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Experimental infection of sheep with ovine and bovine *Dichelobacter nodosus* isolates

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ABSTRACT

The aim of this study was, under experimental conditions, to investigate infection of Norwegian White sheep with ovine and bovine isolates of *Dichelobacter nodosus* of varying virulence. In addition, the efficacy of gamithromycin as a treatment for the experimentally induced infections was examined. The study was performed as a single foot inoculation using a boot. Four groups, each with six lambs, were inoculated with four different challenge strains (Group 1: benign bovine strain; Group 2: virulent bovine strain; Group 3: benign ovine strain; Group 4: virulent ovine strain). The main criterion to determine that infection was transferred was that *D. nodosus* isolate was obtained by culture. After the trial all lambs were treated with gamithromycin. Clinical symptoms of footrot developed in all groups, and when removing the boots two weeks after challenge, *D. nodosus* was isolated from 5 of 24 experimental lambs. All lambs tested negative for *D. nodosus* by PCR within six weeks after treatment with gamithromycin. This study strongly indicates that *D. nodosus* isolates from both sheep and cattle can be transferred to sheep under experimental conditions. The study also indicates that gamithromycin may be effective against *D. nodosus*.

1. Introduction

In 2008, ovine footrot was diagnosed in Norway for the first time in 60 years (Meling and Ulvund, 2009). The outbreak was restricted to Rogaland, a county with a high density of farm animals where co-grazing of sheep and cattle is practised (Vatn et al., 2012). Ovine footrot is a debilitating disease causing lameness and reduced animal welfare (Stewart, 1989; Dwyer and Bornett, 2004). *Dichelobacter nodosus*, a Gram negative anaerobic bacterium, is the main aetiological agent and the bacterium produces extracellular proteases, which are categorized based on their thermostability (Beveridge, 1941; Depiazzi et al., 1991). Benign isolates producing thermolabile proteases are associated with mild interdigital dermatitis which does not progress, whereas virulent isolates producing thermostable proteases tend to cause severe footrot where the keratinous part of the claw horn separates from its underlying tissues (Stewart, 1989). In addition to the virulence of the involved bacterial strain, development of disease depends on environmental conditions and differences in

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the susceptibility between breeds (Beveridge, 1941; Emery et al., 1984; Egerton and Raadsma, 1991; Depiazzi et al., 1998). Due to variations in DNA sequence of the fimbrial subunit gene fimA, *D. nodosus* is divided into ten serogroups (Claxton, 1989; Ghimire et al., 1998).

Antibiotics, such as oxytetracycline and enrofloxacin, are used to treat footrot (Kaler et al., 2012). The more recently introduced macrolid antibiotic gamithromycin has been used as flock treatment and even though highly effective in some areas *D. nodosus* was not eliminated from all sheep in all flocks even though (Forbes et al., 2014).

D. nodosus is commonly isolated from cows with interdigital or digital dermatitis (Laing and Egerton, 1978; Knappe-Poindecker et al., 2013). All isolates tested from cattle in Norway having no contact with sheep produce the heat labile protease and have been defined as benign (Gilhuus et al., 2013; Knappe-Poindecker et al., 2013). The epidemiological importance of these infections for the spread and control of ovine footrot is considered to be low, and Beveridge (1941) concluded that cattle are unlikely to be important as reservoirs for *D. nodosus*. However, two previous Norwegian studies have indicated that cross-infection of *D. nodosus* from sheep to co-grazing cattle did occur (Rogdo et al., 2012; Knappe-Poindecker et al., 2014). In the latter study, two cows stayed infected for at least one housing season, creating a reservoir for virulent *D. nodosus*. The bacterium has previously been transmitted both naturally and experimentally between cattle and sheep (Egerton and Parsonson, 1966; Wilkinson et al., 1970; Laing and Egerton, 1978), but more studies of the possible transfer of different strains from sheep and cattle in Norway are needed.

The aim of this study was, under experimental conditions, to investigate infection of Norwegian White sheep with ovine and bovine isolates of *D. nodosus* of varying virulence. In addition, the efficacy of gamithromycin as a treatment for the experimentally induced infections was investigated.

2. Materials and methods

2.1. Experimental animals

The trial was conducted on 27 weaned lambs of the breed Norwegian White sheep (NKS). The 15 ewe lambs and 12 ram lambs were chosen randomly from the flock belonging to the Norwegian University of Life Sciences. This flock is considered free of footrot based on clinical inspection of the claws in all sheep in the flock and bacterial samples from random sheep as part of the surveillance programme "Healthy Feet" (Vatn et al., 2012). Prior to the start of the trial, all 27 lambs tested negative for *D. nodosus* on PCR and FISH.

Each lamb had tags in both ears with a unique identification number. The lambs were aged 4–5 months at the start of the trial, and had a mean body weight of 44 kg (range 33–56 kg). During the trial, the lambs were kept in the closed animal unit at the Norwegian University of Life Sciences, Campus Sandnes, approved by the Norwegian Food Safety Authority (National Animal Research Authority) for infectivity studies in sheep. The stall contained nine boxes with rib mesh floor with no bedding and they were completely separated from each other (mean size of 3 m^2, range 2.1–3.6 m^2). Each box housed three lambs of the same gender.

The lambs were randomly allocated to four experimental groups, each with six lambs, and a control group with three ewe lambs. Each experimental group consisted of three ram lambs and three ewe lambs. Before the start of the trial, the selected lambs underwent a clinical examination by a veterinary surgeon. The claw health of each lamb was controlled and recorded. Swabs were taken from the interdigital skin on the right hind foot of all lambs, and tested for *D. nodosus* by PCR as described below. From this point, the lambs were isolated from other animals. Care was taken to prevent cross-contamination during handling and feeding and gloves were changed between every lamb at removal of the boots.

Table 1
Origin, virulence and serogroup of the challenge strains.

Group	Origin and breed	Virulence	Serogroup
1	Norwegian Red cattle	Benign	I
2	Norwegian Red cattle	Virulent	A
3	Norwegian White sheep	Benign	G
4	Norwegian White sheep	Virulent	A
5 (Controlgroup)	–	–	–

2.2. Preparation of the bacterial suspensions

Four different indigenous challenge strains were used in this study (Table 1). It was aimed for each experimental lamb to be inoculated with 10 ml of a bacterial suspension containing 10^6–10^7 bacteria/ml. The challenge strains were grown anaerobically for six days on 4% hoof agar (HA) with addition of 1% 'Lab-Lemco' powder (Oxoid, Basingstoke, UK) and 0.2% tryptose (Oxoid). Growth on each agar plate was checked for purity using phase contrast, flushed with 2 ml room-tempered saline and gently scraped with an L-shaped spreader to detach bacteria. The saline containing the bacteria was collected using a Pasteur pipette and gathered in a 15 ml falcon tube (Greiner bio-one, Frickenhousen, Germany). Additional saline was added to each falcon tube to a total volume of 11 ml. After gentle mixing, 1 ml of the suspension was removed and used to prepare ten-fold dilutions in double distilled water. The dilutions were boiled for 1 min and used as template in a real time PCR to detect *D. nodosus* (Frosth et al., 2012). Using a 10 μl inoculation needle, undiluted bacterial suspension from the falcon tube was cultured on 2% HA and incubated anaerobically at 37° C. Two days later confluent growth of *D. nodosus* on the HA plates was confirmed from all the prepared broths used for inoculation of experimental lambs. By real-time PCR, the presence of *D. nodosus* was confirmed in the 10^{-7} dilutions from each inoculation broth.

The remaining 10 ml bacterial suspension in each falcon tube was immediately used to infect the experimental lambs.

2.3. The trial

Fig. 1 illustrates the timeline for the trial. On day 1, no events directly affecting the infection trial were performed, but measurements were done and samples collected to be used in an animal welfare study by Stubsjøen et al. (submitted).

On day 2 of the trial, the claw health of all lambs was controlled. Biopsies were taken with a 3 mm biopsy punch (Miltex, Inc. USA) from the interdigital skin for histopathological evaluation and fluorescence *in situ* hybridization (FISH) for identification of *D. nodosus*. The right hind foot of each lamb was placed in Nordströms rubber boots®, and 10 ml tap water was added to create moist conditions. The boots were secured with an adhesive bandage and left on for seven days. The position of the boots was controlled daily, and the lambs were observed for signs of lameness. Lameness was scored after Morck et al. (1994) as 0 = no limp; 1 = slight limp; 2 = moderate limp and 3 = non-weight bearing.

On day 9 of the trial, the boots were removed and the feet examined and scored for footrot after Egerton and Roberts (1971), as described in Table 2. The boots were replaced and infused with 10 ml bacterial suspension, which was prepared as described above. In the control group, 10 ml tap water was added instead of bacterial suspension. The lambs were monitored daily for the following two weeks to assess lameness and pain, and the position of the boots was controlled. Pain was scored after Ahern et al. (2009) as described in Table 3. Lambs scoring >1 on lameness and/or pain received 0.5 mg/kg live weight meloxicam SC every other day until the pain resolved (Metacam; Boehringer Ingelheim vetmedica GmbH).

On day 23, two weeks after inoculation, the boots were removed and the claws were examined for symptoms of footrot and blisters. Signs of lameness were recorded. Swabs from the interdigital space for culturing and PCR regarding *D. nodosus* were collected. Because a viscid material had formed in the boot, the skin was first wiped with moist paper towels before being dried off with a paper towel. Biopsies were taken and analyzed as described below. Lambs with signs of footrot, lameness or