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Characterisation of *Dichelobacter nodosus* isolates from Norway

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ABSTRACT

An outbreak of ovine footrot in Norway in 2008, the first reported since 1948, prompted action to investigate Norwegian isolates of *Dichelobacter nodosus*. A total of 579 isolates from 124 different farms were characterised. These included 519 isolates from sheep, 52 isolates from cattle and 8 isolates from goats. The potential virulence of the isolates was assessed by the gelatin gel test (GG-test) and the elastin agar test, that test the heat stability and elastase activity of bacterial proteases, respectively. The isolates were also tested for the presence of *intA* by PCR, and allocated to serogroups by differentiation of *fimA* variants using multiplex PCR or sequencing. Thirty of the isolates were also serogrouped by slide agglutination. Three hundred and five isolates were defined as virulent by the GG-test. All these were from sheep from 52 farms located in the county of Rogaland in the south west of Norway. All isolates from cattle and goats were defined as benign by the GG-test. *IntA* was only detected in 6 (2.0%) of the virulent isolates. All serogroups except D and F were detected. Three hundred and seventy-two (64.3%) of the isolates belonged to serogroup A, and 96% of the virulent isolates belonged to this serogroup. On the grounds that virulent isolates were only found in one county, and that the majority belonged to the same serogroup (A), it is believed that a virulent *D. nodosus* strain was introduced to Norway relatively recently and that so far it has only spread locally.

1. Introduction

In 2008, ovine footrot, caused by the Gram-negative anaerobe *Dichelobacter nodosus*, was diagnosed in Norway for the first time in 60 years (Meling and Ulvund, 2009). Footrot is a major challenge for the sheep industries worldwide (Wani and Samanta, 2006). *D. nodosus* can also cause footrot in goats and interdigital dermatitis in cows.

The severity of footrot depends on bacterial virulence, climatic conditions and sheep breed. Clinical signs range from interdigital dermatitis to complete loosening of hoof horn (Stewart, 1989). A few virulence factors, including three extracellular proteases (Thomas, 1964), have been described for *D. nodosus*. Isolates that secrete elastase, as measured by the elastin agar test (Stewart, 1979), or heat stable protease, as measured by the gelatin gel test (GG-test) (Palmer, 1993), are regareded as potentially virulent (Kortt et al., 1982; Depiazzi et al., 1991). Some strains are incapable of causing severe disease despite secretion of thermostable protease. Cheetham et al. (2006) suggested that PCR detection of a genetic element, *intA*, could supplement the GG-test and improve detection of truly virulent strains.

D. nodosus is divided into 10 serogroups (A-I and M), and at least 21 serotypes based on fimbrial antigens (Claxton, 1989; Ghimire et al., 1998; Bhat et al., 2012). This

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is relevant in immunisation, because the effect of vaccination is serogroup specific (Egerton, 1974).

Ovine footrot is notifiable in Norway, and following its discovery in 2008 more than four thousand sheep flocks were examined clinically. Of these, 20% were tested by PCR for *D. nodosus* and 530 (46%) flocks were positive. The majority had very mild symptoms (Vatn *et al.*, 2012). About 70 flocks with severe symptoms were clustered in the south west of Norway.

There are significant differences in sheep breeds, management practices and climatic conditions between Norway and other countries where ovine footrot is a challenge. Therefore, it was important to establish knowledge of the bacteriological agent, and the manifestations of footrot in Norwegian sheep under Norwegian conditions. To better understand the epidemiology of ovine footrot in Norway, it was necessary to first investigate characteristics of Norwegian *D. nodosus* isolates. The aim of this study was to characterise *D. nodosus* isolates with respect to serogroup and virulence and to relate this to geographical location and animal species.

2. Materials and methods

2.1. Bacterial isolates

Five hundred and nineteen *D. nodosus* isolates from sheep, 52 isolates from cattle and 8 isolates from goats were included (Table 1). These had been collected from samples analysed at the Norwegian Veterinary Institute (NVI) between 2008 and 2011. The isolates originated from animals with footrot like lesions (sheep and goats) or interdigital dermatitis (cows), or from healthy animals sampled for management and surveillance purposes.

The major sheep breed in Norway is the meat sheep Norwegian White Sheep. With only few exceptions sampled sheep were of this breed. Bovine samples were all from dairy cows, and the goat samples were from Boer and Mohair goats.

Culturing had been performed on 4% hoof agar (HA) as described by Stewart and Claxton (1993), but with the addition of 1% 'Lab-Lemco' powder (Oxoid, Basingstoke, England) and 0.2% Tryptose (Oxoid, Basingstoke, England). *D. nodosus* suspect colonies were subcultured onto 2% HA. The isolates had been confirmed as *D. nodosus* by real-time PCR (Frosth et al., 2012), and stored at −70 °C in Bacto™ Heart Infusion Broth (BD, Sparks, MD) with 15% glycerol.

All available isolates from 2008 to 2010 were included, with a maximum of 3 isolates per animal. In addition, 2 randomly selected isolates from every new *D. nodosus* culture-positive flock from 2011 were included. Geographically, the isolates originated from 124 farms (flocks or herds) from 10 of the 19 Norwegian counties (Table 1). From 1 farm, isolates from both sheep and cattle were included. From 7 of the farms, isolates had been collected on two or three occasions over 3–12 months. Animals from 4 of these farms had been footbathed with 15% Zinc sulfate ($ZnSO_4$) between two of the samplings, and from one of the farms the second sampling was of animals purchased after the footbathing (Synnøve Vatn, Animalia, Personal communication).

2.2. Gelatin gel test

All isolates were categorised as virulent or benign based on their ability to secrete heat stable or heat labile proteases, respectively, as shown by the gelatin gel test (GG-test). The test was performed essentially as described by Palmer (1993).

D. nodosus was grown anaerobically in HEPES-TAS broth (Stewart and Claxton, 1993) at 37 °C for 48–72 h. Forty ml of a gel containing 1% Agarose NA (GE healthcare, Uppsala, Sweden), 1% Gelatin from porcine skin, Type A (Sigma–Aldrich, St. Louis, MO), 0.02 M TRIS and 0.001 M $CaCl_2$ was prepared. To prevent growth of contaminant bacteria, 2.5 mM Sodium Azide (BHD Laboratory Supplies, Poole, England) was added. The gel was poured onto a 20 cm × 20 cm glass plate and when solid, 4 mm diffusion wells were made. Broth cultures were diluted 1:1 in a dilution buffer (pH 8.5) containing 0.1 M HEPES (Sigma–Aldrich, St. Louis, MO) and 0.04 M $CaCl_2$, and 20 μl were applied to the wells after 0, 8 and 16 min of heating at 68 °C. The gels were incubated in a moist chamber at 37 °C for 18 h. Remaining gelatin was precipitated by immersion in a saturated Ammonium sulphate ($(NH_4)_2SO_4$) solution at 60 °C. The diameter of proteolysis around each well was measured and compared with a standard, which was calibrated according to Palmer (Palmer, 1993).

Table 1
Dichelobacter nodosus isolates with respect to host species, the number of farms (flocks and herds) and geographical location in Norway.

County	No. of isolates	No. of sheep (flocks)	No. of cattle (herds)	No. of goats (herds)
Hordaland	2	2 (1)	0 (0)	0 (0)
Rogaland	447	234 (97)[a]	16 (3)[a]	3 (1)
Telemark	17	9 (4)	0 (0)	1 (1)
Buskerud	9	8 (3)	0 (0)	0 (0)
Østfold	2	2 (1)	0 (0)	0 (0)
Akershus	22	8 (2)	4 (1)	0 (0)
Hedmark	61	28 (7)	0 (0)	0 (0)
Oppland	8	4 (2)	0 (0)	0 (0)
Nordland	5	0 (0)	2 (1)	0 (0)
Troms	6	0 (0)	2 (1)	0 (0)
Total	579	295 (117)[a]	24 (6)[a]	4 (2)

[a] From one farm, isolates from both sheep and cattle were included.