abaxial claw wall.[1,26] In the final stage of the disease, this leads to detachment of the horn shoe from the dermis.[26] Characteristically a cheese-like material can be observed in the IDS, on the dermis separated from the claw capsule, crusts and pathologically altered claw horn, and this emits a pungent, putrid odor.[8] While sheep mainly succumb to foot rot, other animal species usually remain silent carriers of *D. nodosus*.[2] There are some studies available on the prevalence of foot rot in sheep flocks, but unfortunately, they are difficult to compare. Diagnostic

definitions (clinic foot rot vs. pathogen detection), study designs and study populations are frequently interpreted differently.[26] In Germany, 52,2 % of sheep farms have been found affected by foot rot and 42,9 % were *D. nodosus*-positive.[23] In Switzerland, the prevalence at flock level was 16,2 % for virulent and 2,8 % for benign *D. nodosus* strains.[2] A foot rot prevalence of 90,0 % has been reported in the UK, while clinical foot rot was found to affect 5,8 % of slaughtered lambs in Sweden.[12,15] A recent study from Sweden reported a prevalence of foot rot at the flock level of 2,0 % and at the animal level of 0,7 %. Only sheep with a foot rot score ≥2 were counted. The prevalence of *D. nodosus* was 9,1 % at the flock level and 5,7 % at the animal level.[20] However, as yet no data is available on the prevalence of foot rot in Austrian sheep flocks. The most important intervention for containing, controlling and monitoring foot rot is diagnosis.[8] The goal here is to identify clinical foot rot, followed by the detection of *D. nodosus*.[1] The former is based on near characteristic symptoms and claw findings, whereby a foot rot clinical lesion score (Table 1; foot rot score) can be assigned.[9] However, these scores are not definitive, and may overlap with other diseases. For example, a foot rot score of one cannot be clinically distinguished from an OID.[8] Further, CODD cannot be differentiated from foot rot in the advanced course of the disease, which is possible in its early stages, as CODD begins at the coronary band, in contrast to foot rot, which originates from the IDS.[1]

During clinical examination, all four feet of each animal must be examined, which is best performed during claw trimming.[26] However, detection of the pathogen, by bacterial culture or Polymerase-Chain-Reaction (PCR), is more sensitive than clinical examination alone.[16,17,26] Indeed, some infected animals show few[1] or no clinical symptoms, especially in the dry and cold months.[26] Some sheep may also act as silent carriers of benign or even virulent *D. nodosus* strains, i.e., they are subclinically infected.[1,19] Moreover, clinically healed animals may still harbor trapped pathogens in horn gaps or pockets, which can act as reservoirs for further infection.[26]

Bacterial cultivation of *D. nodosus* is particularly demanding as special agar plates, containing hoof particles, are required.[26] Instead, competitive real-time PCR, is the method of choice for the detection of *D. nodosus*.[21] This technique returns fast results and has a higher sensitivity than bacterial culture.[5,16,26] In addition, competitive real-time PCR also permits differentiation of virulent from benign strains of *D. nodosus* based on gene sequences encoding *AprV2* and *AprB2*.[21] The high sensitivity of PCR thereby enables identification of pathogen-positive animals or flocks before clinical symptoms become manifest.[21,26] Pooling a single swab for all four feet as a four-foot-swab (swab rotated 90° after each foot) reduces the number of samples to be tested with negligibly lower sensitivity and specificity of 93,8 % or 98,3 %, respectively.[11] Risk-based sampling (Table 2) can further improve the sensitivity and specificity of PCR testing. By pooling four-foot-samples into a pool of five or ten in risk-based sampling, efficiency and cost savings can be further increased. However, to be tested animals must be carefully selected, which can be very time-consuming.[11] Samples collected on a non-risk basis require at least one sample with a Ct-value < 31 to be identified as positive due to high dilution with negative samples.[11] This strategy, which focuses on detection of virulent *D. nodosus*, is currently being used in the Swiss foot rot control program with an expected cost of around 100 euros per tested flock.[26] For this purpose, in Switzerland pools with up to ten swabs (corresponding to ten animals) in a single PCR batch are used. Three such risk-based pools-of-ten per flock are recommended.[11,16]

Various control programs for foot rot exist, which aim to achieve endemic stability.[4] However, control programs should be adapted to respective local climatic and structural conditions in their respective regions.[1,4]

The aim of this study was to determine the prevalence of *D. nodosus* in Tyrolean sheep farms in western Austria and to investigate its connection with clinical foot rot and the degree of lameness observed. Further, PCR performance for detection of *D. nodosus* is evaluated and compared with

Table 1: Ability on assigning a score to the claws in respect of foot rot in sheep.[9]

Score	Description
Score 0	Healthy claw
Score 1	Limited mild interdigital dermatitis: red skin, hair loss and moist interdigital skin.
Score 2	More extensive interdigital dermatitis with slightly cheesy coating on the interdigital skin and slightly damaged horn on the edge of the inner claw wall.
Score 3	Severe interdigital dermatitis and under-running of the horn of the heel and sole.
Score 4	Further under-running spreading to the walls of the claw.
Score 5	Loose horn capsule.

clinical and bacteriological investigations. Finally, the intra-flock prevalence of positive farms and the optimal pool size for the four-feet-swab detection method are determined. Study results are anticipated to serve as an essential basis for economic and effective control strategies against foot rot in alpine regions.

Materials and methods

Farms and testing procedures

The study was discussed and approved by the institutional ethics and animal welfare committee of the University of Veterinary Medicine Vienna, in accordance with Good Scientific Practice guidelines and national legislation.

In the course of this study, 124 sheep farms located in the Austrian federal state of Tyrol were sampled for foot rot between January and April 2021 as described below. Many Tyrolean sheep farms are testing their animals for endoparasites and footrot in early spring before the animals are brought on alpine pasture. To ensure a representative amount of participating farms, our study had to be performed at the same time, although the footrot prevalence is expected to be higher later during the pasture season.[1,4,8] Sample size was calculated using epitools (https://epitools.ausvet.com.au/prevalencess?page=PrevalenceSS) with an expected prevalence of foot rot between 16–30 %, based on the literature available.[2,10,11] Number of herd and herd size were obtained from Statistik Austria (https://www.statistik.at/statistiken/land-und-forstwirtschaft/betriebsstruktur/betriebsdaten/betriebe). This resulted in 350 samples needed to be evaluated to calculate the prevalence with a precision of 5 % and a confidence interval of 95 %. The average farm size was 30 sheep (median = 25; min = 2; max = 160), representing a study population of 3156 animals. Both ewes and rams were considered. In 66 (53,2 %) of the participating farms, the actual lameness prevalence of the flock was recorded as part of the farm visits, followed by the examination and sampling of individual animals (n = 631). The number and selection of the examined and sampled animals per flock were based on the "Instruction for risk-based sampling of foot rot" which can be viewed in Table 2.[18] Lameness and the degree of lameness (score 0–5) was observed as described in Baumgartner et al. 2018.[3] Lameness refers to the presence of lameness in a sheep and the lameness score describes the severity of the lameness. The sheep were then tipped onto their rump, the dirty claws were cleaned dry and claw trimming were performed with a claw knife and scissors. To avoid possible transmission of claw-associated pathogens, after each sheep the knives and scissors were cleaned with water and placed in an iodine-containing solution. The foot rot clinical lesion score was recorded at this point, as described in Table 1 and the flock clinical status calculated as described in Table 3.[7,9] All investigations in these flocks were performed together by two of the authors (Andreas M. and Eller C.) to standardize the results as far as possible. The remaining 58 (46,8 %) participating farms were sampled for foot rot only, according to the protocol described below, by one of two farm veterinarians, without any further surveys. The farms were enrolled in the study based on willingness of the owner to participate on a voluntary basis. In total, 2173 sheep were sampled for *D. nodosus* by PCR and 188 also by bacteriological culture.

In order to differentiate between *D. nodosus*-infected and non-infected sheep, a dry swab (Regular Floqswab Sterile; SRK Pharma 630, 17th A Main Rd, 6th Block, Koramangala, Bengaluru, Karnataka 560095, India) sample was taken from the interdigital space or, if foot rot present, from the edge of the lesion, following the sampling protocol described by Greber et al. 2018.[11] One swab was used for all four limbs of each animal, with the swab being rotated 90° when changing between claws. The dry swabs were trans-

Table 2: Risk-based sampling of foot rot in sheep flocks.[18]

flock size	N[a]
≤20	16
21≤30	18
31≤40	25
41≤50	26
51≤70	27
71≤120	28
121≤260	29
261≤500	30

[a]N = number of sheep per farm to be sampled for real-time PCR for *D. nodosus* detection.
The sheep were selected in the following order.:
1. Clinical lameness present; 2. Newly purchased sheep; 3. Animal that recently participated in a sheep show;
4. ram; 5. sheep with poor claw condition.

ported without any special cooling to the laboratory within two hours and stored at -20 °C until the analysis was performed. In farms with known cases of foot rot, according to the information of the farmer and in animals with a clinical foot rot score > 0, an additional swab was taken and placed in modified Amies liquid medium (eSwab Regular Floc., Copan Group, Via Francesco Perotti, 10, 25125 Brescia BS, Italy) for culture in the same manner as described for dry swabs. The swabs for bacterial culture were sent to the laboratory of the Austrian Agency for Health and Food Safety (AGES) in Linz immediately after collection without cooling.

Detection of *D. nodosus* by competitive real-time PCR

Detection of *D. nodosus* using competitive real-time PCR is a method described by Stäuble et al. 2014.[21] The procedure was performed for this study by the AGES laboratory in Innsbruck. The frozen swabs were warmed to room temperature, homogenized and underwent automated DNA extraction using the IndiMAG Pathogen Kit384 (INDICAL BIOSIENCE GmbH, Deutscher Pl. 5b, 04103 Leipzig, Germany) and IDEAL96 (ID.vet Genetics, 310, Rue Louis Pasteur, 34790, France), in accordance with user manuals. After DNA isolation, a section of the protease genes *aprV2* and *aprB2* was amplified by using competitive real-time PCR with the primer pair DnAprTM-R/DnAprTM-L (TIB Molbiol Syntheselabor GmbH, Eresburgstrasse 22–23, 12103 Berlin, Germany). The virulent (*aprV2*) and benign (*aprB2*) variants were detected with the TaqMan probes DnAprTM-v and DnAprTm-b (Eurofins Genomics, Anzinger Strasse 7a, 85560 Ebersberg Germany). Amplifications was performed with the PCR device BioRad (CFX96 real-time System C1000 Touch Thermal Cycler, BioRad, 1000 Alfred Nobel Dr, Hercules, CA 94547 USA) using the cycler program dnTM (1 cycle with 95°C for three minutes and 45 cycles with alternating 95°C for three seconds and 60°C for 30 seconds). The linear DNA fragment of the Enhanced Green Fluorescent Protein Gene (EGFP), which was added during the extraction and detected with the corresponding primer-(EGFP-1F/EGFP-10R) probes- (EGFP-1-HEX) mix (TIB Molbiol Syntheselabor GmbH, Eresburgstrasse 22–23, 12103 Berlin, Germany), served as the inhibition and extraction control (fluorescent dye = HEX). The reference strain DSM23057 *D. nodosus aprV2* (fluorescent dye = Fam)-diluted in a feces suspension to imitate a contaminated field sample was used as a positive control. For lack of areference strain of D. nodosus *aprB2* a linear DNA fragment from *aprB2*-gene was used as positive control for aprB2 strains (fluorescent dye = Cy5). The mastermix include six-parts Quanta Perfecta qPCR ToughMix (Quantabio, 100 Cummings Center #407j, Beverly, MA 01915, USA), one-part primer*(aprV2/B2)*-probe (Fam/CY5)-Mix, one part EGFP(HEX)-Mix and two parts RNAse-free water. 10µl mastermix and 2µl sample were used, which corresponds to a ratio of 5:1. The real-time PCR reactions were evaluated using the device's own software (Bio-Rad CFX Maestro) by measuring and evaluating the data from the fluorometric "real-time" detection during amplification using the sequence-specific fluorescence signals (Fam, Cy5, HEX). The assessment made was based on the fluorescence data obtained (amplification plot), taking into account the data obtained from positive and negative controls as well as the inhibition control. Samples with C_t values > 40 were interpreted as negative.[21] When positive results were obtained from the negative controls, or negative results from positive controls or from samples with a deviating course of the amplification curve, the homogenization, extraction, amplification and evaluation steps were repeated.

Detection of *D. nodosus* by bacterial culture

Bacteriological examination was performed in accordance with the guidelines of the ovine foot rot chapter of the Australian and New Zealand diagnostic procedure (2014). https://www.agriculture.gov.au/sites/default/files/sitecollectiondocuments/animal/ahl/ANZSDP-Ovine-foot rot.pdf. The eSwabs were inoculated on hoof agar plates, which were subsequently incubated at 37°C under anaerobic conditions. The culture plates were checked for growth of *D. nodosus* after an incubation period of at least five days.

Comparison of PCR analysis of individual samples with pooled samples.

A total of 35 individual *aprV2*-positive samples analyzed by PCR were pooled after homogenization to 35 pools with five samples per pool (pool-of-five). Each pool contained at least one positive single sample. Homogenization, extraction

Table 3: Definition of the sheep flock clinical status depending on the frequency and severity of clinical foot rot per farm.[7]

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

and amplification were performed in the same manner as described for individual samples. Thereafter results of the pool-of-five were compared with the single samples. Additionally, the C_t-values of undiluted samples with a dilution series (1:5; 1:7; 1:10) were compared. The two different master mixes, Quanta Perfecta qPCR ToughMix (Quantabio, USA) and KAPA PROBE FAST qPCR Master Mix (2x) Universal (Sigma-Aldrich, 3300 S 2nd St #3306, St. Louis, MO 63118, USA), were used in the PCR for undiluted and diluted samples to evaluate their applicability for testing pools. Initially only the master mix KAPA universal was used. However, since many PCR runs had to be repeated due to the failure of the inhibition and extraction control (HEX), we switched to the master mix Quanta perfecta qPCR ToughMix. The C_t-values of the two different master mixes were compared with each other. Homogenization and extraction were performed in the same manner as described above.

Statistical evaluation

The data were statistically processed using SPSS (International Business Machines Corporation [IBM], 1 Orchard Road, Armonk, New York, USA) and Excel (Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-6399, USA). Firstly, a frequency analysis and descriptive statistics for all variables were computed. A classification tree, independent variable importance and crosstabs were then constructed. The significance analysis was performed using the chi-square test (χ^2). The Pearson correlation coefficient (r) and the Pearson contingency coefficient (C_{korr}) were computed. The level of significance was set at $p < 0,05$.

Table 4: Results of Real-Time PCR to detect *Dichelobacter nodosus* compared with the results of bacterial culture, clinical foot rot score, flock clinical status and lameness of the sampled sheep.

	PCR positiv		PCR negativ		total		correlation Ckorr	Chi^2 p-value
	absolut	relativ (%)	absolut	relativ (%)	absolut	relativ (%)		
culture positiv	33	100,0	0	0,0	33	17,9	0,91	<0,001
culture negativ	11	7,3	140	92,7	151	82,1		
footrot score 0[a]	13	2,3	553	97,7	566	89,7	0,87	<0,001
footrot score 1[a]	7	24,1	22	75,9	29	4,6		
footrot score 2[a]	4	80,0	1	20,0	5	0,8		
footrot score 3[a]	12	92,3	1	7,7	13	2,1		
footrot score 4[a]	14	100,0	0	0,0	14	2,2		
footrot score 5[a]	4	100,0	0	0,0	4	0,6		
flock clinical status 1[b]	2	4,5	42	95,5	44	66,6	0,89	<0,001
flock clinical status 2[b]	0	0,0	0	0,0	0	0,0		
flock clinical status 3[b]	3	60,0	2	40,0	5	7,6		
flock clinical status 4[b]	15	88,2	2	11,8	17	25,8		
lameness	39	67,2	19	32,8	58	7,0	0,71	<0,001
no lameness	31	4,0	735	96,0	766	93,0		
lameness score 0	31	4,0	735	96,0	766	93,0	0,78	<0,001
lameness score 1	7	70,0	3	30,0	10	1,2		
lameness score 2	14	63,6	8	36,4	22	2,7		
lameness score 3	17	70,8	7	29,2	24	2,9		
lameness score 4	0	0,0	1	100,0	1	0,1		
lameness score 5	1	100,0	0	0,0	1	0,1		

The first two columns are organized into positive and negative PCR samples and can be conmpared with the other collected parameters. They are further subdivided into absolute and relative values, with the relative values expressing the different frequency of the further parameters collected in relation to the PCR result. The third column shows the whole number of further investigated parameters (culture, clinical foot rot score, flock clinical status and lameness) in absolute and relative values. The relative values of the third column express the distribution of the parameters within their group. The fourth and fifth columns present the p-value and the Pearson contingency coefficient between the PCR and the other parameters.[a =9, b =7]

Results

In the course of this study, *D. nodosus* was detected by PCR in 38 farms (30,7 %). From 2173 sampled sheep, 120 (5,5 %) were PCR-positive. The mean intra-flock prevalence was 10,0 % (min = 0,7 %; max = 34,8 %). All but one (99,2 %) positive sample revealed the virulent (*aprV2*) strain of *D. nodosus* and only one (0,8 %) sheep was positive with a benign (*aprB2*) strain. Results of the bacterial culture, the clinical foot rot score, flock clinical status and lameness compared with the PCR results are displayed in Table 4.
From 44 PCR-positive samples 33 also demonstrated growth of *D. nodosus* in bacterial culture (sensitivity 75,0 %). All 140 PCR-negative animals also demonstrated negative results in bacterial culture (specificity 100,0 %) (Table 4). The clinically determined foot rot score (scored from 0 to 5) was recorded from 631 sheep. Of these, 566 (89,7 %) animals had no clinical foot rot (score 0) and 65 (10,3 %) had foot rot scores ≥1, ranging from interdigital dermatitis to underrunning of the claw horn and loosening of the horn capsule. A positive PCR result was found in 2,1 % of sheep with foot rot score 0, in 24,1 % of sheep with foot rot score 1 and in at least 80,0 % of sheep with a foot rot score ≥2 (Table 4). Overall, 41,3 % (n = 19) of the participating farms had lame sheep in their flock and 7,0 % (n = 58) of the entire examined study population were lame. Of the 585 sheep with clinically healthy claws (score 0), 553 (94,5 %) were not lame at clinical examination. In 14 (77,8 %) of 18 sheep with a lameness score ≥3 underrunning of the claw horn (score ≥3) was present. The presence of lameness (C_{korr} = 0,71; p < 0,001) and lameness scores (C_{korr} = 0,78; p < 0,001) correlated strongly and significantly with the present of clinical foot rot. Further, PCR results significantly correlated with lameness (C_{korr} = 0,71; p < 0,001) as well as the lameness score (C_{korr} = 0,72; p < 0,001; Table 4).

Sample pooling

A total of 35 pools with five samples per pool (pool-of-five) included 35 individual *aprV2*-positive samples, with each pool containing at least one positive sample. All pool-of-fives were PCR-positive. When the C_t-values of individual samples and a pool-of-five were compared, the C_t-values of the individual samples were 1,5 lower (p < 0,05) and they significantly correlated, albeit weakly, with the pool-of-five (r = 0,342; Ckorr = 0,117; p < 0,05). Further investigation of undiluted samples and their dilutions are presented in Table 5. The 1:5 dilutions of all positive samples were still confirmed as positive samples. Even higher dilutions of positive samples could be detected as positive in 95,4 % (1:7) and 90,9 % (1:10) dilutions.

Two different mastermixes were used and there were no significant statistical differences between them (p = 0,08). However, the Quanta Perfecta qPCR ToughMix revealed a tendency to produce higher C_t values than the KAPA PROBE FAST qPCR Master Mix (2x) Universal. The mean C_t-value rose slightly after dilution. The C_t-value of all dilution levels strongly and significantly correlated with C_t-values of undiluted samples (Table 5).

Table 5: Comparison of undiluted and diluted Dichelobacter nodosus-positive real-time PCR samples of *aprV2*-positive swap samples taken from the interdigital space or from the edge of foot root lesions in sheep.

	descriptive			Correlation with undiluted		
KAPA universal[a]	**N[c]**	**mean Ct-value**	**sd**	**r**	**Ckorr**	**p-value**
undiluted	21	30,2	4,1	1		
1:5	19	31,2	3,0	0,89	0,79	<0,001
1:7	10	32,2	3,4	0,96	0,92	<0,001
1:10	12	32,2	3,1	0,87	0,76	<0,001
Quanta perfecta qPCR ToughMix [b]						
undiluted	17	31,9	4,5	1		
1:5	16	32,6	3,8	0,87	0,76	<0,001
1:7	11	32,2	3,0	0,74	0,55	<0,001
1:10	15	33,4	3,6	0,83	0,70	<0,001

[a]Master mix Kappa universal (Sigma-Aldrich, USA), [b]Master mix Quanta perfecta qPCR ToughMix (Quantabio, USA); [c]N = number of samples; sd = standard deviation; r = Pearson correlation coefficient; Ckorr = Pearson contingency coefficient.

Discussion

The 30,65 % prevalence of *D. nodosus* at the flock level found in this study is comparable to other countries. Germany had 42,93 % positive flocks, although prevalence at the flock level in Switzerland was much lower at 16,2 % for virulent and 2,8 % for benign *D. nodosus* strains.[2,23] Sweden reported an even lower *D. nodosus* prevalence of 9,1 % at the flock level.[20] A within-flock prevalence of 36 ± 31 % (min = 0,5 %; max = 100 %) with high variability was determined in Germany compared with our study, which had an intra-flock prevalence of 10,0 % (min. = 0,7 %; max. = 34,8 %).[23] In addition, in our study, 5,52 % PCR- positive samples were detected at the animal level. Sweden recorded a similar prevalence of 5,7 % at the animal level.[20] In contrast, Switzerland had 16,9 % positive virulent *D. nodosus* at the animal level.[2]

Nevertheless, cross-comparison of the various studies on foot rot prevalence is often difficult because of alternative definitions. For example, case definitions (clinic vs. pathogen detection), study designs and study populations are often differently interpreted.[26] In this study 10,2 % of sheep had only a clinical foot rot score ≥1. Animals with a foot rot score of 2 or higher are considered positive for foot rot in Sweden. For example, some studies reported a clinical prevalence of foot rot at the animal level of 5,8 % in 2011 and 0,7 % in 2023.[15,20] If animals with a foot rot score of 1 were counted as negative in our study, then we would obtain a comparable prevalence of 5,7 % for clinical foot rot. Alternatively, 13,8 % of the examined population from the study by Storms et al. from 2021 had a clinical foot rot score ≥2 and 53,2 % had a clinical foot rot score ≥1.[23] Further, husbandry conditions vary in the different countries. One reason for the low incidence of clinical foot rot in our study could be that the samples were taken during the first three month of the year, when all sheep in Tyrol are housed in sheds. The most severe and most common clinical foot rot lesions are usually found during the pasture period and during the wet season.[1,4,8] The sampling time of the present study was chosen due to the regular testing of sheep performed by many farms in early spring, before the animals are brought to alpine pastures, to assure a high number of participating herds. This might have caused an underestimation of the disease prevalence to a certain extend. Bacterial culture had a sensitivity of 75,0 % and a specificity of 100,0 % compared with PCR (C_{korr} = 0,91; p < 0,001). Therefore, only one in four *D. nodosus*-positive animals were recognized as positive bacteriologically. From these results it can be deduced that plated bacterial growth is an indication of infection of the IDS. Conversely, negative findings from the bacteriology laboratory do not mean absence of *D. nodosus.* For this reason, and additional resources required, plate culture is not recommended for the diagnosis of *D. nodosus* infection. The occurrence of clinical foot rot (C_{korr} = 0,86; p < 0,001) and the farm category (C_{korr} = 0,89; p < 0,001) agreed well with the PCR results obtained. Deviations between clinical foot rot and PCR outcomes could be found in animals with a clinical foot rot score of 1, where only 24,1 % of animals were positive using PCR and in two animals with clinical foot rot scores of 2 and 3. This finding might be due to another cause of claw disease, such as OID, which can be mistaken for foot rot at its early stages, or because of the lower sensitivity of 93,8 % using the four-feet-samples.[8,11] Conversely *D. nodosus* was detected in clinically normal animals. This could be explained by subclinical infection, or reflect good farm management.[26] However, the impact of the latter was not examined in detail in our study. In the study from Frost et al. 2015 it was reported that the clinical flock status is a good tool for providing an objective overview of the severity of foot rot in a flock.[7] Furthermore, the course of the infection process can be displayed better in control programs when this scoring system is used. Category 2 could be dispensed with in the case of clinical flock status since no farm fitted this category in our study.

We also observed that several clinical foot rot scores were found on a farm with clinical infection in many animals. The presence of lameness in a flock can be an indication of foot rot on the farm (C_{korr} = 0,71; p < 0,001). In our study 67,2 % of lame animals were also positive for *D. nodosus,* according to PCR (Table 4). The presence of lame sheep also gives an indication of the general claw health of the flock.[24] For example, 32,8 % of lame sheep had some alternative cause of lameness, such as white line disease, separation of the claw wall or interdigital dermatitis.

To decrease the time and cost of diagnosing *D. nodosus*, four-foot-samples were pooled in a pool-of-five or pool-of-ten for risk-based (Table 2) sampling.[11] In our study, we were able to achieve comparable results with the pool-of-five as with the individual samples. Conversely, the pool-of-ten or pool-of-five that are not taken for risk-based analysis are considered impractical due to the high loss of sensitivity.[11] The 1,5 higher C_t-value in the pool-of-five can be explained by dilution of the positive samples with the negative ones. Increases in the mean C_t-value was also observed in further pooled experiments. When performing the pool test, increased susceptibility to errors could be determined with the 1:7 dilutions (sensibility = 95,4 %) and 1:10 dilutions (sensibility = 90,9 %). The 1:5 dilution showed a sensibility of 100 %. This was true for samples with high C_t values in the undiluted state and could be the reason for the lower sensitivity. However, it should be noted that the different sensitivities of the different dilutions or the undiluted samples were not statistically significantly different (p = 0,81). The two master mixes compared showed no statistically significant difference in their results. However, higher C_t -values tended to be achieved with the Quanta Perfecta qPCR ToughMix. This is poten-

tially important as it could lead to negative results if the positive sample is diluted even further or if the samples are weakly positive.

Overall, it can be concluded that the flock level prevalence of *D. nodosus* from this study in western Austria is comparable to other countries. At the individual animal level, we were able to demonstrate a lower prevalence than in other countries. Further, we were able to show that the clinical assessment can give a good overview of the health of the animals regarding foot rot; and the foot rot scores available show a high statistical agreement with PCR results. However, PCR detection of the pathogen is essential for a reliable diagnosis, especially in animals with discrete claw lesions, and remains a tool of choice to establish and promote control strategies. In this study, risk-based pools-of-five can be used to save costs and materials.

Moreover, this study provides an overview of foot rot in Tyrolean sheep flocks and outlines the possibilities and limitations of the various diagnostic tools for *D. nodosus*. We suggest that further studies to document the importance of relevant influencing factors, such as the use of alpine pastures, management factors, biosecurity and others pertaining to foot rot are necessary for effective design of future control strategies and control programs.

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Conflict of interest

This research did not receive any grant from public, commercial or not-for-profit funding agencies.

Prévalence de *Dichelobacter nodosus* dans les troupeaux de moutons de l'ouest de l'Autriche: Comparaison des cultures bactériennes, du piétin clinique et de la boiterie avec la PCR et analyse d'un pool d'échantillons pour le diagnostic par PCR

Le piétin ovin est une maladie des onglons hautement contagieuse et multifactorielle, causée par *Dichelobacter nodosus (D. nodosus)* qui constitue la principale cause de boiterie chez les ovins. L'objectif de cette étude transversale était de déterminer la prévalence de *D. nodosus* dans l'ouest de l'Autriche, tant au niveau de l'animal que de l'exploitation.

La PCR en temps réel a été évaluée en comparaison avec les examens cliniques et bactériologiques effectués à partir d'écouvillons des espaces interdigités pour détecter les animaux infectés par *D. nodosus*. En outre, l'utilisation d'un pool d'écouvillons des quatre membres pour détecter le piétin a été déterminée. Au cours de l'étude, un total de 3156 moutons provenant de 124 fermes ont été examinés pour détecter des boiteries et des signes cliniques de piétin.

La prévalence de *D. nodosus* dans les troupeaux était de 30,65 %, la culture bactérienne montrant une sensibilité de 75 % et une spécificité de 100 % ($p < 0,001$), respectivement, par rapport à la PCR. En outre, les scores cliniques de piétin (Ckorr = 0,87; $p < 0,001$) et les scores de boiterie (Ckorr = 0,71; $p < 0,001$) étaient fortement corrélés avec la détection de *D. nodosus* par PCR. Les résultats montrent que l'examen clinique peut être utilisé pour identifier les animaux infectés par *D. nodosus* dans les troupeaux mais que la PCR doit être utilisée pour confirmer le diagnostic. *D. nodosus* a pu être détecté aussi bien avec des pools de cinq échantillons basés sur le risque qu'avec des échantillons non dilués ($p < 0,001$), ce qui suggère qu'un pool de cinq échantillons pourrait être une méthode appropriée et rentable pour détecter *D. nodosus* dans les troupeaux de moutons.

Cette étude donne un aperçu du piétin dans les troupeaux de moutons tyroliens et souligne les possibilités et les limites des différents outils de diagnostic pour *D. nodosus*. D'autres études visant à examiner les facteurs d'influence possibles, y compris les pâturages alpins, les facteurs de gestion et la biosécurité prédisposant au piétin, sont nécessaires pour la conception de futurs programmes de contrôle efficaces dans les régions alpines.

Mots clés: *Dichelobacter nodosus*, piétin, prévalence dans les troupeaux, PCR, ovins

Prevalenza di *Dichelobacter nodosus* negli allevamenti di pecore dell'Austria occidentale: confronto tra colture batteriche, pododermatite infettiva clinica e zoppia con PCR e analisi del raggruppamento campioni per la diagnosi mediante PCR

Lo scopo di questo studio trasversale era di determinare la prevalenza di *D. nodosus* nell'Austria occidentale sia a livello del singolo animale che a livello dell'allevamento.

La PCR in tempo reale è stata valutata in confronto con indagini cliniche e batteriologiche su tamponi interdigitali per rilevare gli animali affetti da *D. nodosus*. Inoltre, è stata determinata l'efficacia dell'uso di tamponi per quattro arti raggruppati per rilevare la pododermatite infettiva. Nel corso dello studio sono stati esaminati un totale di 3156 pecore provenienti da 124 allevamenti per zoppia e segni clinici di pododermatite infettiva.

La prevalenza dell'infezione da *D. nodosus* nell'allevamento è stata del 30,65 %, con una sensibilità della coltura batterica del 75,0 % e una specificità del 100,0 % ($p < 0,001$) rispettivamente, confrontata con la PCR. Inoltre, i punteggi di pododermatite infettiva clinica (Ckorr = 0,87; $p < 0,001$) e i punteggi di zoppia (Ckorr = 0,71; $p < 0,001$) sono risultati altamente correlati con il rilevamento di *D. nodosus* mediante PCR. Il risultato ha mostrato che l'esame clinico può essere utilizzato per identificare gli animali infetti da *D. nodosus* nelle greggi, ma la PCR deve essere utilizzata per confermare la diagnosi. *D. nodosus* è stato rilevato altrettanto bene con campioni raggruppati a cinque come con campioni non diluiti ($p < 0,001$), suggerendo che un raggruppamento a cinque campioni potrebbe essere un metodo adatto ed economicamente efficace per rilevare *D. nodosus* negli allevamenti di pecore.

Questo studio fornisce una panoramica della pododermatite infettiva negli allevamenti di pecore tirolesi e delinea le possibilità e i limiti dei vari strumenti diagnostici per rilevare la presenza di *D. nodosus*. Ulteriori studi per indagare possibili fattori influenti, tra cui il pascolo alpino, fattori di gestione e predisposizione alla pododermatite infettiva della biosicurezza, sono necessari per la progettazione di futuri programmi di controllo efficaci nelle regioni alpine.

Parole chiave: *Dichelobacter nodosus,* pododermatite infettiva, prevalenza nel gregge, PCR, pecore

Prevalence of Dichelobacter nodosus in western Austrian sheep flocks: Comparison of bacterial cultures, clinical foot rot and lameness with PCR and analysis of sample pooling for PCR diagnosis

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