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Molecular genetic analysis of *Dichelobacter nodosus* proteases AprV2/B2, AprV5/B5 and BprV/B in clinical material from European sheep flocks

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ABSTRACT

Dichelobacter nodosus, the etiological agent of ovine footrot, exists both as virulent and as benign strains, which differ in virulence mainly due to subtle differences in the three subtilisin-like proteases AprV2, AprV5 and BprV found in virulent, and AprB2, AprB5 and BprB in benign strains of *D. nodosus*. Our objective was a molecular genetic epidemiological analysis of the genes of these proteases by direct sequence analysis from clinical material of sheep from herds with and without history of footrot from 4 different European countries. The data reveal the two proteases known as virulent AprV2 and benign AprB2 to correlate fully to the clinical status of the individuals or the footrot history of the herd. In samples taken from affected herds, the *aprV2* gene was found as a single allele whereas in samples from unaffected herds several alleles with minor modifications of the *aprB2* gene were detected. The different alleles of *aprB2* were related to the herds. The *aprV5 and aprB5* genes were found in the form of several alleles scattered without distinction between affected and non-affected herds. However, all different alleles of *aprV5* and *aprB5* encode the same amino acid sequences, indicating the existence of a single protease isoenzyme 5 in both benign and virulent strains. The genes of the basic proteases BprV and BprB also exist as various alleles. However, differences found in samples from affected *versus* non-affected herds do not reflect the currently known epitopes that are attributed to differences in biochemical activity. The data of the study confirm the prominent role of AprV2 in the virulence of *D. nodosus* and shed a new light on the presence of the other protease genes and their allelic variants in clinical samples.

1. Introduction

Ovine footrot is a highly infectious disease caused by the Gram-negative bacterium *Dichelobacter nodosus* (formerly known as *Bacteroides nodosus*). It is present in many countries and has recently been reported in Europe (Moore et al., 2005; Belloy et al., 2007; Zhou et al., 2010; König et al., 2011; Frosth et al., 2012; Gilhuus et al., 2013). This debilitating disease is considered to be one of the most important causes of lameness in sheep flocks (Wassink et al., 2003; Abbott and Lewis, 2005). Apart from the animal ethics issues evoked by the painful condition, the lameness is of considerable economic importance in the alpine area. Here, foraging involves major walking distances. As a consequence, the disease is responsible for losses in meat, wool

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and milk production, and it increases labour and management efforts relating to treatment and eradication (Nieuwhof and Bishop, 2005; Wani and Samanta, 2006; Green and George, 2008).

D. nodosus colonises the damaged interdigital skin and is found in large quantities in the superficial layers of the early footrot lesion (Egerton et al., 1969; Calvo-Bado et al., 2011; Witcomb, 2012). Macroscopically, the condition is characterised by necrotising inflammation of the interdigitial skin; a pasty foul smelling scum accumulates and necrotic separation of the horn wall from underlying tissue occurs (Green and George, 2008). Clinical presentations vary and are classified with different scoring systems (Stewart and Claxton, 1993; Conington et al., 2008). Although the disease has a wide spectrum of severity, generally three forms are recognised; benign, intermediate and virulent footrot (Stewart and Claxton, 1993). Farming management and favourable environmental factors influence the spread and progression of the disease (Depiazzi et al., 1998; Wassink et al., 2003). However, it is the nature of the causative bacterial strain, which is decisive for the initiation, and potential severity of an outbreak (Whittington, 1995; Kennan et al., 2010, 2011). A number of virulence factors have been identified in *D. nodosus*, as *e.g.* the virulence associated gene regions *vap* and *vrl* that are preferentially present in virulent strains and may therefore be indicators of virulence (Katz et al., 1991; Billington et al., 1996). Type IV fimbriae and extracellular proteases are essential for virulence of *D. nodosus* (Kennan et al., 2001, 2010). Fimbrial surface structures act as a bacterial antigen with corresponding classification into 10 serogroups (Claxton et al., 1983; Chetwin et al., 1991). Moreover, type IV fimbriae are virulence factors essential for twitching motility and cell adherence (Han et al., 2008) but also they are directly related to protease secretion *via* a type II secretion-like pathway that utilises the type IV fimbrial apparatus (Han et al., 2007).

Extracellular subtilisin-like serine proteases (or subtilases) are commonly produced as pre-pro-precursors in a wide variety of organisms such as bacteria, archaea, fungi and eukaryotes. They are activated extracellularly by cleavage of the non-catalytic N-terminal pre-pro region and the C-terminal domain. Most of them have a broad substrate specificity and are required for either defence or growth on protein-containing substrates (Siezen and Leunissen, 1997). This protein digesting process, as a source of amino acids and energy precursors, has also been postulated for *D. nodosus* (Myers et al., 2007). More importantly, however, the ability to produce subtilases is a key virulence factor in *D. nodosus* (Kennan et al., 2010; Wong et al., 2011; Han et al., 2012). Isolates are currently routinely distinguished by the elastase test first developed by (Stewart, 1979) and by the gelatine-gel test (Palmer, 1993); they measure quantitative elastase activity and protease thermostability, respectively. Virulent strains produce the more heat stable acidic proteases AprV2 and AprV5 and the basic protease BprV; more benign strains produce the less thermostable enzymes AprB2, AprB5 and BprB. AprV2 is essential for virulence as confirmed recently by construction of isogenic protease mutants of the virulent strain VCS1703A (Kennan et al., 2010).

Analysis of the mutants' phenotypic characteristics *in vitro* and in a pen based trial has confirmed that AprV2 is the major thermostable protease and is responsible for the overall elastase activity of virulent *D. nodosus* strains (Kennan et al., 2010). The mature virulent AprV2 protease differs from the benign AprB2 only by a single amino acid change (Tyr92Arg) (Riffkin et al., 1995) which results in important changes in the three-dimensional structure (Kennan et al., 2010). AprV5 is required for the activation both of itself and of the other two proteases (Han et al., 2012). BprV is significantly more efficient in degrading extracellular matrix components of the hoof horn than the benign BprB (Wong et al., 2011). The three proteases act synergistically (Kennan et al., 2010). Since the 1.4 Mb genome of *D. nodosus* has been fully sequenced (Myers et al., 2007) continuous research on virulence factors, invasion strategies and immunogenic potential has rapidly improved our understanding of the footrot pathogenesis. The introduction of PCR-based approaches has further improved the diagnosis of the disease; yet, the knowledge regarding epidemiology and characteristics of *D. nodosus* strains circulating in the sheep population is, at least in continental Europe, still somewhat limited.

In the present study we have analysed the alleles of the genes of the major extracellular virulent proteases AprV2, AprV5, BprV, and benign proteases AprB2, AprB5, and BprB of *D. nodosus* both from healthy and from footrot-affected sheep flocks in Switzerland, France, Germany and Norway.

2. Materials and methods

2.1. Origin of samples and strains

Within the framework of an epidemiological study on footrot a total of 1715 swab samples were collected from European sheep flocks, predominantly in 2011/2012. In Switzerland, sampling was carried out on 18 different farms. Of these, nine farms (1130 swab samples) were affected with footrot at the time of sampling, with at least one animal with clinical symptoms (a); nine other farms (248 swab samples) were not affected having had no animals with symptoms for at least two years (*n*) (Supplementary Table S1). In France, 17 farms were sampled in 2012. Thereof, nine farms (79 samples) were affected and seven farms were not affected (104 samples). One farm (farm H; 12 samples) was reported to be free from footrot but individuals were affected by foot abscess and hence the status was recorded as unclear (*u*) (Supplementary Table S1). In Germany, three farms were sampled in between 2007 and 2013. Thereof, one farm was not affected (10 samples) and two (131 samples) were affected by footrot at the time of sampling. Purified DNA from four Norwegian *D. nodosus* field isolates categorised as benign by the gelatine gel test (Palmer, 1993) with modifications (Moore et al., 2005; Belloy et al., 2007; Zhou et al., 2010; König et al., 2011; Frosth et al., 2012; Gilhuus et al., 2013) and purified DNA of *D. nodosus* type strain ATCC25549^T as a control for the virulent genotype were included in the study.