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Understanding the Molecular Epidemiology of the Footrot Pathogen *Dichelobacter nodosus* To Support Control and Eradication Programs▿†

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The Gram-negative anaerobe *Dichelobacter nodosus* is the primary etiologic agent of ovine footrot. Few studies of the genetic diversity and epidemiology of *D. nodosus* have been done, despite the economic cost and welfare implications of the disease. This study examined a large collection of Australian isolates; 735 isolates from footrot-infected sheep from 247 farms in Western Australia (WA) were tested by pulsed-field gel electrophoresis (PFGE), and a subset of 616 isolates was tested by infrequent restriction site PCR (IRS-PCR). The genetic diversity of WA isolates was compared to that of 61 isolates from three other Australian states. WA isolates were genetically diverse, with 181 molecular types resolved by PFGE, resulting in a simple diversity ratio (SDR) of 1:4 and a Simpson's index of discrimination value (*D*) of 0.98. IRS-PCR resolved 77 molecular types (SDR = 1:8 and *D* = 0.95). The isolates were grouped into 67 clonal groups by PFGE (SDR = 1:11, *D* = 0.90) and 36 clonal groups by IRS-PCR (SDR = 1:17, *D* = 0.87). Despite the high genetic diversity, three common clonal groups predominated in WA and were found in other Australian states. On some farms, molecular type was stable over a number of years, whereas on other farms genetically diverse isolates occurred within a flock of sheep or within a hoof. This study provides a large database from which to appropriately interpret molecular types found in epidemiological investigations and to identify common and unknown types that may compromise footrot eradication or control programs.

Footrot is a contagious disease affecting the hooves of sheep, goats, and occasionally cattle, with the primary etiological agent being the anaerobic bacterium *Dichelobacter nodosus* (2). The bacterium is an obligate parasite of the hoof and survives for a limited time outside the host.

In Western Australia (WA), a footrot eradication program existed between 1974 and 2006 until a control program was implemented in 2007. Under the programs, infected sheep are identified, quarantined, and treated (8). Understanding the risk factors for disease transmission improves the effectiveness of eradication or control programs. Risk factors include transmissibility of the organism, environmental factors, phenotype, virulence potential of different strains, or as in the case of footrot, clinically expressed virulence that is dependent upon optimal environmental conditions (4). Molecular epidemiology can provide information about the risk factors associated with different bacterial strains—associations between particular genotypes and phenotypes, including strain virulence and transmissibility, interstrain genetic relatedness, and identification of clonal groups—and adds to information gathered for trace-back of infection outbreaks (14). An effective molecular typing system identifies genetic diversity such that organisms isolated at different times from different geographical locations and from different hosts can be differentiated or classified into subtypes of strains (15). The pulsed-field gel electrophoresis (PFGE) method is considered the "gold standard" for molecular epidemiological studies of bacteria because it is reliable, reproducible, highly discriminatory, and sensitive enough to detect recent evolutionary divergence that may occur during a disease outbreak (1). PFGE was used on *D. nodosus* in a small study of three farms in Malaysia, in which 12 isolates were differentiated into eight molecular types (24), suggesting considerable diversity, despite the small number of isolates tested.

The purpose of this study was to use two molecular typing methods (PFGE and infrequent restriction site PCR [IRS-PCR]) to study a large collection of *D. nodosus* isolates and to determine the suitability of these methods for investigation of the molecular epidemiology of *D. nodosus* during infection outbreaks and over the long term. It was anticipated that these methods would assist in ongoing eradication and control programs for ovine footrot in WA.

MATERIALS AND METHODS

Bacterial isolates. A total of 735 *D. nodosus* isolates were obtained from infected sheep on 247 farms in WA and tested by PFGE. A subset of 616 isolates from the same 247 farms was tested by IRS-PCR. Not all isolates from the same farm with the same molecular type as obtained by PFGE were tested by IRS-PCR. A further 61 isolates were tested from three other Australian states: 16 isolates from 13 farms in New South Wales (NSW), 24 isolates from 22 farms in Victoria (VIC), and 21 isolates from 21 farms in South Australia (SA). All isolates were collected between 1976 and 2002 and stored freeze-dried or at −80°C at the National Footrot Reference Laboratory and in the Bacteriology Laboratory, Department of Agriculture and Food Western Australia. The isolates were selected at random from the collection of 10,000 isolates that originated from farms quarantined as part of the eradication program in WA. An

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TABLE 1. Adaptor sequences used for IRS-PCR

Designation[a]	Sequence 5′–3′
AH1	AGA ACT GAC CTC GAC TCG CAC G
AH2	TGC GAG T
AX1	PO_4-CTA GTA CTG GCA GAC TCT
AX2	GCC AGT A
PXT	AGA GTC TGC CAG TAC TAG AT

[a] AH1 + AH2 = adaptor AH; AX1 + AX2 = adaptor AX; PX-T and AH1 are the primers used in the PCR and amplify a subset of XbaI-CfoI restriction fragments.

unequal number of isolates was tested from each farm and from each year and ranged from 1 isolate tested for years 1976, 1978, 1988, and 1989 up to 176 isolates tested for the year 2000, 131 from 2001, and 125 from 1997.

Molecular typing methods. (i) PFGE. The PFGE method (3, 11, 12, 23) was adapted and optimized for *D. nodosus*. Bacteria were lawn inoculated onto a 50-mm plate of Trypticase arginine serine hoof maintenance medium (16) and grown under anaerobic conditions for 3 or 4 days at 37°C. Cells were suspended in 0.6 ml of ice-cold wash buffer (1 M NaCl, 10 mM Tris base [pH 8.0], 10 mM EDTA) in a 1.5-ml microcentrifuge tube, and the opacity was adjusted visually to McFarland turbidity standard opacity 5. Purity was checked by a Gram-stained smear. Agarose plugs were prepared using 300 μl of preheated (55°C) 2% chromosomal-grade agarose (Bio-Rad Laboratories, Sydney, Australia) in wash buffer, bacterial cell suspension (300 μl), and a further 300 μl of wash buffer mixed in a 1.5-ml microcentrifuge tube in a water bath set to 55°C. The final cell suspension equated to 10^9 cells per ml. Cells could be stored in wash buffer at −20°C, and plugs could made at a later date. Solidified plugs were removed from the molds and placed into 3 ml wash buffer in 5-ml tubes and stored at 4°C until required. DNA was extracted by replacing the wash buffer with 3 ml lysis buffer (1 M NaCl, 10 mM Tris [pH 8.0], 100 mM EDTA disodium salt, 0.5% Sarkosyl, 0.2% sodium deoxycholate, 1 mg/ml lysozyme, and 2 μg/ml RNase) and incubating the plugs at 37°C with gentle shaking for 1 or 2 h. The lysis buffer was replaced with 3 ml of ESP buffer (0.5 M EDTA disodium salt, 1% Sarkosyl, 100 μg/ml proteinase K) before incubation in a water bath set to 55°C for 1 or 2 h or overnight. The plugs were washed twice with Tris-EDTA (TE) buffer containing 30 μl of phenylmethylsulfonyl fluoride (PMSF) per 3 ml of TE for 1 h for each wash, subjected to two final washes with TE buffer, and then stored in TE buffer at 4°C. Each agarose plug was cut into one third (3 mm) and used for macrorestriction of the DNA. After equilibration of the plug in two washes of TE buffer, the DNA was restricted with 40 units of ApaI (Promega, Sydney, Australia) in a 100-μl volume containing restriction buffer and milliQ water for 2 h at 25°C. The digestion reaction was stopped by the addition of 0.5 M TBE buffer. The agarose plugs were inserted into a 1% agarose gel (chromosomal grade; Bio-Rad) in 0.5 M TBE buffer (10× TBE is 89 mm Tris base, 89 mM boric acid, and 2.5 mM disodium EDTA), and the restriction fragments were separated in a CHEF DR III electrophoresis system (Bio-Rad) at 6 V/cm, with a pulse time of 0.1 to 16 s for 22 h. The separated fragments were stained for 1 h with ethidium bromide (50 μl/liter) and visualized and photographed over UV light (AlphaImager 1220; Alpha Innotech Corporation, San Leandro, CA). An isolate (AC 7742) nominated as a restriction control received the same treatment as the unknowns. A Lambda ladder (Bio-Rad) molecular weight marker was placed in lanes 1 and 30.

(ii) IRS-PCR. To ensure that the same DNA was used for both typing methods, DNA was extracted from an agarose plug prepared for PFGE. An agarose plug was seated on 0.5 cm of aquarium filter wool packed into the bottom of a 0.6-ml microcentrifuge tube with a hole punctured into the base. This was inserted into a 1.5-ml microcentrifuge tube, both tubes were placed into liquid nitrogen, and the plug was snap-frozen for 3 min. The DNA (∼20 μl) was collected by centrifugation (Heraeus Biofuge Pico) at 3,500 × *g* (6,000 rpm). The IRS-PCR was performed according to Mazurek et al. (12). A 12.5-μl aliquot of restriction mixture (80 U/μl XbaI, 10 U/μl CfoI [HhaI] enzyme, and 10× multicore buffer [Promega]) was added to 2.5 μl DNA and incubated at 37°C for 1 h. The adaptors (oligonucleotides AH and AX; purchased from Invitrogen, Australia) (Table 1) were prepared as described previously (12). The AH and AX adaptors were ligated to the restriction fragments (12.5 μl) in a 7.5-μl mixture containing each adaptor (20 pmol), T4 ligase (1 U/μl) (Boehringer Mannheim, Roche Diagnostics, Castle Hill, Australia), ligase buffer (Boehringer Mannheim), and ultrapure water and incubated for 1 h at 16°C. T4 ligase was denatured at 65°C for 20 min. The restriction fragments were recut with 1 unit of each enzyme for 15 min at 37°C to ensure that no restriction fragments had reformed during ligation. A subset of fragments with an XbaI and CfoI recognition site at either end was preferentially amplified using the AHI and PXT primers (Table 1) in a 25-μl PCR mixture containing 5 μl of DNA, 20 pmol of each primer, 10× PCR buffer, and AmpliTaq (Applied Biosystems, Melbourne, Australia) at a final concentration of 1 unit 2 mM deoxynucleoside triphosphate (dNTP) mix (final concentration 100 μM), 2 μl of 25 mM $MgCl_2$, and ultrapure water. For each new batch of primers, adaptors, or dNTP, the concentration of dNTPs was reoptimized and was either 100 μM or 200 μM. Amplification of the fragments was carried out on a DNA engine (Bio-Rad) with cycling parameters of 94°C for 5 min; 30 cycles of 95°C for 2 min, 55°C for 1 min, and 72°C for 2 min; and an end cycle of 72°C for 10 min. DNA bands were separated in a 4% high-resolution agarose (MetaPhor agarose; Cambrax Bio Science Rockland) gel set in a wide minisub cell GT (Bio-Rad) using 150 V for 150 min, with the gel bed packed in ice to prevent overheating. Bands were stained and photographed as described previously. A DNA-free control and two isolates (AC6 and AC6034) nominated as restriction and amplification controls were treated the same as the unknowns. A 100-bp molecular-size marker (Geneworks, Adelaide, Australia) was placed in lanes 1 and 30.

Interpretation of fingerprints. The restriction banding patterns obtained by both techniques were examined and interpreted according to guidelines (20) that classify banding patterns as closely related, possibly related, and unrelated, based on the visual pattern of the bands compared to the outbreak strain. An isolate was closely related to the outbreak strain if the banding pattern differed by no more than two or three bands and was possibly related when there was a difference in four to six bands yet the patterns were visibly similar, and an unrelated isolate had a difference of seven or more bands and no visible similarity to the outbreak strain. Isolates were assigned a prefix, such as PFA, with PFA7a, PF7b, etc. indicating subtypes (closely related and possibly related) for fingerprints generated by PFGE, and in a similar manner the prefix IrsT was assigned to fingerprints generated by IRS-PCR. Isolates classified as closely related and possibly related were considered to form a clonal group; for example, PFA7, PFA7a, and PFA7b formed the clonal group PFA7. Cluster analysis using a dendrogram was performed with BioNumerics version 5 software (Applied Maths, Saint-Martins-Latem, Belgium) set with the Dice unweighted pair group method, with arithmetic averages and a position tolerance of 5%.

Analysis of diversity. A simple diversity ratio (SDR) was calculated by dividing the number of isolates by the number of molecular types or number of clonal types.

Simpson's index of diversity (*D*) (18) was used to estimate the probability that a single strain belonged to the *j*th type (9) such that if *D* was 0.8, then there was an 80% likelihood that any two strains in that population would belong to two different molecular types. These calculations were performed in an Excel spreadsheet (Microsoft).

RESULTS

Genetic relatedness of isolates from the four Australian states. The total of 796 isolates from 303 farms in four Australian states were resolved into 214 PFA types and further classified into 82 PFA clonal groups by PFGE, whereas the IRS-PCR, applied to a subset of 677 isolates from the same farms, resolved these into 94 IrsT types, with a further classification into 48 IrsT clonal groups. Both techniques indicated high genetic diversity in *D. nodosus* as measured by *D* and the SDR (Table 2). Overall, the PFGE method detected greater genetic diversity than the IRS-PCR ($D = 0.98$ for PFA types and $D = 0.96$ for IrsT types), and this was reflected by both methods of analysis when applied to molecular types and clonal groups.

Although a small number of isolates was investigated from each of three states (NSW, SA, and VIC), both typing methods again indicated genetic diversity between isolates when calculated using the SDR (Table 2). Due to the small numbers tested, *D* could not be calculated.

Molecular typing of 735 isolates from 247 farms in WA identified 181 PFA types and 67 PFA clonal groups. A subset of 616 isolates tested by the IRS-PCR method identified 77 IrsT types and 36 IrsT clonal groups (Table 2). The SDR was

TABLE 2. Summary of results for PFGE and IRS-PCR molecular typing of Australian isolates of *D. nodosus*

State	No. of farms	PFGE results							IRS-PCR results						
		No. of isolates	No. of PFA types	No. of clonal groups	SDR of PFA types	SDR of clonal groups	Simpson's diversity (*D*) for molecular type	Simpson's diversity (*D*) for clonal type	No. of isolates	No. of IrsT types	No. of clonal groups	SDR of IrsT types	SDR of clonal types	Simpson's diversity (*D*) for molecular type	Simpson's diversity (*D*) for clonal type
WA	247	735	181	67	1:4	1:11	0.98	0.9	616	77	36	1:8	1:17	0.95	0.87
VIC	22	24	21	18	1:1.14	1:1.33	NC	NC	24	17	13	NC	NC	NC	NC
SA	21	21	19	15	1:1.10	1:1.40	NC	NC	21	17	12	NC	NC	NC	NC
NSW	13	16	14	10	1:1.14	1:1.60	NC	NC	16	13	11	NC	NC	NC	NC
Total	303	796	214	82	1:3.7	1:9.7	0.98	0.91	677	94	48	1:7.2	1:14	0.96	0.89

IrsT, molecular type using primer PXT and IRS-PCR method; NC, not calculated; NSW, New South Wales; PFA, molecular type using restriction enzyme ApaI and PFGE method; SA, South Australia; VIC, Victoria; WA, Western Australia.

1:4 for PFA types and 1:8 for IrsT types, and *D* values were 0.98 and 0.95, respectively, for each typing method. The SDR for clonal groups was 1:11 for PFA clonal groups and 1:17 for IrsT clonal groups, and *D* values were 0.90 and 0.87, respectively (Table 2).

A dendrogram constructed from one of each of the 181 standard PFA types from WA showed that all types had a genetic relatedness greater than 50% (see Fig. S1 in the supplemental material). At a level of 75% similarity, 39 clusters were identified; at 70% similarity, 26 clusters; and at 65% similarity, 18 clusters. At 65% similarity, two main clusters containing 32% (n = 58) and 35% (n = 64) of the isolates were identified. The remaining 13% of isolates belonged in one of the remaining 16 clusters. A dendrogram of IrsT types was not constructed.

Distribution of clonal groups throughout Australia. The distribution of clonal groups throughout the four Australian states is summarized in Table 3. PFGE typing identified 82 clonal groups from 796 isolates, and 3 of these clonal groups (PFA 7, 9, and 11) were common to isolates tested from the four Australian states. Clonal groups PFA 3, PFA 6, and PFA 46 were common to three of four states (but not common to the same three states). The results from the IRS-PCR method confirmed these findings of common clonal groups between states. IrsT clonal groups 1, 13, and 29 were common to all four states, and clonal group IrstT 2 was common to three states (NSW, SA, and WA). Despite the small number of isolates tested from three states, IRS-PCR identified 11 clonal groups common among two or more of the states.

Distribution and persistence of clonal groups within WA. Of the 735 isolates tested from WA, 387 (52.6%) belonged to one of the three PFA clonal groups (PFA 7, 9, and 11) common to all four Australian states. The remaining 348 isolates (47.3%) occurred at rates of less than 5% in one of the other 64 PFA clonal groups. The most common clonal group was PFA 11, with 158 (21.5%) isolates, which contained 16 PFA molecular types. The SDR for isolates in the PFA 11 clonal group was 1:9, and *D* was 0.87. Clonal group PFA 7 was the next most common group and contained 135 (18.4%) isolates, which were resolved into 15 PFA types. The SDR was 1:10, and *D* was 0.8. The third major clonal group, PFA 9, contained 94 isolates (12.8%) (SDR = 1:5 and *D* = 0.90) and contained 18 PFA molecular types.

The remaining 64 PFA clonal groups contained less than 5% of the isolates. Five clonal groups (PFA 12, 19, 21, 24, and 8) harbored 4.9%, 4.3%, 4.0%, 2.6%, and 2.6% of the isolates, respectively, and the remaining 59 clonal groups harbored even fewer isolates.

The 735 isolates tested from WA were obtained from the period 1976 to 2002. Despite the inconsistent numbers tested from each year, some clonal groups persisted throughout the eradication program. Clonal group PFA 9 was identified every year from 1976 up to 2002. Only seven isolates from 1976 to 1988 were tested, and although clonal group PFA 7 was identified from the one isolate tested from 1988, it was isolated in subsequent years up to 2002. Clonal group PFA 11 was identified from an isolate tested from 1985 and was identified in isolates up to 2002. Other clonal groups, such as PFA 3, 15, 60,

TABLE 3. Clonal groups of *D. nodosus* common between the four states[a]

State	Clonal group no.																	
	PFA						IrsT											
WA	3	6	7	9	11		1	2	3	5	13	14	15	23	25	29		60
NSW	3	6	7	9	11	46	1	2			13			23		29	39	
SA		6	7	9	11	46	1			5	13		15			29		60
VIC	3		7	9	11	46	1	2	3	5	13	14			25	29	39	
Total no. of types				214									94					
Total no. of clonal groups				82									48					

[a] IrsT, molecular type using primer PXT and method IRS-PCR; PFA, molecular type using restriction enzyme ApaI and PFGE method; NSW, New South Wales; SA, South Australia; VIC, Victoria; WA, Western Australia.

TABLE 4. Distribution of isolates on 133 WA farms according to genetic similarity based on molecular type

Genetic classification of isolates on farms	No. (%) of farms for indicated typing method[a]	
	PFA	IrsT
Genetically identical isolates	41 (30.8)	31 (26.3)
Genetically similar isolates (clonal)	27 (20.3)	24 (20.3)
Genetically dissimilar isolates	65 (48.9)	63 (53.4)

[a] For PFA, n = 133 farms; for IrsT types, n = 118 farms. PFA, molecular type using restriction enzyme ApaI and PFGE method; IrsT, molecular type using primer PXT in the IRS-PCR method. Data taken from 133 farms from which more than one isolate was selected for molecular typing.

66, 84, and 85, were detected in the 1990s and earlier but were not identified subsequently. The genetic diversity of isolates from different years was examined from years in which there were 20 or more isolates from 20 or more farms. This applied to isolates obtained in 1990 and from 1994 to 1997 and 2000 to 2002. The SDR ranged from 1:2.3 to 1:6.3. In 1996, a total of 79 isolates were typed using PFGE, resulting in 11 PFA clonal groups (SDR = 1:7.2). In later years (2000 and 2001), 176 and 131 isolates were typed, with resulting SDRs of 1:6.1 and 1:5.9, respectively.

Genetic diversity of molecular types at the farm level and at the hoof level. More than one isolate was tested from 133 (53.8%) of the 247 WA farms by the PFGE method, and 118 (47.8%) farms had more than one isolate tested by the IRS-PCR method. Although unequal numbers of isolates were tested from each farm, these data were used to estimate the genetic diversity of the isolates from infected sheep on a farm and between farms. Three classifications of farms were identified based on the genetic similarity of the isolates obtained from the infected sheep on the farm: (i) farms that had sheep infected only with genetically identical isolates; (ii) farms that had sheep infected only with genetically similar isolates, that is, clonal isolates originating from the parent strain; and (iii) farms that had sheep infected with isolates that were genetically dissimilar (Table 4). In the first classification, of the 133 farms from which more than one isolate was tested by PFGE, 41 farms (30.8%) had only genetically identical isolates from infected sheep. Only two isolates were tested by PFGE from 73.2% of the farms with genetically identical isolates, but the remaining farms had three or more isolates tested, and four farms had seven to nine isolates tested, and all these isolates from the respective farms were genetically identical. A similar result was found for the IRS-PCR (Table 4). The second grouping comprised farms from which only genetically similar isolates were detected and constituted a clonal group, as seen by the presence of subtypes of the parent strain. The detection of genetically similar isolates occurred with 20.3% (n = 27) of farms when tested by both PFGE and IRS-PCR. The third classification was farms with genetically dissimilar or a diverse range of *D. nodosus* strains from a particular farm and included 48.9% (n = 65) of farms, as detected by PFGE, and 53.4% (n = 63), as detected by the IRS-PCR method.

An individual hoof could contain more than one molecular type. Up to three different molecular types were found with the individual hooves of 30 (4.1%) sheep on 15 (6.1%) of the 247 farms tested from WA. The isolates were either genetically similar (belonged to the same clonal group) or were genetically dissimilar. From the 30 sheep, 68 isolates were obtained and comprised 40 different PFA types and 17 PFA clonal groups. A subset of 39 isolates was tested by IRS-PCR, and 26 IrsT types were identified. These belonged to 15 IrsT clonal groups.

DISCUSSION

This study used two typing methods to determine the genetic diversity of a large number of epidemiologically related and unrelated *D. nodosus* isolates collected over almost 3 decades, with the aim of assessing the suitability of the methods for long-term epidemiological investigation of the organism and to assist in interpretation of typing results. Both methods met the recommended guidelines (22): high discrimination ($D > 0.90$) between unrelated isolates (9, 18), clearly distinguished clonal types originating from a single isolate, reproducible results, and amenability to computerized analysis, which allows longitudinal comparison of a large number of isolates to enable the tracking of the spread of genetic types over time. PFGE was more discriminatory than IRS-PCR and twice as likely to detect a genetic change. These findings agree with numerous studies in which PFGE was found to be the more sensitive typing method for detecting genetic change for use in epidemiological studies (6, 17, 19). The use of two methods gives greater confidence in the interpretation of the results because the PFGE method, which detects rapid change, can indicate genetic differences occurring during an outbreak, yet the results from the IRS-PCR, with its reduced ability to detect change, can signify genetic relationship to the outbreak strain. Some isolates had the same PFGE and IRS-PCR profiles, indicating recent infection from the source of the outbreak; other isolates had the same PFA type but a different IrsT type or vice versa, which might indicate recent divergence from the outbreak strain, or infection with a common clonal group from an unrelated epidemiological source.

Common clonal groups (PFA 7, 9, and 11) were present in all four states, despite the small numbers of isolates tested from three of the states. Footrot may have been introduced into Australia soon after British colonization in the late 1700s when sheep were imported by the early settlers (13). The finding of the same clonal groups throughout Australia validates this report and suggests a common ancestor. A different hypothesis is that the presence of major clonal groups common to the various states might be due to importation of one or more groups to individual states, followed by transmission to other states over time. A combination of these two scenarios is likely to have occurred. This hypothesis is purely conjecture but may be tested in the future by methods such as multilocus sequence typing (MLST). The origin of genetic diversity observed with Australian isolates may be resolved by examination of strains of *D. nodosus* from Spain, as Spanish Merinos were the first sheep imported to Australia, but also from Portugal and France, where the disease also has been present for more than 200 years. A technique such as MLST would provide phylogenetic data that would complement the data obtained from molecular typing. *Campylobacter coli* has been reported to have a diversity index of 0.98 using PFGE (21), the same as that for *D. nodosus*. In that study PFGE was more discriminatory than MLST, although both methods provided valuable

information about epidemiology and phylogeny. As yet MLST has not been applied to *D. nodosus* but needs to be done to more fully understand genetic diversity and phylogeny of the organism.

Despite the eradication program in WA, half the isolates tested belonged to one of three predominant clonal groups (PFA 7, 9, and 11), which have persisted over the 26-year period. This persistence may be due to the wide dispersal of these clonal groups throughout the state, or the genetic types associated with these clonal groups may have the ability to persist longer than others. Wide dispersal might have been due to "infected" farms unwittingly selling infected sheep to a large number of other farms. Prolonged dispersal prior to the introduction of the eradication program in 1974 might explain the presence of these strains throughout the state. In addition, the eradication program policy prevented footrot-infected sheep from entering WA, and although some disease incursion did happen, it was usually detected and eradicated before disease transmission occurred. In this manner the policy may have contributed to the development of the emergence of common clonal groups by preventing new molecular types from entering WA.

In the present study, farms (with sheep infected with *D. nodosus*) were categorized based on the molecular types found on each farm, that is, genetically identical, genetically similar, or genetically diverse in molecular type. Approximately half the farms had sheep infected with genetically diverse isolates of *D. nodosus*, as detected by both methods. In part, this reflects trading practices in which some farmers may buy sheep (inadvertently infected with footrot) from a number of sources and from a number of geographically diverse areas, whereas others buy sheep less often or from a reduced number of sellers. The category of farms containing genetically similar isolates may indicate prolonged infection and the rise of genetically similar isolates from the same clonal group. Within the category of farms containing genetically identical isolates, some farms had infected sheep for greater than a 2-year period but showed no development of genetic diversity within the isolates.

This study also found multiple molecular types in a single infected hoof. Isolates were either genetically closely related and belonged to the same clonal group or were genetically unrelated. Clonal types found within a hoof indicate recent genetic divergence and may be due to infections that persist over time or due to environmental stressors that induce genetic divergence. Unrelated molecular types in the same hoof might indicate transmission from other sheep infected with that particular molecular type or clonal group. These results have implications for eradication and control programs, as they highlight the importance of culture, the need for identification of different strains, and the testing of more than one isolate from a hoof. In a number of these cases, isolates that were identified as being of a different molecular group had a slight difference in colonial morphology, such as a rough or smooth appearance or dry or mucoid appearance.

Results of PFGE from the current study are consistent with work done in Malaysia, where 12 isolates of *D. nodosus* from three epidemiologically unrelated farms had eight different molecular types (24), indicating a high genetic diversity in that *D. nodosus* population. A study in New Zealand investigated the genetic diversity of *D. nodosus* using PCR to amplify regions of the fimbrial gene (*fimA*) and found considerable genetic diversity, with 11 of 15 strains showing differences in fimbria sequences (25). These same serotypes were identified with sheep flocks in the United States (10), and it is likely that *D. nodosus* in the United States is also genetically diverse.

Field studies suggest that the expression of virulence severity in *D. nodosus* is associated with environmental factors, such as temperature and moisture (4), and the high genetic diversity seen with *D. nodosus* may be due to the organism undergoing constant genetic change as a result of environmental stressors. Although the rate of change cannot be determined from this type of study, the results indicate that typing of isolates for infection trace-back purposes should be done close to the time of the outbreak. The results of SDR for PFGE showed that every fourth isolate was of a different molecular type. Therefore, based on this figure, at least four or more isolates should be tested from each farm when investigating trace-backs and outbreaks of footrot to reliably detect all genetic types present on the farm. After prolonged infection, the clonal groups of *D. nodosus* may evolve, and therefore epidemiological investigations should be undertaken relatively quickly to positively identify a link between infected farms. All available data, such as sale yard and farmer records, should be used in conjunction with molecular typing when undertaking epidemiological investigations into footrot.

PFGE is highly discriminatory for outbreak situations, but it has been argued that the stability of PFGE may not be reliable for long-term epidemiological studies, as high degrees of genetic variation, possibly due to selective pressure or large chromosome fragments undergoing more rapid changes than small fragments, leads to multiple PFGE profiles in pandemic clones and makes the technique unsuitable for long-term studies (7). In the current study the three predominant WA clonal groups, PFA 7, 9, and 11, were isolated at the beginning and at the end of the 26-year period. Over this time, minor genetic changes led to an increase in the number of molecular types within each clonal group; however, the parent strain was still detected for the duration. Our results indicate that PFGE is suitable for long-term studies of *D. nodosus*.

A dendrogram constructed of PFA molecular types showed that strains clustered at between 50% and 100% similarity. Clusters of isolates were differentiated at particular levels of similarity, and these clusters contained isolates with a range of clonal types. These levels of similarity between unrelated strains and clonal strains are similar to findings for other bacteria. Results for *Pseudomonas aeruginosa* showed that unrelated strains had a similarity between 20% and 60%, whereas clonal strains clustered at levels above 80%. Clonal relatedness was similar for *Acinetobacter*, but the overall relatedness of all strains was as low as 50% (22). In our study, the initial assignment of molecular type to an isolate was done visually rather than by dendrogram. This ensured no bias in allocation of molecular type to an isolate based on dendrogram analysis alone. A subtype was allocated to an isolate when a minor genetic change (20) was seen from the parent molecular type on the same farm. Visual allocation of molecular type may lead to misidentification of molecular type, but any doubt about allocation of molecular type was overcome by testing isolates on the same electrophoresis gel with the "standard" type as a

control. In a number of cases, the dendrogram identified some molecular types as identical, yet visual examination of the fingerprints clearly indicated a shift in one band position relative to the other. Fingerprint types that showed two or three bands close together or the absence of a closely aligned band were not always differentiated by the software. This is dependent upon the band comparison position tolerance, which in this study was set at 5%, as recommended (5); therefore, visual comparison is vital.

In conclusion, *D. nodosus* isolates in WA were genetically diverse yet were dominated by three clonal groups, which were also found in infected sheep from three other Australian states. This study provides a database of molecular type information from which to interpret specific epidemiological situations, such as infection outbreaks and trace-backs to the source of the infection.

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