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Genomic heterogeneity of Dichelobacter nodosus within and between UK sheep flocks and between age groups within a flock

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Research article

Keywords: ,

Posted Date: August 21st, 2019

DOI: https://doi.org/10.21203/rs.2.13309/v1

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Version of Record: A version of this preprint was published at BMC Microbiology on May 1st, 2020. See the published version at https://doi.org/10.1186/s12866-020-01769-9.

Abstract

Footrot and interdigital dermatitis are endemic infectious diseases in all sheep farming regions, impairing welfare and production. The development of efficacious vaccines against the primary causative pathogen has been hampered by the extensive antigenic diversity of Dichelobacter nodosus. Understanding the heterogeneity of the pathogen within and between farms is essential if the feasibility of bespoke vaccine production is to be assessed. In this study 56 ewe and lamb isolates from 9 farms were compared by D. nodosus serogroup and Multi Locus Sequence Type which provides significantly enhanced discriminatory power for molecular epidemiology. . Within farm genomic heterogeneity was significantly lower than between farms. Serogroup heterogeneity between flocks ranged from two to five unique serogroups per flock. Three flocks contained isolates of two serogroups, two flocks contained isolates of three serogroups and one flock included isolates of five serogroups. Analysis of 25 isolates from one flock with high prevalence of lameness, identified that serogroup and sequence type was significantly correlated with age. Significantly higher proportion of lambs were infected with serogroup B (principally ST85) as opposed to serogroup H (principally ST86), which predominated amongst adult sheep. This indicates that host-pathogen dynamics and susceptibility to particular D. nodosus strains may be age dependent.

Background

Footrot is one of the most important infectious diseases affecting the welfare and productivity of sheep globally. Understanding the population structure of the pathogens involved, particularly *Dichelobacter nodosus* is critical to the development of efficacious vaccines for footrot control. Vaccines based upon a broad range of *D. nodosus* serotypes, (*Footvax,* MSD) have been shown to provide moderate protection to new infections and reduction in clinical disease severity (Duncan *et al.,* 2012). The limited efficacy observed has been attributed to antigenic competition (O'Meara, *et al;* Hunt *et al.,* 1995) and prompted the development of 'bespoke' vaccines specific to the serotypes present on individual farms in Australia with greater success (Dhungyel *et al.,* 2013). Understanding the population structure of the pathogen at farm level helps us to understand the challenges and opportunities for the implementation of similar farm-specific vaccination in the UK disease context. Serotyping (Serogroups A-I and M) has historically been used for classification of *D. nodosus* and has demonstrated significant variation between countries and between farms (Claxton et al 1983; Cagatay and Hickford, 2005; Moore *et al.,* 2005). However, genomic techniques such as multi locus sequence typing (MLST) or core genome multi locus sequence typing (cgMLST) allow greater discrimination between isolates and can provide more insights into the dynamics of the disease and its transmission within and between flocks or farms, as has been demonstrated previously in molecular epidemiological studies in other species, such as bovine mastitis using MLST (Phuektes *et al.,* 2001; Davies *et al.,* 2016). The aim of this study was to compare the heterogeneity and population structure of *D. nodosus* using two alternative discriminatory methodologies, serotype and cgMLST sequence type, within and between flocks in the UK.

Methodology

Samples (n = 2126) were collected from 10 sheep farms situated within Nottinghamshire, Derbyshire and Northamptonshire. All veterinary practices within the east midlands region of England were approached to invite their sheep clients to participate in the research project. 15 farms responded and the ten largest commercial sheep flocks were selected. All flocks were classified as lowland lamb producers ranging in size from 200—1100 ewes. Farmers were requested to present all of their lame ewes on the day of sampling all of which were swabbed (each foot) along with an equal number non-lame sheep selected randomly. The following individual animal data was collected on each animal, age by teeth eruption, body condition score (1–5), breed, gender, hoof heath status (Healthy—no abnormality, Interdigital dermatitis (mild to severe inflammation of the interdigital skin), Footrot—inflammation of the interdigital skin with underrunning of the hoof horn) and diagnosis inc CODD. Farm level variables were recorded including stocking density, ewe replacement policy (open vs closed flock replacement policy) and number of source flocks for current breeding ewe population. management system, husbandry practices specifically related to hoof hygiene and lameness treatment and prevention practices; Footvax vaccination, footbath protocols, antibiotic treatment protocol.

Within Farm A, where the largest number of isolates were generated, only serogroup B and H were identified. 50 lambs and ewes from the flock of 264 ewes and 387 lambs were selected at random. The ewes and lambs had been co-grazed on the same pasture since lambing. The lambs age ranged from 6—8 weeks old. None of the lambs from which D.nodosus was successfully cultured were the progeny of the ewes from which D. nodosus was also cultured.

Bacterial Isolation

Interdigital Swabs (E-swabs 480CE, Copan U.S. A.) were taken from 2,126 ewes and lambs. After collection the swabs were stored in liquid Amies solution at 5°C overnight. Hoof Agar plates containing 4% w/v Bacto Eugon agar (BD, U.S. A.), 0.5% w/v Difco Yeast Extract (BD, U.S. A.), 1.5% w/v BBL Beef Extract (BD, U.S. A.), 1% Sodium Chloride and 6.6% w/v ovine hoof powder (Parker et al., 2005) were inoculated from the swabs and incubated anaerobically at 37°C. Pure colonies were collected from plates in sterile PBS, washed by centrifugation and resuspended in molecular biology grade water (ThermoFisher, UK).

DNA Isolation and Sequencing

DNA was isolated using the Qiagen Cador Pathogen Mini Kit, following the manufacturers guidelines and eluted in 60 μl of elution buffer. DNA was sent to MicrobesNG (Birmingham University, U. K.), for sequencing using the Illumina MiSeq at 2 × 250 bp [Raw data is available in the Short Read Archive (PRJNA386733)].

Analysis of Sequence Data

Sequence reads were assembled using the A5-MiSeq pipelines, (Coil et al., 2015). Briefly, raw reads were analysed for overall quality and sequence adaptors using trimmomatic (Bolger et al., 2014). The reads were then error corrected using the SGA k-mer based approach (Simpson et al., 2012). The quality checked paired and unpaired reads were assembled using IDBA-UD (Peng et al., 2012). These were then scaffolded and extended using SSPACE (Boetzer et al., 2011) before having the clipped and corrected reads realigned using

BWA (Li et al., 2009). The scaffolds were then checked for discordant reads indicative of misassembles and scaffolded again using SSPACE (Boetzer et al., 2011).

Serogroup and Phenotype Determination

The assembled contig files were used as the input for IPCRESS (Slater et al., 2005) a part of the exonerate pipeline. In silico Serogroup determination was completed using the PCR primers developed by (Zhou et al., 2001) and phenotypic (aprV2/aprB2) determination made use of the PCR primers created by (Frost et l., 2015).

Statistical analysis was conducted in *Minitab18 (*Minitab 18Inc, 2018) using moods median test for continuous cgMLST distance matrix data comparison of genetic similarity between bacterial isolates between and within flocks. Fisher's exact tests were used for comparison of proportions of isolates for within flock analysis.

Results

Serogroups and MLST classification was performed on 56 isolates from 9 flocks of the 10 sampled flocks were analysed (Table 1) no isolates could be cultured from the remaining flock. In total 6 serogroups and 26 unique MLST sequence types were identified (Table 2). Two sequence types (ST88 & ST91) were represented in two different serogroups while the remaining sequence types were serogroup specific (Table 3). Up to seven separate MLST sequence types were identified per farm (range 1—7).

Isolates of *D. nodosus* were identified from 36 ewes and 9 lambs. Seven ewes and four lambs generated two isolates from different feet. Five of the seven ewes produced identical MLST sequence types while greater diversity was observed in the lambs with multiple isolates where and two of the four lambs produced dissimilar MLST sequence type isolates.

Genetic heterogeneity within and between flocks was assessed by comparison of cgMLST distance matrix distributions from flocks with at least four isolates from at least 3 individuals (Figure 1). The inter-flock heterogeneity was significantly greater compared to that observed within any single flock ($p < 0.0001$) with median distance matrix values of 542 (IQR: 484, 586) and 491 (IQR: 5, 537) respectively. At the level of the individual flock, 3 of the 5 flocks were each significantly less genetically heterogeneous than the distribution of distance matrix values for isolates compared between different flocks. Of the two remaining flocks, isolates from one flock were significantly more genetically heterogeneous than those isolates compared between flocks.

Within farm analysis of MLST and Serogroup distribution by age.

Serogroup heterogeneity within flocks ranged up to 5 serogroups per flock (Table 1). In two flocks the same two serogroups B & H were the only two identified. All isolates originating from lambs in Farm A were typed serogroup B (7 isolates from 4 lambs, six ST85 and one ST111). Within this flock serogroup B was recovered significantly more frequently from lambs compared to adult ewes ($p = 0.0325$) (4 or 4 lambs vs 5 of 15 ewes). Within the lambs serogroup B and sequence type ST85 was dominant representing a

significantly larger proportion of the sequence types recovered from lambs compared to the adult sheep (p = 0.0181). In contrast, in adult ewes Serogroup H was the most common and within this serogroup ST86 was the dominant sequence type identified.

Of the flocks studied, three purchased replacement females, three flocks bred their own female replacements and the remainder practiced a combination of the two policies. Of those flocks purchasing replacements, 2 - 6 different flocks of origin were recorded from sampled sheep per flock. Neither the MLST sequence type or serogroup diversity at the farm level correlated with flock replacement purchasing policies. At the individual ewe level, neither the MLST sequence type or serogroup diversity correlated with the clinical state of the sampled foot (Figure 2). The isolate diversity determined by sequence type or serogroup did not, in this sample of flocks, correlate with either the flock level prevalence of clinical lameness at the time of sampling or with flock management policy towards purchased ewe replacements as opposed to home reared replacements. There was also no correlation between clinical lesion severity and either serogroup or sequence type. All but one isolate was classified as 'virulent' phenotype by aprV2 gene carriage.

Discussion

Whilst the data presented here represent a small number of lowland flocks and are not intended to provide a definitive description of the *D. nodosus* population structure that would be generalizable to all UK flocks or all farming systems, it does identify a number of interesting and important factors worthy of further investigation. In particular, the limited number of serogroups apparently present on the majority of flocks sampled indicates that bivalent or trivalent vaccines may be appropriate and potentially efficacious in some UK flocks. Serotypes B & H have been used in bivalent vaccines in Australia and may be particularly appropriate for some UK flocks on the basis of the results of this study. However, currently there is no commercially available method of identifying serogroup identity or prevalence in UK flocks and this would need to be addressed if a more sophisticated, targeted approach to *D. nodosus* vaccination is to be attempted.

Whilst serotypes B & H have previously been identified in UK flocks (Moore et al, 2005), this is the first time that these serotypes and the MLST sequence types ST 85 and ST86 have been shown to be disproportionately associated with specific age groups of sheep (ewes vs lambs). This suggests that host pathogen interactions may change or develop with age causing variation in relative susceptibility to different *D. nodosus* strains. The degree of contact through the use of shared pastures between ewes and lambs may be important factors in determining patterns of colonisation and transmission between these different classes of stock. In this study the ewes and lambs had been co-grazed since the lambs birth 6–8 weeks prior to the sample collection date so the lambs would have been exposed to all of the strains from the ewes and yet were not equally colonised by them.

Similar to the findings reported by (Smith et al., 2017) there was no correlation with lesion or severity (normal, interdigital dermatitis, footrot), additionally, the apparent lack of correlation between the diversity of the *D. nodosus* population and the purchasing policies/biosecurity policies of the flocks may indicate that the bacterial population on the foot changes over time, influenced primarily by the farm environment rather

than by the establishment of host populations which are stable over sustained periods. However, larger, multi-flock, longitudinal studies would be required to robustly address these questions with sufficient statistical power to fully elucidate the transmission and colonisation dynamics at the ewe and flock level over time.

The interaction between host and pathogen genetics, environmental conditions and management practices, including antibiotic use and footbathing, are important to understand the influence on hoof microbiome stability over time. This is outside the scope of the current study. The greater discriminatory power of MLST compared to serogroup (115 sequence types compared to 10 serogroups) enhances our ability to understand the transmission of the bacteria between individuals and the wider molecular epidemiology on the hoof and in the pasture or bedding environment. However, the difficulty in isolating and culturing the bacteria prior to DNA extraction results in low recovery rate of usable data compared to serogroup testing methods. Improved techniques in bacterial culture and DNA sequencing would substantially improve the quantity of usable data for epidemiological studies into infectious ovine lameness.

Declarations

Ethical Approval

This study was reviewed and approved by the University of Nottingham, School of Veterinary Medicine and Science ethical review committee ERN: 1144 140506 (Non ASPA).

Data Availability statement

All sequence data generated for this study is held in the NCBI SRA and EMBL ENA under the accession number PRJNA386733 and scripts used are available at https://github.com/ADAC-UoN/MLST.

Author contributions

PD selected and recruited the study flocks, AB created and populated the MLST database and performed all the bioinformatics analysis and generated figures for the manuscript. PD conducted the epidemiology analysis and wrote the manuscript. AB and TC designed the MLST database. AB, PD, and NB collected the swabs to isolate D. nodosus from the U. K. farms. AB and CS processed the swabs from the U. K. farms. CS purified the isolates and prepared the samples for sequencing. All authors have read the manuscript and provided input.

Acknowledgments

The authors would like to thank the farmers for their time and cooperation in this study.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1. Serogroup diversity by farm. The number of isolates of each D.nodosus serogroup isolated from each of the 9 study flocks from which isolates were successfully cultured. No isolates could be cultured from the tenth flock.

Farm id	Serogroup						Total
	A	B	C	E	H	I	
A		13			12		25
B	2	3	1	2		3	11
C		4			1		5
D	1		2				3
E		1	1		2		4
F	1	2				1	4
G						1	1
H		1					1
I	1	1					2
Total number of farms	4 of 9	7 of 9	3 of 9	1 of 9	3 of 9	3 of 9	

Table 2 MLST sequence type diversity by flock. Numbers of ST's identified per farm.

MLST Sequence Type	Farm id								
	A	B	C	D	E	F	G	H	I
75		1							
76									1
78		2							
79		3							
80		2							
81		1							
82		1							
83				1					
84				2					
85	11				1				
86	9				2				
87	2								
88		1			1				
91			3						
98			1						
99						1			
103							1		
104						1			
105								1	
106									1
107			1						
108	1								
109						1			
110						1			
111	1								
112	1								
Total number of MLST ST's per farm	6	7	3	2	3	4	1	1	2

Table 3 MLST sequence type diversity by serogroup. Numbers of ST's identified per serogroup.

MLST Sequence Type	Serogroup					
	A	B	C	E	H	I
75		1				
76	1					
78	2					
79						3
80				2		
81		1				
82			1			
83			1			
84	1		1			
85		12				
86					11	
87					2	
88		1	1			
91		2			1	
98		1				
99		1				
103						1
104						1
105		1				
106		1				
107		1				
108		1				
109		1				
110	1					
111		1				
112					1	

Figures

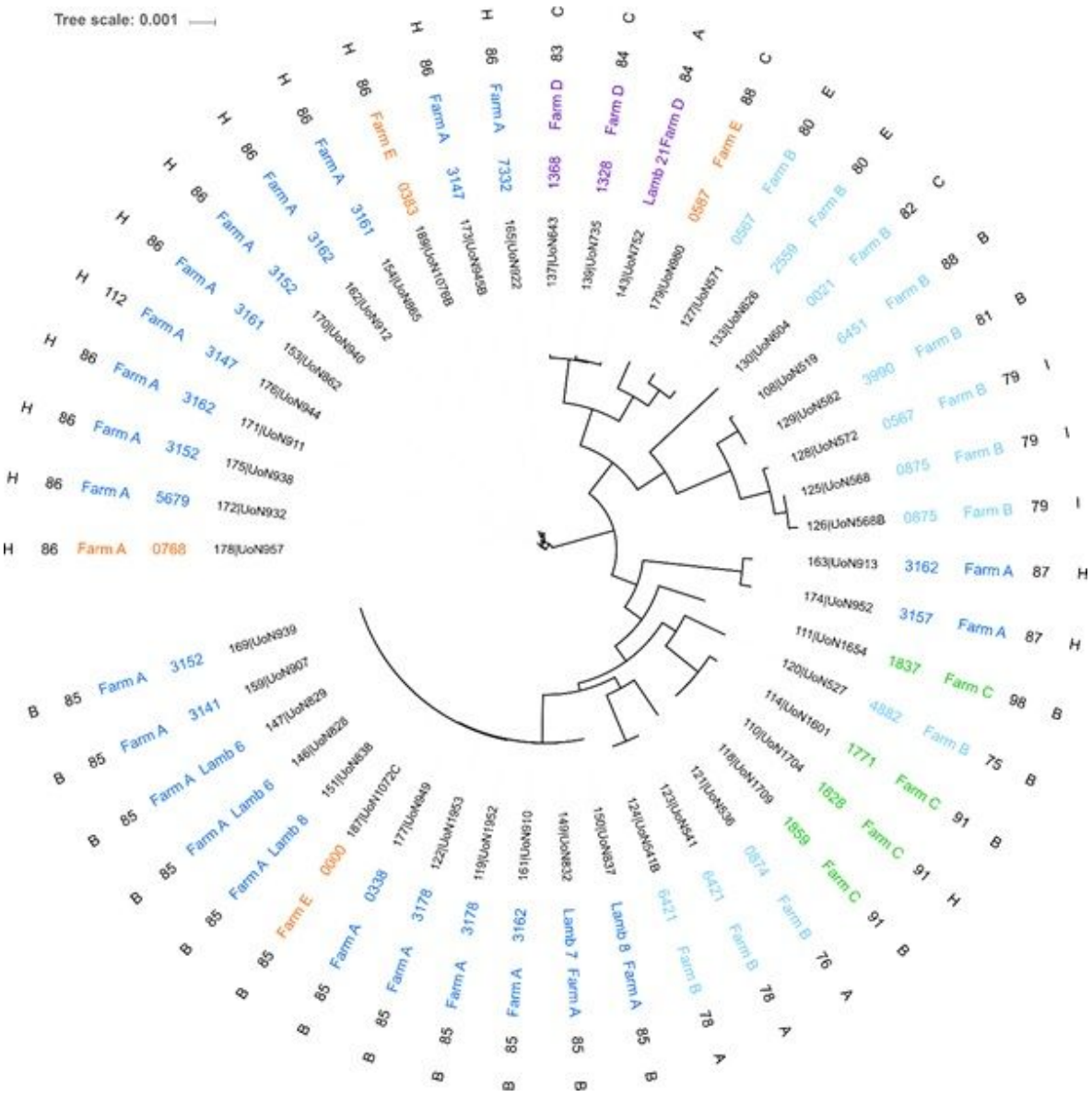

Figure 1

Figure 1 Core genome MLST of D. nodosus. Phylogeny inferred using maximum-likelihood double precision, implemented in FastTree Labels from leaf tips outwards are Isolate ID and name, sheep identification number, farm identifier, Sequence type and Serogroup.

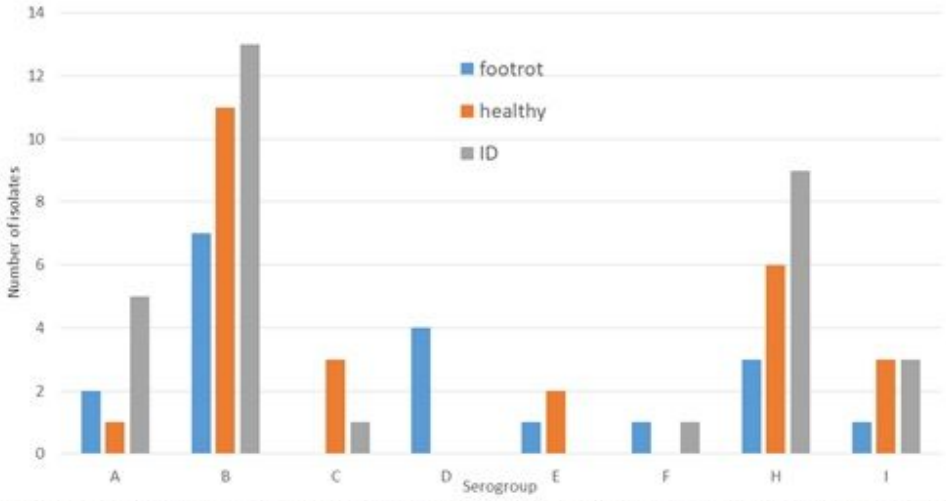

Figure 2 The frequency distribution of isolates by serogroup for each of the three hoof heath categories (Heathy – no abnormalities, Interdigital Dermatitis (ID), Footrot – ID plus underrunning of the hoof capsular horn)

Figure 2