Ovine footrot: new insights into bacterial colonisation

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Ovine footrot is characterised by interdigital dermatitis (ID) and by the separation of the skin and hoof horn (under-running footrot). *Dichelobacter nodosus* is the essential pathogen causing footrot; the role of other microorganisms in this disease remains unclear. The aims of this study were (i) to investigate the colonisation of *D nodosus*, *Fusobacterium necrophorum* and *Treponema* species in biopsies from the ovine interdigital skin of healthy, ID and footrot-affected feet and (ii) to characterise the virulence of *D nodosus* strains in those biopsies. Postslaughter biopsy samples (n=241) were collected and analysed by real-time PCR to determine prevalence and load of the different bacterial species. The highest prevalence and load of *D nodosus* were found on feet with ID. The vast majority of samples contained virulent *D nodosus* and some samples contained both virulent and benign *D nodosus*. Notably, the more pathogenic subspecies of *F necrophorum* was found in samples from UK sheep. Our findings provide further insights into the role bacterial colonisation may play in the early stage of ID and in the progression towards footrot.

Ovine footrot is a major cause of lameness affecting sheep welfare worldwide (Goddard and others 2006); it is characterised by two different clinical presentations, interdigital dermatitis (ID) and under-running footrot. ID is an initial inflammation of the interdigital skin where the superficial epidermal layers are inflamed, damaged and slough off irregularly and it may develop into under-running footrot, which is characterised by the separation of the hoof horn from the sensitive underlying tissue (Beveridge 1941, Egerton and others 1969). In Australia, mild/benign footrot is also used synonymously for ID and under-running footrot is called virulent footrot (Raadsma and Dhungyel 2013).

Footrot is a complex disease with *Dichelobacter nodosus*, a Gram-negative anaerobic bacterium, as the essential pathogen causing under-running footrot (Egerton and others 1969, Kennan and others 2001, 2010, Han and others 2008). *D nodosus* load was found to be already increased in ID before the development of under-running footrot, thereby suggesting that *D nodosus* load drives the early stages of infection (Witcomb and others 2014, 2015). Additionally, the occurrence of this disease is associated with different factors such as the virulence of *D nodosus* strains (Kennan and others 2010), farm management (Green and others 2007), environmental conditions (Wassink and others 2005, Muzafar and others 2016) and initial damage in the epithelium of the interdigital skin (Beveridge 1941, Egerton and others 1969).

Whole-genome sequencing demonstrated that *D nodosus* has a global conserved bimodal population, correlating with virulent and benign phenotypes (Kennan and others 2014). A large number of virulent *D nodosus* strains were identified in Australia (Kennan and others 2014), while in Scandinavian countries, such as Sweden, mainly benign strains have been found (Frosth and others 2015). In UK flocks, virulent *D nodosus* was more prevalent than benign in swabs from sheep with ID and footrot (Moore and others 2005). Virulent and benign *D nodosus* strains differ in their ability to degrade the extracellular matrix of the host due to enzymatic activity of extracellular proteases (Rifkin and others 1995). The acidic protease AprV2 is responsible for the overall elastase activity of virulent strains and was shown to be essential for the development of footrot, while the acidic protease AprB2 is associated with a benign phenotype (Kennan and others 2010). Importantly, presence of virulent *D nodosus* strains does not always correlate with severity of clinical presentations since virulent *D nodosus* has also been identified in sheep without any clinical sign and in ID cases (Moore and others 2009, Stäuble and others 2014).

In addition, *Fusobacterium necrophorum* and *Treponema* species and a range of other bacterial genera have been identified in the ovine interdigital skin (Roberts and Egerton 1969, Bennett and others 2009, Calvo-Bado and others 2011, Frosth and others 2015). The role of *F necrophorum* in this disease still needs to be fully understood, with two hypothesis currently discussed: (i) *F necrophorum* is important to establish ID before *D nodosus* infection and hence initiates the disease (Egerton and others 1969) or (ii) *F necrophorum* is involved in the persistence and severity of footrot, once the under-running lesion has developed, playing a role as an opportunistic, secondary pathogen (Witcomb and others 2014, 2015). *F necrophorum* is divided into subspecies *necrophorum* and *funduliforme*, the first is described to be more pathogenic (Tan and others 1996).
T. pallidum pallidum has been reported in footrot lesions (Calvo-Bado and others 2011) and were found in a small number of samples across all clinical conditions (Table 1). In contrast, benign *D. nodosus* strains were detected in 15 per cent (36/241) of the samples (Fig 1b). This highlights the presence of virulent strains which contribute to the development of footrot. The prevalence of *D. nodosus*, *F. necrophorum*, Treponema species and eubacteria was investigated in the ovine interdigital skin biopsies. All samples were positive for eubacteria (100 per cent of prevalence). The total number of footrot samples were significantly more prevalent in mild ID (*P*<0.05 and <0.01, respectively), moderate/severe ID (*P*<0.001, respectively) and footrot (*P*<0.05 and <0.01, respectively) in comparison with healthy feet, with highest prevalence in moderate/severe ID samples. Moreover, total bacterial load was quantified using real-time PCR based on 16S rRNA gene for eubacteria (Frosth and others 2012) and the intergenic region spacers were identified using a multiplex PCR assay for *T. pallidum pallidum* (Frosth and others 2015). Real-time PCR for F. necrophorum and *D. nodosus* species-specific genes was carried out in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific).
subspecies. *F. necrophorum* was significantly more prevalent in footrot than in healthy feet (P<0.05) (Fig 1c). Presence of both *D. nodosus* and *F. necrophorum* in the same tissue sample or virulent *D. nodosus* and *F. necrophorum* in the same tissue was significantly higher in footrot compared with healthy feet (P<0.01 and <0.01, respectively). *Treponema* species prevalence was very low (8 per cent, 20/241) and similar across all clinical conditions (Fig 1d) (see online supplementary appendix 1).

Similar proportion of eubacterial DNA was detected at around 0.06 per cent±0.020 (mean±se of the mean) of total DNA for all samples, with 0.07 per cent±0.041 for healthy samples, 0.028 per cent±0.007 mild ID, 0.027±0.011 for moderate/severe ID and 0.073±0.039 for footrot samples. *D. nodosus* load was significantly higher in moderate/severe ID and footrot in comparison to healthy feet (P<0.001 for both) (Fig 2a). Virulent *D. nodosus* load was significantly increased in mild ID, moderate/severe ID and footrot compared with a healthy feet (P=0.001, P<0.0001 and F<0.0001, respectively), with highest load in moderate/severe ID (Fig 2b). *F. necrophorum* load was significantly increased in footrot but not in ID samples (P=0.022) (Fig 2c). The highest *Treponema* species load was found in footrot followed by healthy feet (Fig 2d).

In summary, while eubacterial loads were similar in all feet, both prevalence and load of total and virulent *D. nodosus* were highest in moderate-to-severe ID, while *F. necrophorum* were highest in footrot samples.

**Discussion**

In this study, the authors provided further insights into the bacterial colonisation present in healthy, ID and footrot ovine feet. The authors found similar patterns regarding the prevalence and load of *D. nodosus* and *F. necrophorum* in postslaughter biopsies from the interdigital space as previous studies in UK sheep flocks using swabs and biopsies (Moore and others 2005, Calvo-Bado and others 2011, Witcomb and others 2014, 2015).

As expected, the highest *D. nodosus* prevalence and load found in this study was in ID samples, hence supporting the hypothesis that *D. nodosus* drives the development of the early stages of footrot (Witcomb and others 2014, 2015). Interestingly, *D. nodosus* was found in a large proportion of biopsy samples from healthy feet (58 per cent, 46/79), suggesting it might be present in the stratum corneum (horny layer) but not necessarily causing disease. It is also possible that these visibly healthy feet might have had subclinical footrot and may have developed
under-running lesions in the following days. Risk factors for the development of under-running footrot in addition to the presence of virulent *D. nodosus* include poor foot conformation (Kaler and others 2010), superficial skin damage (Egerton and others 1969), sheep breed (Emery and others 1984) and presence of co-infecting bacteria such as *F. necrophorum* (Egerton and others 1969, Roberts and Egerton 1969).

In this study, the majority of *D. nodosus* present in the ovine interdigital skin biopsies were virulent strains. Similarly, high prevalence of virulent *D. nodosus* in the UK sheep was identified previously using gelatinase gel protease assay (Moore and others 2005). Therefore, these studies demonstrate that virulent strains are currently circulating in UK flocks. In contrast, in Sweden where under-running footrot is not endemic, most of the *D. nodosus* were found to be benign (Frosth and others 2015). The authors found a mixed population of benign and virulent *D. nodosus* strains in the same feet; a potential synergistic role of benign and virulent strains still needs to be investigated.

*F. necrophorum* prevalence and load were higher in footrot than in ID and healthy samples. These results, together with other published data that also found an increased presence of *F. necrophorum* in footrot lesions (Beveridge 1941, Bennett and others 2009a, Witcomb and others 2014, 2015), suggest that the prevalence of *F. necrophorum* in healthy feet varies according to sampling structure and collection methods. *F. necrophorum* is a commensal in the alimentary tract (Smith and Thornton 1997) and might be present in faeces contaminating ovine feet. Moreover, it was also detected on swabs taken from the oral cavity of sheep and suggested it might be transmitted from the mouth of sheep to the paddock (Bennett and others 2009a); hence, the significance of *F. necrophorum* in healthy ovine interdigital skin remains unclear; it may colonise healthy skin as a commensal microorganism without causing any skin disease, while in damaged skin, *F. necrophorum* colonisation may initiate ID and, thus, predispose the invasion of *D. nodosus*. Whether *D. nodosus* essentially requires *F. necrophorum* colonisation to facilitate its skin invasion remains unclear.

*F. necrophorum* is divided into subspecies *necrophorum* and *funduliforme*, the first is described to be more pathogenic due to a higher lipopolysaccharide content and higher production of leukotoxin (Tan and others 1996). In this study, the majority of positive samples for *F. necrophorum* was subspecies *necrophorum*. Previous studies investigating this bacterium in UK flocks did not differentiate the subspecies of *F. necrophorum* (Witcomb and others 2014, 2015). Therefore, despite the fact that this sample size is small, this is the first study suggesting that *F. necrophorum* subspecies *necrophorum* may be the more prevalent subspecies circulating in UK flocks. Since *F. n* subspecies *necrophorum* is described to be more virulent than *F. n* subspecies *funduliforme* (Tan and others 1996) and considering the fact that this bacterium may exacerbate footrot lesions, there might be an association between the high prevalence of severe footrot lesions in the UK and the presence of *F. n* subspecies *necrophorum*. In contrast, in Swedish flocks where most of the footrot lesions were associated with mild footrot, *F. n* subspecies *funduliforme* was more prevalent than *F. n* subspecies *necrophorum* (Frosth and others 2015).

Spirochaetes have also been identified in ID and/or footrot lesions (Beveridge 1941, Calvo-Bado and others 2011, Frosth and others 2015). In the present study, a small number of biopsies...
were positive for Treponema species with similar prevalence in healthy, ID and footrot feet. Similarly, low detection of Treponema species in ovine biopsies from UK sheep was also reported by Calvo-Bado and others (2011); moreover, no significant association between Treponema species and footrot was reported by Frosth and others (2015). Hence, whether the low detection of Treponema species reflects its importance in the footrot pathogenesis remains an open question to be further elucidated. The authors amplified treponemal DNA using a genus-specific qPCR and not a species-specific assay, hence detecting free living as well as pathogenic Treponema species; therefore, more studies are warranted to characterise the Treponema species commonly present in ovine footrot. In contrast to early investigations reporting that an initial infection with D nodosus is often followed by an infection with Treponema species (Beveridge 1941, Thomas 1962), the authors only found 3 per cent of the biopsies (8/241) positive for both virulent D nodosus and Treponema species.

A limitation of using abattoir samples is that it is impossible to investigate the progression of the disease and thus verify whether healthy or ID feet positive for D nodosus would develop footrot lesions. On the other hand, an advantage of using abattoir samples is the ability to collect biopsies from animals that are slaughtered for other purposes than this study and detect bacteria localised within tissues.

Conclusions

The results presented in this study, together with other published data, confirm that D nodosus is mainly associated with ID stage and F necrophorum with footrot stage, thereby supporting that D nodosus drives the early stages of footrot and F necrophorum plays a role in the pathogenesis of ovine footrot. Moreover, virulent D nodosus population is more prevalent than benign in UK abattoir footrot samples. Treponema species were detected in few samples; hence, further studies are warranted to provide more detailed information about the role Treponema species may have in ovine footrot. Additionally, this study reports novel results regarding the higher prevalence of F necrophorum subspecies necrophorum than subspecies funduliforme in sheep from the UK, and a mixed population of virulent and benign D nodosus present in the same skin biopsy. The detection and characterisation of Dichelobacter nodosus from cases of ovine footrot in England and Wales. Veterinary Microbiology 150, 57–67. 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