Veterinary Microbiology 176 (2015) 378–381

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Identification and characterization of serogroup M *Dichelobacter nodosus* from sheep with virulent footrot

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ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form 5 January 2015
Accepted 16 January 2015

Keywords:
Sheep
Footrot
Dichelobacter nodosus
Serogroup M

ABSTRACT

As part of an outbreak-specific footrot vaccination field trial a total of 1282 footrot lesion samples were collected from 2 sheep flocks on King Island, Tasmania. Breeding rams were shared between the two flocks, suggesting a common source of infection. All samples were tested for *Dichelobacter nodosus*. A total of 1047 *D. nodosus* isolates were obtained in pure culture (490 from 670 lesion samples from flock 1, and 557 from 612 lesion samples from flock 2) were tested by agglutination and PCR tests for the 9 common Australian serogroups A to I. After the first rounds of a specific vaccination program, a significant proportion of the isolates of *D. nodosus* from these flocks were found to be negative in the serogrouping tests and the prevalence of the disease remained high in both. Those isolates were tested retrospectively against New Zealand and Nepal serogroup M antisera and found to be positive. Fimbrial gene (*fimA*) sequences of three isolates collected over three years were identical indicating that these strains belonged to one serogroup and were most closely related to New Zealand and Nepal serogroup M sequences. More than 40% of the *D. nodosus* isolates from these flocks belonged to serogroup M and were virulent in tests for protease activity. The next most prevalent serogroup was A (23%). This study reports the identification and characterization of serogroup M isolates of *D. nodosus* from Australia, and led to routine testing for serogroup M in flocks where specific vaccination will be applied for control, treatment and eradication of the virulent footrot.

1. Introduction

Antigenic variation among isolates of the bacterium *Dichelobacter nodosus*, the essential causative agent of ovine footrot, was first observed using a slide agglutination test (Egerton, 1973). The fimbria or pilus of *D. nodosus* is involved in the K agglutination reaction and is the major known protective immunogen (Egerton et al., 1987; Thorley and Egerton, 1981). Strain variation lies in the fimbrial proteins and their subunit genes, and this feature has been used to classify isolates into 9 serogroups (A–I) in Australia (Claxton, 1986; Claxton et al., 1983; Dhungyel et al., 2002; Schmitz and Gradin, 1980). The serogroup classification system provides the basis for the success of outbreak-specific vaccination (Dhungyel et al., 2013; Dhungyel et al., 2008; Gurung et al., 2006; Egerton et al., 2002; Egerton and Morgan, 1972). Depending on the homology of the fimbrial sequence, *D. nodosus* fimbriae have been classified into 2 groups, namely Class I or A set *fimA*1 and Class II or D set *fimA*2 (Elleman, 1988; Mattick et al., 1991). Class I consists of serogroups A, B, C, E, F, G, I, and M, and Class II consists of fimbriae of serogroups D and H.

Alternative fimbrial classification schemes have been proposed: British (Day et al., 1986) and American (Gradin et al., 1993; Schmitz and Gradin, 1980). Within the British

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http://dx.doi.org/10.1016/j.vetmic.2015.01.014
0378-1135/

system was a proposal for extension of the Australian system to accommodate a further nine serotypes, J–R. This was based on a tube agglutination test using absorbed rabbit antisera, and isolates with similar agglutination patterns were regarded as a serotype. The American system was also based on a tube agglutination test but using non-absorbed sera; isolates reacting within a dilution of the homologous reaction were considered to belong to a serotype. The British and American systems classified isolates into 17 serotypes using tube agglutination tests while the Australian system using slide agglutination created 9 serogroups. Three of the British (Chetwin et al., 1991b) and three of the American (Gradin et al., 1993) serotypes did not react with antisera for any of the nine Australian serogroups. Even though the British and the American systems were able to demonstrate fine antigenic variation (into serotypes) this did not correlate to cross-protection between the serotypes, unlike the Australian "within serogroup" cross-protection (Claxton, 1981). An additional serotype M, different from the common Australian prototypes was described in Australia and New Zealand (Day et al., 1986; Chetwin et al., 1991a) and in Nepal (Ghimire et al., 1998).

Virulent footrot is endemic in some flocks of sheep on King Island, Tasmania. For many years management of the disease was attempted using conventional methods like footbathing and parenteral antibiotics, without any success. High rainfall and green pastures all year round in most years made control difficult. For these reasons outbreak-specific vaccination was attempted in 3 flocks as part of a larger trial. For the purpose of outbreak-specific vaccination, intensive investigation and characterization of isolates of *D. nodosus* from affected animals was carried out on each farm (Dhungyel et al., 2013). In this study we report the identification and characterization of previously unidentified serogroup M isolates of *D. nodosus* from two of the trial flocks. To our knowledge this is one of the very few studies to report prevalence of serogroup M isolates in Australian sheep flocks.

2. Materials and methods

Two flocks were investigated as part of an outbreak-specific footrot vaccination trial (Dhungyel et al., 2013). They were infected with *D. nodosus* through use of a common set of rams. A total of 1282 foot swab samples (670 from flock 1 and 612 from flock 2) were collected between 2005 and 2010 using standard protocols for diagnosis and collection of samples for culture of *D. nodosus* (Dhungyel et al., 2013; Stewart and Claxton, 1993). All the samples were cultured, and *D. nodosus* isolates were serogrouped using slide agglutination tests initially against 9 serogroups (A to I) using prototype rabbit antisera. From 2007, after the first round of vaccinations, isolates with inconclusive agglutination reactions were tested with New Zealand M antisera (Claxton, 1986; Claxton et al., 1983). These isolates were also tested by serogroup specific (A to I) PCR (Dhungyel et al., 2002). All isolates were tested using two in vitro virulence tests: gelatin gel test (Palmer, 1993) and elastin agar test (Stewart, 1979).

D. nodosus isolates were identified phenotypically by their in vitro growth, colony characteristics, and cell morphology (Stewart, 1989). Those isolates not reacting or showing very weak cross reaction in slide agglutination tests (Claxton et al., 1983) and negative results in serogrouping A–I PCR tests (Dhungyel et al., 2002) were subjected to a *D. nodosus* species specific PCR test based on the 16S RNA gene (La Fontaine et al., 1993). Whole cell samples of some of those isolates were run on a SDS PAGE gel alongside similar preparations of prototype strains of serogroup A to check for the presence of the fimbrial protein (Whittington et al., 1997).

For the production of new antisera to serogroup M, 2 New Zealand white rabbits were vaccinated with 2 doses of whole cell serogroup M vaccine of a representative strain (SVC 09/096/1-1); the primary and boosters doses were administered 4 weeks apart. Antisera were collected 2 weeks post booster vaccination. Antisera against Nepal serogroup M isolate (Nep 115) were prepared at the University of Sydney (Ghimire et al., 1998) and New Zealand isolate by Day et al., 1986, provided by Dr. K. Cooper, Mallickrodt Veterinary, Upper Hunt, New Zealand.

Fimbrial subunit gene regions of three representative isolates (SVC 07/097/5-1, 08/251/4-1 and 09/096/1-1) of serogroup M isolated in three different years (2007, 2008 and 2009) from sheep in flock 1 were amplified by *FimA* PCR using the forward primer PTC 830 and the reverse primer PTC 5 (Cox, 1992). PCR products were purified using a Promega PCR purification kit and sequenced at the Gandel Charitable Trust Sequencing Centre, Monash University. New sequences were compared with other published *D. nodosus* sequences by a Blastn Search (NCBI). Sequence alignments and analyses were done in Clustal W version 2 (Larkin et al., 2007) and molecular phylogenetic analysis by the Maximum Likelihood method in Mega 6 (Tamura et al., 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura–Nei model (Tamura, 1993). The tree with the highest log likelihood (−1214.1750) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 413 positions in the final dataset.

3. Results and discussion

A total of 1047 *D. nodosus* isolates were obtained from 1282 swab samples collected from 1282 animals between 2005 and 2010. There were 490 isolates from 670 lesion swabs from flock 1 and 557 isolates from 612 lesion swabs from flock 2. All isolates were identified as *D. nodosus* by their in vitro growth, colony characteristics, and cell morphology. Those isolates not reacting (non-typeable) in