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Detection and Serogrouping of *Dichelobacter nodosus* Infection by Use of Direct PCR from Lesion Swabs To Support Outbreak-Specific Vaccination for Virulent Footrot in Sheep

Andrew S. McPherson,[a] Om P. Dhungyel,[a] Richard J. Whittington[a]

[a]Farm Animal Health, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camden, NSW, Australia

ABSTRACT Virulent footrot is an economically significant disease in most sheep-rearing countries. The disease can be controlled with vaccine targeting the fimbriae of virulent strains of the essential causative agent, *Dichelobacter nodosus*. However, the bacterium is immunologically heterogeneous, and 10 distinct fimbrial serogroups have been identified. Ideally, in each outbreak the infecting strains would be cultured and serogrouped so that the appropriate serogroup-specific mono- or bivalent vaccine could be administered, because multivalent vaccines lack efficacy due to antigenic competition. If clinical disease expression is suspected to be incomplete, culture-based virulence tests are required to confirm the diagnosis, because control of benign footrot is economically unjustifiable. Both diagnosis and vaccination are conducted at the flock level. The aims of this study were to develop a PCR-based procedure for detecting and serogrouping *D. nodosus* directly from foot swabs and to determine whether this could be done accurately from the same cultured swab. A total of 269 swabs from the active margins of foot lesions of 261 sheep in 12 Merino sheep flocks in southeastern Australia were evaluated. DNA extracts taken from putative pure cultures of *D. nodosus* and directly from the swabs were evaluated in PCR assays for the 16S rRNA and *fimA* genes of *D. nodosus*. Pure cultures were tested also by the slide agglutination test. Direct PCR using extracts from swabs was more sensitive than culture for detecting and serogrouping *D. nodosus* strains. Using the most sensitive sample collection method of the use of swabs in lysis buffer, *D. nodosus* was more likely to be detected by PCR in active than in inactive lesions, and in lesions with low levels of fecal contamination, but lesion score was not a significant factor. PCR conducted on extracts from swabs in modified Stuart's transport medium that had already been used to inoculate culture plates had lower sensitivity. Therefore, if culture is required to enable virulence tests to be conducted, it is recommended that duplicate swabs be collected from each foot lesion, one in transport medium for culture and the other in lysis buffer for PCR.

KEYWORDS *Dichelobacter nodosus*, diagnosis, footrot, serogroup, sheep, vaccines

Footrot is an economic and animal welfare concern in most sheep-rearing countries (1, 2). The clinical disease is the result of complex interactions between the essential causative agent, *Dichelobacter nodosus*, and the host and its environment (3). It is a highly infectious disease that manifests as painful separation of the horny epidermis of the hoof, exposing the sensitive underlying dermal tissues. *D. nodosus* possesses an abundance of fine, filamentous appendages called fimbriae (4) which constitute the primary surface (K) antigen (5). Isolates are divisible into 10 serogroups (A to I and M) by means of the slide agglutination test, which classifies strains according to the presence of the major, group-specific fimbrial epitopes (6–8) that are encoded by the *fimA* gene (9). *D. nodosus* strains are further divisible into two classes based on the

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Address correspondence to Richard J. Whittington, richard.whittington@sydney.edu.au.

arrangement of the fimbrial gene region: class I, which consists of serogroups A, B, C, E, F, G, I, and M, and class II, which consists of serogroups D and H (8, 10).

Fimbriae are highly immunogenic, and vaccines incorporating fimbrial proteins are protective (11, 12), but immunity is serogroup specific, with little or no cross-protection between serogroups (13). Up to 7 serogroups may be present in a flock of sheep (7, 11). Multivalent vaccines targeting up to nine serogroups have been investigated in previous trials (14, 15) and remain commercially available in some countries but provide only limited protection due to antigenic competition (16). However, outbreak-specific mono- and bivalent vaccines can be used successfully to treat, prevent, and eradicate virulent footrot (11, 12) as they avoid antigenic competition. Direct comparisons of multivalent vaccine and bivalent vaccine have not been reported.

Currently, in order to target the appropriate *D. nodosus* strain(s) with a mono- or bivalent vaccine, the infecting *D. nodosus* strain(s) must be cultured from lesion material and serogrouped using a slide agglutination test (5) or multiplex *fimA* PCR (17). These culture-based methods are slow, requiring up to 6 weeks for a result. This can delay the implementation and, potentially, the success of outbreak-specific vaccination programs (11). Furthermore, culture-dependent testing is unlikely to detect all serogroups present in a flock unless there is an intensive-sampling strategy (18). Consequently, there is a need for a more rapid and more sensitive testing procedure.

Strains of *D. nodosus* differ in virulence, a phenotype that is independent of serogroup, and this leads to forms of the disease that differ in severity. In Australia, these are dichotomously classified as benign footrot and virulent footrot (19). It is not economically justifiable to impose quarantine restrictions or use vaccine or other treatments unless the diagnosis is virulent footrot. Importantly, diagnosis is always made at the flock level. In some states (New South Wales, South Australia, and Western Australia), quarantine and mandatory disease control are imposed but only in flocks with virulent footrot. While a clinical diagnosis of virulent footrot is often obvious, sometimes the environment is not conducive to disease expression and a virulence test based on the protease activity of pure cultures of *D. nodosus* is used (19). Such a test is mandatory in Western Australia, and the results take priority over clinical diagnosis. The only currently approved virulence tests in Australia require culture and assessment of protease activity, because real-time PCR tests for detection of the *aprV2* protease gene lack specificity in Australian sheep flocks (20).

While direct (culture-independent) tests for *D. nodosus* infection had long been sought (21) and, more recently, have been reported using both conventional and real-time PCR platforms targeting the 16S rRNA, *pnpA*, *rpoD*, and *aprV2* and *aprB2* genes (22–29), the diagnostic performances of these tests can vary (20, 23, 25, 30), and there are no reports of direct PCR-based serogrouping methods validated against a reference test at the flock level. That these could be developed is suggested by reports of PCR amplification followed by cloning and sequencing (31, 32) or of PCR–single-strand conformational polymorphism analysis (33) of class I- and class II-specific regions of the *fimA* gene. However, *fimA* has not been validated previously as a target for detection of *D. nodosus per se*.

The primary objectives of this study were to develop and validate a procedure for the direct detection and serogrouping of *D. nodosus* from foot lesion swabs and to determine whether this could be done accurately from the same swab that is cultured. Previously published conventional multiplex PCR assays targeting the single-copy *fimA* gene (17) and the triple-copy 16S rRNA gene (24) were compared to the reference tests of culture of *D. nodosus*, the slide agglutination test, and serogroup-specific PCR on pure cultures.

MATERIALS AND METHODS

Collection of foot swabs. Twelve Merino sheep flocks with preexisting diagnoses of virulent footrot that were located in southeastern Australia (Tasmania and New South Wales) were included in this study. Flock-level diagnosis of virulent footrot was based on the proportion of sheep with at least one severe (score 4) lesion (34). Each sheep examined in each flock was placed in dorsal recumbency, and each foot was examined. Foot swabs were collected from sheep with footrot lesions by one of us or by an

experienced veterinarian for diagnostic purposes. A score (0 to 4) was assigned to each foot of each sheep according to a published previously scoring system (35). Foot swabs were collected from the active zone of the interdigital skin lesion or the active margin of a lesion beneath the horn of the hoof using a sterile, cotton-tipped swab (CLASSIQSwabs; Copan Italia, Brescia, Italy). Two swabs were collected from each foot lesion by operator 1, who handed them to a second operator, operator 2, who had no knowledge of which swab was which and who placed one of the two swabs, chosen in no particular order, into a 5-ml serum vial (Techno Plas, St Marys, Australia) containing approximately 5 ml of modified Stuart's transport medium (mSTM) (36) for microbiological culture and DNA preparation and the other swab into a 1.5-ml screw-cap microcentrifuge tube (SSIBio, Lodi, CA, USA) containing 500 μl of lysis buffer (LB) (Buffer RLT; Qiagen, Hilden, Germany) for DNA preparation only. All swabs were transported to the laboratory on ice.

Isolation of *D. nodosus*. *D. nodosus* was isolated from each lesion swab collected in mSTM, as described previously (19). Individual *D. nodosus* colonies, identified by colony morphology (19), were picked from the primary culture plate using a sterile inoculation loop and subcultured onto 2% hoof agar (HA) (37), as described previously (19). This process was repeated until a pure culture of each *D. nodosus* isolate was obtained.

Slide agglutination test. Each *D. nodosus* isolate was serogrouped using the slide agglutination test as described previously (7). Briefly, individual *D. nodosus* colonies were subcultured on 2% HA (37) and incubated as described above. Each pure culture was harvested by flooding the surface of the agar plate with 500 μl of sterile phosphate-buffered saline (PBS; pH 7.4) (Astral Scientific, Taren Point, Australia) with 0.5% (wt/vol) formalin (Fronine, Riverstone, Australia), scraping the *D. nodosus* colonies from the surface of the agar with a sterile scalpel blade, and collecting the suspended culture into a 1.5-ml screw-cap microcentrifuge tube (SSIBio, Lodi, CA, USA). Each suspension was mixed for 10 s in a vortex mixer and visually assessed to ensure an even suspension. Antisera, which were prepared in rabbits for each of the 10 *D. nodosus* prototype serogroup antigens as described previously (7) and stored at −20°C, were brought to room temperature. Twenty microliters of the harvested *D. nodosus* suspension was mixed with 5 μl of undiluted rabbit antiserum on a clean glass microscope slide. The slide was gently rocked for 10 s and examined. A reaction was regarded as representing a positive result when a substantial coarse agglutination reaction result was observed within 10 s of the serum and *D. nodosus* suspension being mixed. A slide agglutination test result was classified as ambiguous when a very fine (low-titer) or delayed agglutination reaction was observed; a result was classified as negative if no reaction was observed.

Elastase test. The virulence of a *D. nodosus* isolate was assessed using the elastase test, as described previously (38). Virulent *D. nodosus* type strain A1001 (elastase positive at 4 to 8 days postinoculation) was used as a virulent control. Plates were incubated in anaerobic jars containing an anaerobic gas pack (Gas Pak; BD, Cockeysville, MD, USA) and an anaerobic indicator (Oxoid, Hampshire, United Kingdom) at 37°C. Plates were examined after 4, 8, 12, 16, 20, 24, and 28 days of incubation and reincubated anaerobically after each examination. An isolate was classified as elastase positive (virulent) if growth was observed and a zone of clearing was observed within 12 days. If a zone of clearing was observed later or not at all, the isolate was classified as elastase negative (benign), in accordance with the dichotomous classification system used by Australian animal health agencies (19).

DNA extraction. DNA was extracted from a pure culture of a *D. nodosus* isolate by boiling and centrifugation, as described previously (17). DNA was extracted from swabs by magnetic bead separation using a BioSprint 96 (BS96) One-For-All Vet kit (Qiagen, Hilden, Germany) in accordance with the BS96 Vet 100 protocol. DNA was stored at −20°C prior to testing.

Direct PCR testing of swabs collected in mSTM (PCR-mSTM) and LB (PCR-LB). mSTM is recommended for the transport of specimens of lesion material for microbiological culture by Australian animal health agencies (19). It would be advantageous if a single swab collected in mSTM could be used for microbiological culture for virulence tests and then for direct PCR testing. Therefore, we compared microbiological culture and direct PCR using a single swab collected into mSTM. Immediately after being used to inoculate a HA plate (see above), each mSTM swab was transferred to a 1.5-ml screw-cap microcentrifuge tube (SSIBio, Lodi, CA, USA) with 500 μl of LB and incubated at 4°C overnight. The microcentrifuge tube was mixed in a vortex mixer at high speed for 30 s to separate material from the swab, and the swab was discarded. DNA was extracted from the lysate via magnetic bead separation. DNA was prepared from the lysate of the LB swabs in the same manner.

PCR detection of *D. nodosus* 16S rRNA gene. *D. nodosus* was detected by PCR amplification of a variable region of the 16S rRNA gene after optimizing a published assay (24). Primers and reaction conditions were as reported previously (24); however, a customized touchdown thermal cycling program was developed consisting of an initial denaturation step of 95°C for 3 min, followed by 2 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; 2 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; 10 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 15 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s and a final extension step of 72°C for 4 min. This was prompted by previous reports of poor sensitivity and nonspecific PCR products associated with the use of these primers under the cycling conditions reported by La Fontaine et al. (24) or under modified cycling conditions (23, 25). Amplification was performed in a Bio-Rad T100 thermal cycler (Bio-Rad, Gladesville, Australia). PCR products were visualized on a 2% agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, South Korea) and viewed under UV light. DNA prepared from virulent *D. nodosus* prototype strain A1001 and nuclease-free water were included in each run as positive and negative controls, respectively. A successful PCR run was defined as one in which (i) there was amplification of the positive control

(indicated by the presence of an amplicon of the appropriate molecular weight on the 2% agarose gel) and (ii) there was no amplification of the negative control.

PCR detection of *D. nodosus fimA* gene. PCR serogrouping was undertaken by conventional PCR amplification of serogroup-specific variable regions of the *fimA* gene, as described previously (17). Amplification was performed in a Bio-Rad T100 thermal cycler (Bio-Rad, Gladesville, Australia). A serogroup M-specific PCR assay was not available. DNA extracted from pure cultures of each of the appropriate *D. nodosus* serogroup prototype strains and sterile nuclease-free water were included in each run as positive and negative controls, respectively. PCR product was visualized on a 2% agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, South Korea) and viewed under UV light. A successful PCR run was defined as one in which (i) there was amplification of the positive controls (indicated by the presence of three amplicons of the appropriate molecular weights on the 2% agarose gel) and (ii) there was no amplification of the negative control.

Statistical analyses. The level of agreement between the results from microbiological culture and those from a PCR procedure was evaluated using Cohen's kappa statistic (39) and interpreted using previously proposed standards for strength of agreement (40). McNemar's chi-square test for paired observations (41) was performed to test differences in the sensitivities of the two tests. The influence of the relevant factors on the detection of *D. nodosus* by microbiological culture or 16S rRNA gene PCR was evaluated using a generalized linear mixed model (GLMM). A binary outcome for the detection of *D. nodosus* via microbiological culture (1 = positive [one or more *D. nodosus* colonies were obtained], 0 = negative [no *D. nodosus* colonies were obtained]) or PCR (1 = positive [the *D. nodosus* 16S rRNA gene was amplified and a band of the appropriate size visualized via gel electrophoresis], 0 = negative [the *D. nodosus* 16S rRNA gene was not amplified]) was coded for analysis. The type of lesion (active or inactive), the lesion score (0 to 4, as described by previously [35]), and the degree of soil/fecal contamination (low, moderate, or high) were accounted for in the fixed model, including interactions between these three factors where computationally possible, while the farm of origin was accounted for in the random model. Categories in which there were fewer than five observations were collapsed prior to analyzing the data. All analyses were conducted in GenStat 16th Edition (VSN International Ltd., Hemel Hempstead, United Kingdom).

RESULTS

A total of 269 foot swabs collected from 269 foot lesions from 261 sheep in 12 Merino flocks in southeastern Australia between September and December 2014 were placed into lysis buffer (LB) and into modified Stuart's transport medium (mSTM) (36) (Table 1). Swabs were collected from one foot of each sheep, except for flock 2, for which two feet were sampled from each of 8 sheep and one foot from the other 34 sheep. Subcultures of 290 *D. nodosus* isolates obtained from culture of the mSTM swabs were analyzed by the slide agglutination test, and subcultures of 275 of these were also analyzed by *fimA* PCR. Fifteen subcultures that failed to grow were not tested by *fimA* PCR. A total of 62 foot swabs collected into mSTM from three flocks (flocks 10 to 12) were not retained after being used to inoculate HA plates; therefore, direct PCR results for foot swabs collected into mSTM were available for 207 foot swabs collected from flocks 1 to 9. DNA extracts from each of the foot swabs were analyzed in the various PCR assays, which are named below according to the type of swab and the gene target.

Comparison of sample collection methods for culture-independent serogrouping. In order to undertake culture-independent serogrouping directly from foot swabs using PCR, we first needed to ensure that the extraction and detection methodologies were satisfactory for the detection of *D. nodosus per se*. This was done because microbiological mSTM is commonly used for sample collection, because culture may be required by regulatory authorities for virulence testing, because it would be advantageous to have to collect only one swab for both tests, and because *fimA* had never been used before as the sole test for the presence of *D. nodosus* and has unknown diagnostic specificity. Therefore, assays were conducted on swabs placed into the mSTM after they had been used to inoculate hoof agar plates and on swabs collected into LB. Culture and PCR for the *D. nodosus* 16S rRNA gene were then used as reference tests.

16S rRNA gene PCR testing of 207 swabs collected into mSTM was more sensitive than culture of these swabs (McNemar's $\chi^2 = 7.04$, $P = 0.0082$); there were 32 foot swabs on which *D. nodosus* was detected by 16S rRNA gene PCR only and 14 foot swabs on which *D. nodosus* was detected by culture only. *D. nodosus* was detected by PCR on approximately 86% of foot swabs compared to 77% detected by culture. The level of agreement between the two methods was fair (kappa = 0.282).

A similar analysis was done using 269 foot swabs collected into LB. 16S rRNA gene PCR testing of swabs in LB was more sensitive than culture of mSTM swabs (McNemar's

TABLE 1 Four different methods of serogrouping *D. nodosus* compared at the flock level[a]

				Culture based serogrouping (pure cultures)				Culture independent serogrouping (lesion swabs)			
				Slide agglutination		*fimA* PCR		*fimA* PCR on mSTM swabs		*fimA* PCR on LB swabs	
Flock	No. of sheep examined	No. of sheep sampled	No. of feet sampled	No. of isolates tested	Serogroup(s) detected	No. of isolates tested	Serogroup(s) detected	No. of swabs tested	Serogroup(s) detected	No. of swabs tested	Serogroup(s) detected
1	51	24	24	25	A, (D), (G)	25	A	24	A	24	A
2	52	42	50	46	A, B, H, I, (D), (F), (G)	36	A, B, H, I	50	A, B, G, H, I	50	A, B, G, H, I
3	42	20	20	36	C, D, E, G, I, (H), (M)	34	C, D, E, G, H, I	20	D, G, H	20	D, E, G, H
4	33	20	20	36	A, B, E, H, I, (C), (D)	36	A, B, E, H, I	20	A, B, E, G, H	20	A, B, E, G, H, I
5	34	20	20	33	A, B, D, G, H, (C), (M)	33	A, E, D, G, H, I	20	A, B, E, G, H	20	A, B, D, E, G, H
6	28	20	20	19	A, B, (D), (E), (H), (I)	19	B	20	A, B	20	A, B
7	13	13	13	13	A, B	13	B	13	A, B	13	A, B
8	27	20	20	20	E, G, H, (B)	20	E, G, H	20	G, H	20	E, G, H
9	20	20	20	14	A, B, E, G, H	14	A, B, E, H	20	A, B, E	20	A, B, E, G
10	12	12	12	11	G	10	G	0	Not tested	12	E, F, G, H
11	26	25	25	22	C, H, (B), (D), (E), (F)	21	H	0	Not tested	25	A, C, D, E, G, H
12	26	25	25	15	E, (C), (G)	14	E	0	Not tested	25	E
Total	364	261	269	290		275		207		269	

[a]Ambiguous slide agglutination test results are shown in parentheses. mSTM, modified Stuart's transport medium; LB, lysis buffer.

TABLE 2 Comparison of results of 16S rRNA gene PCR conducted on DNA extracts from mSTM swabs and LB swabs for the detection of *D. nodosus*[a]

PCR-LB result	No. of PCR-mSTM results		Total
	Negative	Positive	
Negative	10	4	14
Positive	20	173	193
Total	30	177	207

[a]Analyses are performed at the swab/foot level. McNemar's $\chi^2 = 10.67$; $P = 0.0011$; kappa = 0.399; 95% CI = 0.201 to 0.588.

$\chi^2 = 34.32$, $P < 0.0001$); there were 52 foot swabs on which *D. nodosus* was detected by PCR only and 7 foot swabs on which *D. nodosus* was detected by culture only. *D. nodosus* was detected on approximately 95% of foot swabs by PCR and 78% of foot swabs by culture. The level of agreement between the two method was poor (kappa = 0.118).

A comparison of 16S rRNA gene PCR results for foot swabs collected from 207 feet into both mSTM and LB confirmed that use of LB swabs was superior (McNemar's $\chi^2 = 10.67$, $P = 0.0011$) (Table 2). Thus, detection of *D. nodosus* using 16S rRNA gene PCR of swabs collected into LB was a significantly more sensitive procedure than culture.

The direct use of *fimA* PCR on LB swabs was compared with culture and 16S rRNA gene PCR on LB swabs for the detection of *D. nodosus*. *fimA* PCR was more sensitive than culture (McNemar's $\chi^2 = 9.94$; $P = 0.0017$) to the extent that the level of agreement between the two tests was poor (kappa = 0.092; 95% confidence interval [CI], −0.038 to 0.221). The procedure that used 16S rRNA gene PCR on LB swabs was more sensitive than the *fimA* PCR on LB swabs (McNemar's $\chi^2 = 10.53$, $P = 0.0012$), leading to poor agreement between these tests (kappa = 0.070; 95% CI, 0.070 to 0.211). Nevertheless, there was agreement between *fimA* PCR and 16S rRNA gene PCR for 84.5% of the LB swabs tested.

Impact of lesion characteristics on the detection of *D. nodosus*. The impact of three foot lesion characteristics (foot score, lesion type, and fecal/soil contamination) on the detection of *D. nodosus* by culture and direct PCR was evaluated. Data representing the proportion of sheep assigned to each category in each flock are provided in Table 3. A relatively small number of inactive lesions were sampled, as we were attempting to sample mostly active lesions; however, inactive lesions were found to

TABLE 3 Frequency of observations for each lesion characteristic in each flock at the time of collection of swabs[a]

Factor	Flock no.											
	1	2	3	4	5	6	7	8	9	10	11	12
Foot score												
1	11	1	2	0	2	0	2	1	1	0	1	2
2	2	2	2	0	2	1	2	4	10	0	9	14
3	0	7	11	2	11	9	2	8	4	7	4	6
4	11	40	5	18	5	10	7	7	5	5	11	3
Total	24	50	20	20	20	20	13	20	20	12	25	25
Lesion type												
Active	18	46	18	20	20	20	13	19	20	12	25	22
Inactive	6	4	2	0	0	0	0	1	0	0	0	3
Total	24	50	20	20	20	20	13	20	20	12	25	25
Contamination												
Low	5	21	14	19	14	15	3	6	0	10	4	8
Moderate	19	17	6	1	5	5	7	11	11	2	14	17
High	0	12	0	0	1	1	3	3	9	0	7	0
Total	24	50	20	20	20	20	13	20	20	12	25	25

[a]Foot scores were assigned using a scoring system (35). "Contamination" refers to the presence of soil and feces on the foot lesion.

TABLE 4 Frequency of positive microbiological culture and direct 16S rRNA gene PCR results for the detection of *D. nodosus*[a]

Variable	Microbiological culture		16S rRNA gene PCR on mSTM swabs		16S rRNA gene PCR on LB swabs	
	% positive swabs	Total no. of swabs	% positive swabs	Total no. of swabs	% positive swabs	Total no. of swabs
Foot score						
1	45.7	25	45.5	22	84.0	25
2	75.0	44	95.2	21	97.7	44
3	90.7	75	98.3	58	100	75
4	75.0	125	84.8	106	92.7	125
Lesion type						
Active	79.8	253	88.1	194	96.0	253
Inactive	50.0	16	46.2	13	75.0	16
Contamination						
Low	89.8	118	94.8	96	99.2	118
Moderate	75.7	115	80.5	82	93.0	115
High	47.2	36	69.0	29	86.1	36

[a]Data are arranged according to the fixed terms in the GLMM. Foot scores were assigned according to a scoring system (35). "Contamination" refers to the presence of soil and feces on the foot lesion.

have a significant effect on the detection of *D. nodosus* in that they were less likely to yield a positive test outcome. Lesion scores were assigned to each foot using a previously described scoring system (35). Score 4 lesions were distributed across all 12 flocks, which was consistent with a clinical diagnosis of virulent footrot. Contamination of foot lesions with soil and fecal material was present in all flocks. The frequencies of positive test outcomes, arranged according to each lesion characteristic, are provided in Table 4 and reveal that the lesion type and the degree of contamination had a marked impact on the likelihood of test positivity for each test and that foot score also appeared to be important for culture and PCR performed with mSTM swabs but not with LB swabs. These features were confirmed with statistical analysis using a generalized linear mixed model (GLMM). Using microbiological culture, the values corresponding to the odds of detecting *D. nodosus* in a score 2, score 3, and score 4 lesion were 1.72, 7.32, and 1.90 in comparison to detecting *D. nodosus* in a score 1 lesion, respectively, with the significant difference ($P = 0.021$) being due to the comparison of score 3 to reference category score 1. The value corresponding to the odds of detecting *D. nodosus* in an inactive lesion was 0.26 in comparison to detecting *D. nodosus* in an active lesion ($P = 0.027$), while the values corresponding to the odds of detecting *D. nodosus* in lesions with moderate and low degrees of contamination were 4.99 and 11.21 in comparison to detecting *D. nodosus* in a lesion with a high degree of contamination, respectively ($P < 0.001$) (see Table S1 in the supplemental material). Interactions between these factors were not significant ($P > 0.05$).

Using 16S rRNA gene PCR on mSTM swabs, the values corresponding to the odds of detecting *D. nodosus* in a score 2 lesion, a score 3 lesion, and a score 4 lesion were 40.13, 25.25, and 12.74 in comparison to detecting *D. nodosus* in a score 1 lesion, respectively. The value corresponding to the odds of detecting *D. nodosus* in an inactive lesion was 0.09 in comparison to detecting *D. nodosus* in an active lesion, while the values corresponding to the odds of detecting *D. nodosus* in lesions with moderate and low degrees of contamination were 14.32 and 25.15 in comparison to detecting *D. nodosus* in a lesion with a high degree of contamination, respectively. These results were statistically significant ($P < 0.001$) (see Table S2). Interactions between these factors were not significant ($P > 0.05$).

Using 16S rRNA gene PCR on LB swabs, the value corresponding to the odds of detecting *D. nodosus* in an inactive lesion was 0.10 in comparison to detecting *D. nodosus* in an active lesion ($P = 0.008$). The value corresponding to the odds of detecting *D. nodosus* in a lesion with low or moderate soil and fecal contamination was

8.50 in comparison to detecting *D. nodosus* in a lesion with a high degree of contamination ($P = 0.007$), while the results of comparisons of the lesion scores, which had been collapsed into two categories due to sample size (score 1 and 2 versus score 3 and 4), were not significant ($P = 0.422$) (see Table S3). The data from the model did not converge with the interactions included, so they were omitted. Examination of the results for each foot score category (Table 4) suggested little difference in positive rates between foot scores and substantial differences due to lesion type and contamination, consistent with the results of the GLMM.

Culture-dependent serogrouping. Subcultures of 290 *D. nodosus* isolates were tested with the slide agglutination test, and subcultures of 275 of the same isolates were tested with the multiplex *fimA* PCR (17). The number of *D. nodosus* serogroups detected in each flock using a combination of the slide agglutination test and *fimA* PCR testing of pure cultures ranged from one to seven (Table 1). The number of *D. nodosus* serogroups detected on a single swab/foot using a combination of the slide agglutination test and the *fimA* PCR ranged from one to three. There were five instances in which a serogroup was detected at the flock level by the slide agglutination test only and two instances in which a serogroup was detected at the flock level by *fimA* PCR only.

Ambiguous slide agglutination test outcomes were observed for 158 isolates originating from 9 of the 12 flocks. These are shown in parentheses in Table 1. In contrast, the serogrouping outcomes from the multiplex *fimA* PCR assays were unambiguous—that is, there was a clear serogroup result for every culture that was tested in the PCR.

The slide agglutination test results and the *fimA* PCR results were compared at the foot level for the detection of *D nodosus* serogroups. Given that there was only one sampling event per foot, this comparison was also a swab-level comparison. There was complete agreement between these tests for 67.4% of swabs/feet tested. In the remaining 32.6% of swabs/feet tested, one or more different serogroups were detected in one or other of the tests.

Elastase test. A total of 162 of the 290 *D. nodosus* isolates, distributed across all 12 flocks, were subjected to the elastase test. Approximately 78% of the isolates tested were elastase positive. Even though 107 isolates were not tested, in most flocks there were elastase-positive isolates within each of the serogroups detected (data not shown).

Direct serogrouping. DNA prepared from 269 lesion swabs collected into LB from sheep in 12 flocks and DNA prepared from 207 swabs collected into mSTM from sheep in 9 flocks were analyzed by multiplex *fimA* PCR (17) (Table 1). The number of serogroups detected in each flock by *fimA* PCR of mSTM swabs ranged from one to five. The number of serogroups detected in each flock by *fimA* PCR of LB swabs ranged from one to six. Up to four additional serogroups were identified in each of these flocks by direct *fimA* PCR on swabs collected into LB compared with those identified by slide agglutination and *fimA* PCR testing of pure cultures, but only one additional serogroup was detected in two flocks by direct *fimA* PCR testing of swabs collected into mSTM. In five of nine flocks, more serogroups were detected by direct *fimA* PCR testing of swabs collected into LB than were detected by direct *fimA* PCR testing of swabs collected into mSTM. Up to four serogroups were detected on one foot by *fimA* PCR of mSTM swabs, and up to five serogroups were detected on one foot by *fimA* PCR of LB swabs.

The results obtained with culture performed using mSTM swabs and with direct *fimA* PCR using LB swabs were compared at the swab/foot level for the detection of *D. nodosus* serogroups. There was complete agreement between culture and the direct *fimA* PCR for 33.8% of swabs/feet tested. In the remaining 66.2% of swabs/feet, one or more different serogroups were detected by one or other of the tests.

DISCUSSION

The success of outbreak-specific vaccination programs for virulent footrot is underpinned by accurate and timely detection and serogrouping of the infecting *D. nodosus* strain(s) (11, 12, 42). We developed a sensitive procedure for direct detection and

serogrouping of *D. nodosus* in specimens of lesion material on cotton swabs, based on existing conventional PCR assays targeting the 16S rRNA and *fimA* genes (17, 24). This study presents the first report of direct PCR-based serogrouping (17) of *D. nodosus* with validation against a reference test at the flock level and the first extensive comparison of the slide agglutination test and serogroup-specific PCR testing (17) of *D. nodosus* field isolates.

Procedures for sample collection, sample transport, and DNA preparation were optimized first for a PCR assay targeting the 16S rRNA gene of *D. nodosus*. Previously reported primers (24) were used; however, the assay was modified and a customized touchdown thermal cycling program was developed to enhance its sensitivity and specificity; this was prompted by reports of poor sensitivity and of nonspecific PCR products in previous studies (23, 25).

Two procedures for handling lesions swabs were compared, one of which enabled prior culture of the swab (mSTM swabs). 16S rRNA gene PCR conducted on DNA prepared from both types of swabs resulted in detection of *D. nodosus* that was more sensitive than culture detection. Notably, *D. nodosus* was detected by direct 16S rRNA gene PCR in 66% to 88% of lesions that were culture negative, which could be explained by the detection of both viable and nonviable organisms by the PCR. DNA extracts from mSTM swabs were less likely to yield positive PCR results than those from LB swabs, possibly because lesion material had been dislodged when the former were used to inoculate culture plates. If culture is not required, swabs collected in LB are recommended for direct PCR detection of *D. nodosus*. If culture is required, duplicate swabs should be collected, one in mSTM for culture and the other in LB for PCR.

With respect to the detection of *D. nodosus per se*, on the basis of these results, we undertook a three-way comparison of the results obtained with direct *fimA* PCR and 16S rRNA gene PCR (both using DNA extracts from swabs collected into LB) and with culture of swabs collected into mSTM. The direct *fimA* PCR was more sensitive than culture but was less sensitive than the 16S rRNA gene PCR. This is not surprising, since the *D. nodosus* genome includes three copies of the 16S rRNA gene (43) but only one copy of the *fimA* gene (10). Logically, the 16S rRNA gene PCR would be run in tandem with the *fimA* PCR to signal possible false-negative test outcomes in the latter. However, given that there was agreement between the two tests for 84% of samples tested, and because multiple samples need to be tested for accurate flock level diagnosis (18), use of the direct *fimA* PCR alone may be sufficient for detection of *D. nodosus* as well as for serogrouping at the flock level.

The rates of detection (86% to 95%) achieved using the direct conventional PCR procedures that were optimized in this study were higher than those reported in previous studies using conventional (25) or real-time (23) PCR amplification of the *D. nodosus* 16S rRNA gene. Although real-time PCR is generally regarded as being more sensitive than conventional PCR, these results demonstrate that high rates of detection, along with high specificity, can be achieved with a conventional PCR assay with an appropriate procedure for sample collection and DNA preparation. The difference between culture detection and direct PCR detection of the 16S rRNA gene reported here is less marked than the differences that have been reported before (23, 25); however, the rates of detection by culture reported in those studies (27% and 43%, respectively) were much lower than the rates achieved in this study (76.8% to 78.0%). We opted to use a conventional 16S rRNA gene PCR assay (24) because the objective of this study was to develop a direct testing procedure to use with the conventional *fimA* PCR (17), and the products of the two reactions could be visualized conveniently on the same agarose gel. Although the conventional 16S rRNA gene PCR assay was reported to be capable of testing specimens of lesion material directly (24), the authors concede that they were unable to detect *D. nodosus* in samples collected from score 2 interdigital lesions and speculated that the *D. nodosus* load may have been too low. Recent studies have shown that the *D. nodosus* load is highest on feet with interdigital lesions (25, 44–46), so the failures to detect *D. nodosus* in score 2 lesions were probably

due to other factors. Subsequently, the sensitivity of the 16S rRNA gene PCR test was also reported to be low when applied to direct testing of lesion material, and the reaction conditions were modified (25); that modification increased sensitivity by up to 17% compared to microbiological culture, and the false-negative rate (culture-positive results/PCR-negative results) was only 0.8% (2/263) of feet tested. However, there were concerns about the specificity of this modified form of the test, due to the presence of nonspecific PCR products (23). Consequently, we introduced further modifications to the 16S rRNA gene PCR test to enhance both sensitivity and specificity.

We undertook an extensive comparison of the slide agglutination test and the serogroup-specific PCR (Table 1). Ambiguous slide agglutination test outcomes, defined as delayed or fine-agglutination reaction results (reported in parentheses in Table 1), were frequently observed. Interpreting such results can be challenging because there are several possible causes. Fine (low-titer) agglutination reactions have been ascribed to reactions between antisera and nonfimbrial antigens (47), minor cross-reactivity between closely related serogroups that share common fimbrial epitopes can occur (6, 7), and the strength of an agglutination reaction is known to vary according to the degree of fimbriation of an isolate (48).

PCR test results from putatively pure cultures are typically given priority when slide agglutination test results are ambiguous (11); this strategy is intended to enhance the specificity of the serogrouping procedure. The slide agglutination and PCR tests are typically performed on different subcultures of the same *D. nodosus* primary culture, and multiple serogroups may be present on a single foot, so the results of the two tests do not always align (18). There were five flocks in this study in which a serogroup was detected by the slide agglutination test but not by PCR testing of pure cultures (Table 1), reinforcing the reports of limitations of culture-dependent testing that were highlighted in a previous study in which even the most intensive sampling strategies failed to identify all serogroups present in a flock (18). Substantially increasing the number of sheep examined and the number of isolates tested would increase the accuracy of current serogrouping procedures (7, 18), but intensive sampling is not practical and the cost would be prohibitive. Direct PCR testing of lesion swabs increased the number of serogroups detected in a flock compared to culture. Additional serogroups (range, one to four) were detected in four flocks (Table 1).

Using the most sensitive method of testing swabs collected into LB, *D. nodosus* was most likely to be detected in active lesions, and on feet with only minor fecal or soil contamination, but lesion score results were not significant (Table 4; see also Table S3 in the supplemental material), even though the *D. nodosus* load is known to be highest in interdigital lesions (44–46). Interestingly, *D. nodosus* was most likely to be detected by culture on feet with score 3 lesions (Table S1). This may have been due to the physical characteristics of score 3 lesions rather than to the *D. nodosus* load; at the score 3 stage, the horn of the epidermis has begun to separate only recently, forming a small cavity that is relatively free of debris and of the necrotic material from which a noncontaminated specimen can be collected. Although the *D. nodosus* load was reported to be higher on feet with interdigital lesions than on feet with severe, underrun lesions (44, 45, 49), in those studies the specimens were collected from the interdigital skin rather than from the active margin of the lesion.

Conclusions. Serogroup-specific vaccines can be used to treat and control virulent footrot, but their success is contingent upon detecting and serogrouping the infecting *D. nodosus* strain(s) that is present in the flock. Current culture-dependent methods are unlikely to detect all serogroups present in a flock. We have developed and validated a sensitive, culture-independent procedure for the detection and serogrouping of *D. nodosus* directly from lesion swabs collected into a lysis buffer. A duplicate swab for microbial culture should be collected if a culture-based virulence test is also required. Samples should be collected from active lesions that are relatively free of contamination with soil and feces. This procedure will enhance the

detection and serogrouping of *D. nodosus* and expedite the administration of serogroup-specific vaccines.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01730-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare that we have no conflicts of interest.

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