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Simultaneous Detection and Discrimination of Virulent and Benign *Dichelobacter nodosus* in Sheep of Flocks Affected by Foot Rot and in Clinically Healthy Flocks by Competitive Real-Time PCR

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Ovine foot rot caused by *Dichelobacter nodosus* is affecting sheep worldwide. The current diagnostic methods are difficult and cumbersome. Here, we present a competitive real-time PCR based on allelic discrimination of the protease genes *aprV2* and *aprB2*. This method allows direct detection and differentiation of virulent and benign *D. nodosus* from interdigital skin swabs in a single test. Clinically affected sheep harbored high loads of only virulent strains, whereas healthy sheep had lower loads of predominantly benign strains.

Dichelobacter nodosus is the essential pathogen involved in the multifactorial disease of ovine foot rot. The Gram-negative bacterium causes painful inflammation and necrotizing lesions of the interdigital skin, a characteristic odor, and undermining of the hoof wall resulting in severe lameness in more advanced cases (1, 2). The clinical presentation depends on the virulence of the *D. nodosus* strain involved and on environmental factors. Diagnosis by cultivation and subsequent typing of this fastidious anaerobe is difficult, laborious, and not routinely done in diagnostic laboratories, and it is often not fast enough to implement the necessary measures to segregate infected animals and prevent the disease from spreading (3). Moreover, the gelatin-gel and elastase assays to test for protease activity, traditionally carried out to detect and differentiate virulent and benign *D. nodosus* strains, is difficult to perform and is strongly dependent on the quality of the growth medium and hence may yield inconsistent results (4). PCR-based approaches have markedly improved sensitivity and speed of detection of *D. nodosus* (3, 5–9). However, a method of fast, reliable and, ideally, simultaneous detection and virulotyping does to date not exist.

The acidic protease 2 (AprV2) has been identified as a key virulence factor of *D. nodosus* (10). In virulent strains, the *aprV2* gene encodes a thermostable protease involved in foot rot tissue damage, whereas benign strains contain the homologous gene *aprB2* encoding a thermolabile protease. The *aprV2* and *aprB2* alleles differ by a 2-bp substitution, TA/CG, at position 661/662 (11). These single nucleotide polymorphisms (SNPs) result in a distinct amino acid change (Tyr92Arg), defining elastase activity in the mature protein (10). We have recently shown by sequencing that these SNPs in *aprV2*/*B2* fully correlated with the clinical status of the individual sheep or the foot rot history of the herd, and the TA and CG nucleotides were consistent with the virulent and the benign *D. nodosus* strains, respectively (12). In contrast, other SNPs in the *aprV2*/*B2* and in the protease genes *aprV5*/*B5* and *bprV*/*B* showed no consistent relation to strain virulence (12).

To rapidly assess infections by virulent or benign *D. nodosus*, we developed a competitive real-time PCR method permitting simultaneous detection and allelic discrimination of *aprV2* and *aprB2* directly from clinical samples. One primer pair and two 3′-minor groove binder (MGB) probes allowing allelic discrimination were designed using Primer Express software v 3.0 (Table 1). The two probes DnAprTM-vMGB (FAM) and DnAprTM-bMGB (VIC) cover the SNPs and are specific for *aprV2* and *aprB2*, respectively. This kind of approach is rarely applied to bacteria but has several advantages, especially in the case of *D. nodosus*. First, in the absence of the specific target but in the presence of a highly similar target, PCR detection and discrimination based on mismatches could lead to false-positive results. This happens when either benign or virulent *D. nodosus* (or even both simultaneously) is present in sheep populations and even in individual animals (13). With the use of two probes competitively, the specific probe will prevent the nonspecific one from binding, thereby raising the overall specificity of the assay. Second, this PCR method allows detection of both the virulent and the benign allele in a single reaction, indicating the presence of either one or both, including their relative amounts. Optimized assay conditions consisted of a 25-μl reaction mixture containing 1× TaqMan Genotyping MasterMix (Applied Biosystems, Foster City, CA), 300 nM primers, 100 nM DnAprTM-vMGB and 250 nM DnAprTM-bMGB, pyrogen-free water, and 2.5 μl of DNA template (Table 1). Amplification was done in a 7500 Real-Time PCR-System instrument (Applied Biosystems), using cycles of 2 min at 50°C and 10 min at 95°C followed by 40 cycles with 15 s at 95°C and 1 min at 60°C. Results were analyzed using the Sequence Detector 7500 software (v 2.0.5.) with the threshold set at 0.015. Samples showing no probe-specific fluorescent signal were considered to be negative ($C_T = 40$), and samples resulting in probe-specific fluorescent signals were defined as being positive ($C_T < 40$). Purified genomic DNA samples from the *D. nodosus* type

Received 16 December 2013 **Returned for modification** 11 January 2014
Accepted 15 January 2014
Published ahead of print 22 January 2014
Editor: E. Munson
Address correspondence to Peter Kuhnert, peter.kuhnert@vetsuisse.unibe.ch.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.03485-13.

doi:10.1128/JCM.03485-13
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TABLE 1 Primers and probes for allelic discrimination of *aprV2/B2*

Target gene	Primer/probe name	Sequence (5′ to 3′)[c]	Position (nucleotides)
aprV2/B2	DnAprTM-L[a,b]	CAATAGCCAAATTTCTTTAGATGGTGAT	573–600
aprV2/B2	DnAprTM-R[a,b]	CAAGAGCTGTCGCTTCTTTCTTT	676–698
aprV2	DnAprTM-vMGB[a]	FAM-CGGTGGTTATCCTGAT-MGB	654–669
aprB2	DnAprTM-bMGB[b]	VIC-TGGTCGTCCTGATC-MGB	657–670

[a] Accession number L38395.
[b] Accession number FN674446.1.
[c] Single nucleotide polymorphisms used for allelic discrimination are underlined.

strain ATCC 25549^T for the virulent genotype *aprV2* and from the Norwegian field strain Nor11B for the benign genotype *aprB2* (14) were used as reference strains for assay optimization and as controls. Pyrogen-free water was applied to DNA and clinical sample preparation and also used as a nontemplate control.

The approximate detection limit and reproducibility of the *aprV2/B2* real-time PCR assay were determined using 10-fold serial dilutions of DNA from reference strains ATCC 25549^T and Nor11B. Mean C_T values were plotted against DNA concentrations on a log scale and the efficiency (E) was calculated based on the slope of the resulting regression curve using the formula $E = (10^{-1/\text{slope}} - 1) \times 100$.

For the virulent strain ATCC 25549^T, positive signals from the *aprV2*-specific probe but not from the *aprB2*-specific probe were observed. The opposite was true for the benign isolate Nor11B. The assay therefore allows discrimination of *D. nodosus* virulotypes. Linearity over a tested range of six 10-fold dilutions was high, with R^2 values of 0.992 and 0.971, as were the calculated efficiencies of 100% and 92% with ATCC 25549^T (*aprV2*) and Nor11B (*aprB2*), respectively. The detection limit, as calculated based on the DNA concentration of the type strain and the known genome size, was around 10 genome equivalents per reaction.

Using sequence data from the study of Stäuble et al. (12) as a gold standard to further test the performance of the competitive real-time PCR, the latter correctly identified all 45 samples containing the *aprV2*, and all 19 field samples containing the *aprB2* allele were properly recognized (see Table S1 in the supplemental material). No false positives or false negatives were detected, and thus the assay proved to be 100% specific and 100% sensitive, and it correctly identified virulent and benign *D. nodosus*.

One hundred samples from flocks affected by foot rot, 92 samples from flocks not affected, and 4 samples from a farm with flocks of unclear status were collected from 16 farms in Switzerland, 2 farms in Germany, and 8 farms in France (see Table S1 in the supplemental material). They originated from affected flocks with a spectrum of disease severity (indicated by foot rot scores from 1 to 5) and from nonaffected flocks with healthy sheep (score 0), based on the Australian scoring system of Stewart and Claxton (15), whereby flock diagnosis is based on the most severe lesions present. Analogously, scores per sheep were determined by the highest-rated foot, and a sample from this foot was taken for subsequent analysis. Specimens were taken from the interdigital skin and, if present, from the margin of a lesion by a cotton swab. This turned out to be better than sampling by biopsy punch or by ring curette (data not shown). Cotton swabs were directly transferred into 1 ml SV lysis buffer (4 M guanidine thiocyanate, 0.01 M Tris-HCl [pH 7.5], and 1% β-mercaptoethanol), soaked for 30 to 60 s, and then removed. Samples in this buffer can be stored for several days without cooling, thus permitting easy transport to the laboratory for further processing using a quick DNA extraction

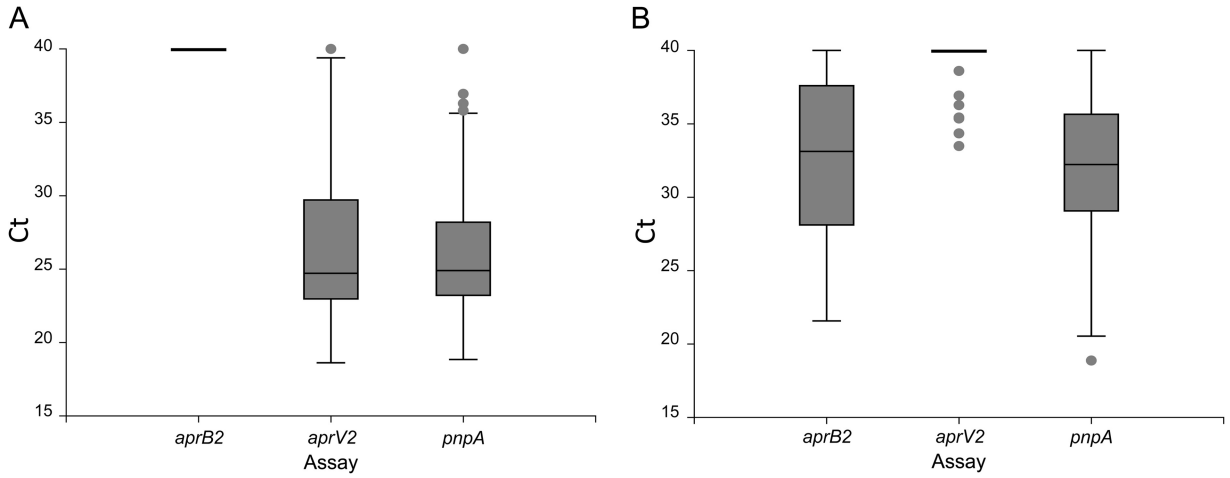

FIG 1 Comparison of C_T values and the distributions of on clinical sample results from the *aprV2/B2* and the *pnpA* real-time PCR assays. Box-whisker plots of C_T values of field samples from clinically affected ($n = 100$; panel A) and nonaffected flocks ($n = 92$; panel B) are shown (*pnpA* assay detecting the presence of *D. nodosus* without distinction of virulence, *aprV2* assay detecting virulent, and *aprB2* detecting benign *D. nodosus* strains). The median and the upper and the lower quartiles are given in the box. The other two quartiles are indicated by lines and the outliers by dots (defined as values 1.5 times below or above the interquartile range). Based on the Kruskal-Wallis test, there were no significant differences ($P < 0.05$) between C_T values from *pnpA* and *aprV2* or *aprB2* within the groups of affected or nonaffected flocks, respectively. However, C_T values are significantly lower in the group of affected than in the group of nonaffected flocks.

TABLE 2 Mean and median C_T values of real-time PCR results at the flock and animal levels

Category (*n*)	C_T values for:					
	aprV2		*aprB2*		*pnpA*	
	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
Flocks affected (100)	26.1 (4.8)	24.5	40.0 (0.0)	40.0	25.8 (4.5)	24.7
Flocks healthy (92)	39.7 (1.2)	40.0	32.8 (5.4)	32.9	31.7 (4.8)	31.0
Animal score 0 (105)	37.2 (5.5)	40.0	35.0 (5.3)	36.6	31.4 (5.0)	31.7
Animal score 1 (9)	25.0 (5.4)	22.7	40.0 (0.0)	40.0	24.7 (5.6)	23.2
Animal score 2 (16)	25.8 (5.2)	24.5	40.0 (0.0)	40.0	25.4 (5.2)	24.3
Animal score 3 (10)	23.2 (2.6)	21.9	40.0 (0.0)	40.0	23.4 (3.8)	22.3
Animal score 4 (17)	27.4 (4.0)	27.4	40.0 (0.0)	40.0	26.4 (3.6)	25.4
Animal score 5 (12)	28.0 (5.5)	26.5	40.0 (0.0)	40.0	27.0 (4.6)	26.6

method (12). The 196 samples were tested for the presence of virulent and benign *D. nodosus* with the competitive real-time PCR, and the results were compared to results from the nondiscriminatory *pnpA*-based real-time PCR assay, which was used to estimate the load of *D. nodosus* in the samples (12).

All of the samples from affected flocks tested positive for the virulent genotype *aprV2* but were negative for *aprB2*. Two samples from a French flock with foot abscesses but unclear foot rot status tested positive for the virulent genotype, and this result was confirmed by sequencing. The assay detected the virulent genotype in animals of affected flocks without clinical symptoms at the time of sampling. Therefore, a simple swabbing of the interdigital skin in clinically healthy sheep may contain high concentrations of virulent *D. nodosus* readily detectable at this early stage by the new assay. This is consistent with histological studies of bacterial invasion revealing the highest *D. nodosus* cell load in early onset cases, which might not be clinically evident at this point (16, 17). These findings underline the fact that scoring systems and clinical assessments do not necessarily correlate with bacterial load and *D. nodosus* virulotype.

In nonaffected flocks, over 80% of the samples tested positive for the benign genotype *aprB2*, as they also did in the *pnpA* assay. Three samples were negative, as in the *pnpA* assay. Nine others were negative for *aprV2* and *aprB2*, although they were weakly positive in the *pnpA* assay. Seven samples were positive for *aprV2*, however, with high C_T values indicating small amounts of virulent *D. nodosus*. Four of these showed positive results only for *aprV2*. The three others were positive for both *aprV2* and *aprB2*, indicating the presence of both virulent and benign *D. nodosus*. Such mixed infections were observed only in animals from two flocks out of the 8 designated "nonaffected" (farms 9 and 15). In these flocks, other samples with only benign and three with only virulent *D. nodosus* were also found. These two flocks had recurrent problems with foot rot during the grazing period following sampling.

There were good correlations of C_T values based on *pnpA* and those of the allele-specific *aprV2*/*aprB2* assay (Fig. 1 and Table 2). Generally, C_T values of animals from affected farms were clearly below 30, whereas those from nonaffected flocks were above 30, indicating that nonaffected animals had a much smaller quantity of *D. nodosus*. An exception was flock 11, which had low C_T values of around 25 for *aprB2* only, indicating a high bacterial load of benign *D. nodosus*. The owner bandaged the feet of the sampled sheep due to slight lameness and very mild interdigital redness and exudates. We assume that this created favorable conditions for the growth of *D. nodosus*. This case shows that pure cultures of benign *D. nodosus* at high loads may lead to a milder form of disease that is clearly detectable by the competitive real-time PCR. There were no significant differences in C_T values of the samples with scores of 1 to 5 (Table 2), indicating that once the disease is clinically evident loads of *D. nodosus* are independent of the severity of the disease.

In conclusion, we present a rapid and sensitive diagnostic tool for early detection and virulotyping of *D. nodosus* directly from simple, noninvasive interdigital swabs of sheep. This assay will help to elucidate the epidemiology of *D. nodosus* and support efforts to combat the disease.

ACKNOWLEDGMENTS

This work was supported by grant 1.13.04 from the Swiss Federal Veterinary Office.

We thank Marianne Gilhuus (Oslo, Norway), Dominique Bérgonier (Toulouse, France), Martin Ganter (Hannover, Germany), and Hendrik Müller (Leipzig, Germany) for the provision of valuable strains and samples. We are grateful to Sibylle Bürki, Amandine Ruffieux, and Lea Normand (Institute for Veterinary Bacteriology, Bern, Switzerland) for excellent technical support. We also acknowledge R. Lüchinger, G. Bearth, R. Hanimann, J. Eitel, and C. Haerdi, who supported the study in Switzerland. We are particularly grateful to Ruth Parham for linguistic revision of the manuscript and to Marcus Doherr for help with statistical analysis.

REFERENCES

1. **Egerton JR, Roberts DS, Parsonson IM.** 1969. The aetiology and pathogenesis of ovine foot-rot. I. A histological study of the bacterial invasion. J. Comp. Pathol. **79:**207–215.
2. **Kennan RM, Han X, Porter CJ, Rood JI.** 2011. The pathogenesis of ovine footrot. Vet. Microbiol. **153:**59–66. http://dx.doi.org/10.1016/j.vetmic.2011.04.005.
3. **Wani SA, Samanta I.** 2006. Current understanding of the aetiology and laboratory diagnosis of footrot. Vet. J. **171:**421–428. http://dx.doi.org/10.1016/j.tvjl.2005.02.017.
4. **Dhungyel OP, Hill AE, Dhand NK, Whittington RJ.** 2013. Comparative study of the commonly used virulence tests for laboratory diagnosis of ovine footrot caused by *Dichelobacter nodosus* in Australia. Vet. Microbiol. **162:**756–760. http://dx.doi.org/10.1016/j.vetmic.2012.09.028.
5. **Belloy L, Giacometti M, Boujon P, Waldvogel A.** 2007. Detection of *Dichelobacter nodosus* in wild ungulates (*Capra ibex ibex* and *Ovis aries musimon*) and domestic sheep suffering from foot rot using a two-step polymerase chain reaction. J. Wildl. Dis. **43:**82–88. http://dx.doi.org/10.7589/0090-3558-43.1.82.
6. **Moore LJ, Wassink GJ, Green LE, Grogono-Thomas R.** 2005. The detection and characterisation of *Dichelobacter nodosus* from cases of ovine footrot in England and Wales. Vet. Microbiol. **108:**57–67. http://dx.doi.org/10.1016/j.vetmic.2005.01.029.
7. **La Fontaine S, Egerton JR, Rood JI.** 1993. Detection of *Dichelobacter*

nodosus using species-specific oligonucleotides as PCR primers. Vet. Microbiol. **35:**101–117. http://dx.doi.org/10.1016/0378-1135(93)90119-R.

8. **Frosth S, Slettemeas JS, Jørgensen HJ, Angen O, Aspán A.** 2012. Development and comparison of a real-time PCR assay for detection of *Dichelobacter nodosus* with culturing and conventional PCR: harmonisation between three laboratories. Acta Vet. Scand. **54:**6. http://dx.doi.org/10.1186/1751-0147-54-6.
9. **Dhungyel OP, Whittington RJ, Egerton JR.** 2002. Serogroup specific single and multiplex PCR with preenrichment culture and immunomagnetic bead capture for identifying strains of *D. nodosus* in sheep with footrot prior to vaccination. Mol. Cell Probes **16:**285–296. http://dx.doi.org/10.1006/mcpr.2002.0427.
10. **Kennan RM, Wong W, Dhungyel OP, Han X, Wong D, Parker D, Rosado CJ, Law RH, McGowan S, Reeve SB, Levina V, Powers GA, Pike RN, Bottomley SP, Smith AI, Marsh I, Whittington RJ, Whisstock JC, Porter CJ, Rood JI.** 2010. The subtilisin-like protease AprV2 is required for virulence and uses a novel disulphide-tethered exosite to bind substrates. PLoS Pathog. **6:**e1001210. http://dx.doi.org/10.1371/journal.ppat.1001210.
11. **Riffkin MC, Wang LF, Kortt AA, Stewart DJ.** 1995. A single amino-acid change between the antigenically different extracellular serine proteases V2 and B2 from *Dichelobacter nodosus*. Gene **167:**279–283. http://dx.doi.org/10.1016/0378-1119(95)00664-8.
12. **Stäuble A, Steiner A, Normand L, Kuhnert P, Frey J.** 2014. Molecular genetic analysis of *Dichelobacter nodosus* proteases AprV2/B2, AprV5/B5 and BprV/B in clinical material from European sheep flocks. Vet. Microbiol. **168:**177–184. http://dx.doi.org/10.1016/j.vetmic.2013.11.013.
13. **Jelinek PD, Depiazzi LJ, Galvin DA, Spicer IT, Palmer MA, Pitman DR.** 2000. Occurrence of different strains of *Dichelobacter nodosus* in new clinical lesions in sheep exposed to footrot associated with multi-strain infections. Aust. Vet. J. **78:**273–276. http://dx.doi.org/10.1111/j.1751-0813.2000.tb11756.x.
14. **Gilhuus M, Vatn S, Dhungyel OP, Tesfamichael B, L'Abée-Lund TM, Jørgensen HJ** 2013. Characterisation of *Dichelobacter nodosus* isolates from Norway. Vet. Microbiol. **163:**142–148. http://dx.doi.org/10.1016/j.vetmic.2012.12.020.
15. **Stewart DJ, Claxton PD.** 1993. Ovine foot rot: clinical diagnosis and bacteriology, p 1–27. *In* Corner LA, Bagust TJ (ed), Australian standard diagnostic techniques for animal diseases. CSIRO Publications, Victoria, Australia.
16. **Calvo-Bado LA, Green LE, Medley GF, Ul-Hassan A, Grogono-Thomas R, Buller N, Kaler J, Russell CL, Kennan RM, Rood JI, Wellington EM.** 2011. Detection and diversity of a putative novel heterogeneous polymorphic proline-glycine repeat (Pgr) protein in the footrot pathogen *Dichelobacter nodosus*. Vet. Microbiol. **147:**358–366. http://dx.doi.org/10.1016/j.vetmic.2010.06.024.
17. **Witcomb L.** 2012. The in situ analysis of the microbial community associated with footrot of sheep. PhD thesis. University of Warwick, Coventry, United Kingdom.