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The role of the environment in transmission of *Dichelobacter nodosus* between ewes and their lambs

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A B S T R A C T

Dichelobacter nodosus (*D. nodosus*) is the essential causative agent of footrot in sheep. The current study investigated when *D. nodosus* was detectable on newborn lambs and possible routes of transmission. Specific qPCR was used to detect and quantify the load of *D. nodosus* in foot swabs of lambs at birth and 5–13 h post-partum, and their mothers 5–13 h post-partum; and in samples of bedding, pasture, soil and faeces. *D. nodosus* was not detected on the feet of newborn lambs swabbed at birth, but was detected 5–13 h after birth, once they had stood on bedding containing naturally occurring *D. nodosus*. Multiple genotypes identified by cloning and sequencing a marker gene, *pgrA*, and by multi locus variable number tandem repeat analysis (MLVA) of community DNA from swabs on individual feet indicated a mixed population of *D. nodosus* was present on the feet of both ewes and lambs. There was high variation in *pgrA* tandem repeat number (between 3 and 21 repeats), and multiple MLVA types. The overall similarity index between the populations on ewes and lambs was 0.45, indicating moderate overlap. Mother offspring pairs shared some alleles but not all, suggesting lambs were infected from sources(s) other than just their mother's feet. We hypothesise that *D. nodosus* is transferred to the feet of lambs via bedding containing naturally occurring populations of *D. nodosus*, probably as a result of transfer from the feet of the group of housed ewes. The results support the hypothesis that the environment plays a key role in the transmission of *D. nodosus* between ewes and lambs.

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1. Introduction

Footrot is an economically important disease of sheep. The aerotolerant anaerobe *Dichelobacter nodosus* (*D. nodosus*) is the essential causative agent (Beveridge, 1941) and *Fusobacterium necrophorum* has been suggested as a secondary bacterium after the development of disease (Beveridge, 1941; Witcomb et al., 2014). The disease is present worldwide and accounts for annual losses of between £24 and £84 million to the UK sheep industry alone (Nieuwhof and Bishop, 2005; Wassink et al., 2010). The severity of ovine footrot can vary from mild interdigital dermatitis (synonymous with benign footrot in Australian research) to virulent footrot causing severe under-running of the hoof horn with separation from the underlying tissue (Stewart, 1989). *D. nodosus* can be detected on the feet of sheep with no sign of disease (Calvo-Bado et al., 2011b; Witcomb et al., 2014) but the load is higher both before and

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during episodes of interdigital dermatitis and virulent footrot than on healthy feet (Witcomb et al., 2014).

Temporal clustering of footrot between mothers and offspring was observed in a state transition study of factors associated with development of, and recovery from, footrot. Given that families cluster spatially this suggests spatiotemporal transmission of *D. nodosus* between family members (Kaler et al., 2010). *D. nodosus* has been isolated from pasture and barns where sheep are kept, indicating that contamination of the environment occurs (Witcomb, 2012). Contaminated holding areas have also been shown to cause disease in sheep put into such environments up to 2 weeks from initial seeding (Beveridge, 1941; Whittington, 1995). Recent work has indicated that *D. nodosus* can survive up to 14 days at 5 °C in soil, and at least 24 days when hoof material was present (Cederlof et al., 2013) and under certain conditions, *D. nodosus* has survived for at least 40 days in soil microcosms (unpublished data), however, further work is required to determine if survival is at a dose that could cause disease in sheep.

Multiple strains of *D. nodosus* detected by serogroup typing have been reported to co-exist in individual feet during subclinical and clinical infections (Claxton et al., 1983; Hindmarsh and Fraser, 1985; Jelinek et al., 2000; Moore et al., 2005). Molecular detection of strain differences is now possible using typing the *pgr* locus and by MLVA of *D. nodosus* (Calvo-Bado et al., 2011a; Russell et al., 2014).

The aims of this study were to investigate whether *D. nodosus* was present on the feet of newborn lambs at or after birth and the potential role played by the environment in pathogen transmission.

2. Materials and methods

2.1. Selection of animals

In April 2011 10 ewes with no clinical signs of disease and one lamb per ewe were convenience selected from a flock of 99 Mule and Suffolk crossbred ewes. Ewes were housed on the 28th March 2011, and samples collected on the 1st–6th April 2011 (Supplementary Table 1). Lambs were born in a large communal straw bedded pen, ewes and their lambs were moved to individual pens once the ewe had given birth to all her lambs. Sampled lambs were marked with tape so they could be identified for subsequent sampling.

2.2. Collection of environmental and foot swab samples

Environmental samples were taken in March prior to lambing and included swab samples of 30 fresh hoof prints in soil, four soil samples from the area around water containers, 10 samples of faecal material on the ground and compacted in the interdigital space and three straw samples collected from the storage area. In April, 10 straw bedding samples were collected from the communal pen where pregnant ewes were housed. All samples were stored at 4 °C for transportation and at −80 °C until analysed. All four feet of each lamb was swabbed using sterile cotton swabs (EUROTUBO collection swab; Delta lab, Rubi, Spain) directly after birth and before the lamb touched the ground. The lamb and its dam were sampled 5–13 h later once the lamb had stood and been transferred, with its mother, to an individual pen. Swabs were stored at 4 °C for transportation and at −80 °C on arrival at the laboratory.

2.3. Detection limit assay by direct PCR and nested PCR from swabs

The *D. nodosus* strain VCS1703A was used as a positive control for all PCR reactions. To determine the PCR detection limits, cells were harvested from a 5 d culture grown on 2% hoof agar, and 10-fold serial dilutions (10^{-1} to 10^{-10}) were made in triplicate in sterile phosphate buffered saline (PBS). The numbers of cells in the initial concentration and all dilutions were counted using a haemocytometer. Sterile swabs were inoculated with 500 μl of each dilution, and frozen at −20 °C to produce swabs containing a known bacterial load. Microbial DNA was extracted from swabs as described below and the DNA used to determine assay detection limits.

2.4. DNA extraction from swabs

Total genomic DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, GmbH and Co, Düren, Germany) with modifications. Swabs were thawed at 4 °C and 400 μl of buffer T1 was added followed by 40 μl of proteinase K. The samples were vortexed twice for 5 s and incubated for 10 min at 56 °C. The mixtures were transferred to microcentrifuge tubes and 400 μl of buffer B3 was added. The samples were vortexed twice for 5 s and incubated for 5 min at 70 °C then allowed to cool before adding 400 μl of 100% ethanol. The samples were again vortexed twice and the supernatant transferred to a NucleoSpin Tissue column and centrifuged at 11,000 × *g* for 1 min. The flow-through was discarded, the membrane was washed with 500 μl of buffer B5 and centrifuged at 11,000 × *g* for 1 min. The flow-through was again discarded, the column was washed with 600 μl of buffer B5 and centrifuged at 11,000 × *g* for 1 min. The flow-through was again discarded and the membrane dried by centrifugation at 11,000 × *g* for 1 min to remove residual ethanol. The DNA was eluted into 40 μl of elution buffer, warmed to 70 °C and centrifuged at 11,000 × *g* for 1 min and the resultant DNA was stored at −20 °C.

2.5. DNA extraction from soil and faeces

DNA was extracted from soil and faecal samples (one gram each) using the Fast DNA Spin Kit for soil (QBiogene, Carlsbad, CA, USA) according to the manufacturer's instructions, and eluted in 70 μl DES (DNase/Pyrogen Free Water). Sterile soil (autoclaved twice at 121 °C for 15 min) was used as negative controls for each set of extractions. The resultant DNA was stored at −20 °C.

2.6. DNA extraction from bedding

One gram of each of 10 bedding samples was thawed, and suspended in 40 ml of transport buffer (sterile PBS

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