final report

Virulent footrot diagnosis

Abstract

Virulent footrot costs the sheep industry $32M pa. This project delivered new, fully validated diagnostic approaches: i) PCR for serogroup M ii) all conventional PCR serogrouping tests upgraded to a modern real time format and multiplexed, and iii) direct PCR detection of *D. nodosus* serogroups from lesion swabs, all of which will improve sensitivity and reduce test times and costs. A validation study of recently published qPCR virulence tests based on protease genes surprisingly revealed that they should not be used due to risk of false positive diagnoses. In an attempt to further develop new molecular virulence tests, single nucleotide polymorphisms in 11 potential virulence-associated genes were evaluated but were found to be not suitable virulence markers. Collectively, these studies indicate that our understanding of the molecular basis of virulence is incomplete, which hinders the development of a simple diagnostic test. Future diagnostic approaches await better understanding of the microbiome of footrot lesions. We trained post graduate students to meet future need for expertise to service industry and we conducted a National Workshop. Producers should demand application of validated diagnostic procedures by the state jurisdictions. There are considerable risks in application of non-validated novel technologies. Furthermore there is a risk that footrot is driven underground and subsequently spread more widely through lack of reporting by producers (due to stigma, risk of quarantine being applied) unless the quality of diagnosis is improved nationally and the means of diagnosis harmonised.

Executive summary

Virulent footrot is an important disease of sheep in southern Australia, costing the industry about $32M pa according to MLA data in 2015. As well as production impacts there are very serious animal welfare considerations. This project mainly concerns diagnosis of footrot in an environment where there is lack of agreement between the State jurisdictions on how to apply laboratory tests for the diagnosis of footrot. While this is partly due to different states being in different phases of control or eradication, it is also because tests have not been standardised and their accuracy has been questioned. Thus NSW places most emphasis on clinical examination of feet, while Western Australia relies on several lab tests to provide a definitive diagnosis. Regardless, all current lab tests for footrot rely on microbiological culture of *Dichelobacter nodosus* and this takes about 35 days. Furthermore, accurate diagnosis of footrot is expensive: up to $3000 per flock based on the testing of up to 20 isolates of *D. nodosus* obtained from up to 10 affected sheep. Historically, fewer sheep/isolates have been recommended to be tested by some state jurisdictions, and subsidies have been applied to reduce the costs to producers. However, epidemiological research and modelling has shown that testing fewer isolates/sheep reduces diagnostic accuracy (Hill et al., 2010). The diagnostic procedure is highly specialised and there are only a few laboratories in Australia with the capacity to perform the entire procedure. Modern technologies exist which might be able to be applied to improve diagnosis.

None of the current diagnostic tests are completely accurate. Historically there are many examples where laboratory results suggest a diagnosis of virulent footrot, but examination of sheep on farm suggests otherwise. None of the diagnostic tests have been properly validated, and it is assumed that all cases of virulent footrot are due to the presence of certain "virulent" strains of *D. nodosus.* The possibility that other bacterial species contribute to the development of severe footrot lesions has not been investigated due to lack of suitable methodologies. While *D. nodosus* is essential, other bacteria may complement so called low-virulent strains of *D. nodosus* and lead to severe lesions, or alternatively, they may compete with so-called highly virulent strains of *D. nodosus* and inhibit the development of severe lesions.

The objectives of this project were to:

1. Develop new modern diagnostic tests for virulent ovine footrot that determine whether the disease outbreak is virulent and which serogroups of *D. nodosus* bacteria are present in an outbreak.

2. Determine whether other bacterial species are preferentially associated with virulent footrot.

3. Conduct a national workshop on footrot diagnosis.

4. Build human capacity by training a PhD student and honours students.

These objectives were met through a systematic investigation, utilising samples that were purposely collected for the project between June 2014 and August 2016 from 40 flocks of sheep from different parts of Australia: 24 flocks with clinically virulent footrot and 16 flocks with clinically benign footrot. The diagnosis on each farm was determined using conventional methods including full clinical

examination and laboratory testing, and this enabled meaningful comparison with newly developed tests.

After commencement of this project two real-time PCR assays were reported in Europe as being capable of differentiating virulent and benign strains of *D. nodosus* (Stauble et al, 2014; Frosth et al, 2015). These were intended gene targets in the present project, and the project was directed to validate them using pure cultures and lesion swab samples collected from footrot outbreaks in Tasmania, South Australia, New South Wales and Western Australia. Based on preliminary evaluation data the assay of Frosth et al. (2015) was subjected to a larger test evaluation. An outbreak was more likely to be classified as virulent with the qPCR test than clinically and the level of agreement between the two approaches was only fair; the diagnostic specificity of the qPCR was only 31%. In contrast there was no significant difference between the proportion of outbreaks classified as virulent by clinical diagnosis or by the elastase test. The level of agreement between clinical diagnosis and the elastase test was almost perfect and the specificity of the elastase test was 79%. Similarly the level of agreement between the gelatin gel test and the qPCR test was poor. Overall, the elastase test was least likely, the gelatin gel test more likely and the qPCR most likely to result in false positive diagnoses of virulent footrot. In an attempt to further develop new molecular virulence tests, single nucleotide polymorphisms in 11 potential virulence-associated genes were evaluated but were found to be not suitable virulence markers. Collectively, these studies indicate that our understanding of the molecular basis of virulence is incomplete, which hinders the development of a simple diagnostic test.

Vaccination has been used successfully to control and eradicate virulent footrot from individual flocks and entire geographic regions. However, immunity is serogroup-specific. To apply vaccine against footrot in an effective way it is necessary to determine the serogroup of the strains of *D. nodosus* that are present on the farm. We previously developed serogroup-specific PCR assays for serogroups A, B, C, D, E, F, G, H and I but not for serogroup M which was identified comparatively recently in Australia. Consequently serogroup M-specific forward and reverse primers were designed and evaluated. The sensitivity and specificity of one set of primers out of many sets that were tested was suitable for routine use. This test can be run as a stand-alone conventional PCR test, in addition to the existing serogroup-specific multiplex PCR assays.

Historically these PCR assays have been applied only to pure cultures of the infecting *D. nodosus* strain(s). Direct (culture-independent) serotyping from lesion swabs has not been reported. Consequently, swab extraction methods were optimised using lesion swabs collected from sheep from twelve flocks with clinically virulent footrot. Detection of *D. nodosus per se* via PCR was significantly more sensitive than detection of the organism by culture. The slide agglutination test on pure cultures was then compared with PCR serotyping directly from lesion swabs. There were no significant differences in sensitivity between the slide agglutination test and serogroup-specific PCR, and the latter test resolved ambiguous test results from the former test. Lesion characteristics had an important effect: lesions with low levels of faecal contamination and active lesions were more likely to yield good test outcomes than dirty or dry lesions.

With advances in technology and to improve the efficiency of the tests, the possibility of adapting current convention as serogroup-specific PCR assays to a real-time PCR platform was evaluated after the design of new sets of primers. Serogroup specific real time (RT) PCRs were successfully

developed and evaluated for all 10 serogroups (A to I, and M) and were further developed into multiplex RT PCRs. In accordance with the technical capacity of the ABI 7500 real time PCR platform, which is the current industry standard used in all of the relevant animal health diagnostic laboratories in Australia, our strategy was to multiplex up to 3 targets in one reaction (ABC, DEF, and GHI into three reactions, and serogroup M with a future candidate virulence PCR). The practical application will be to run all 4 multiplex reactions against each test sample. These new multiplex real time PCR tests were evaluated with samples from pen trials and also with direct lesion swabs collected on-farm. These new RT PCR tests were more sensitive than the conventional serogroup specific PCRs on which they were based.

Footrot is a mixed bacterial disease, and microbial species other than *D. nodosus* present on the foot are critical to the disease process. For example *Fusobacterium necrophorum*, which is a constituent of the normal gastrointestinal flora, has been shown to initiate the disease by enabling *D. nodosus* to invade the epidermis, and to enhance the severity of lesions. Much of what is currently known about the aetiology of ovine footrot was determined using classical microbiological techniques and examination of histological sections. These studies were limited to a small number of microbial species that could be cultured in the laboratory, or those with distinct cell morphologies that were readily identifiable in smears or histological sections. Consequently, amplicon-based metagenomics was applied to determine which genera of bacteria are most abundant in active lesions and might therefore contribute to the disease process. The bacterial community present on the feet of clinically healthy sheep was more phylogenetically diverse than that of sheep with clinical footrot. The dominant phyla in specimens from the feet of clinically healthy sheep were Actinobacteria, Firmicutes and Proteobacteria compared to Firmicutes, Bacteroides, and Actinobacteria and Tenericutes in sheep with virulent footrot.

This project has delivered new diagnostic approaches that are fully validated. A new PCR for serogroup M will improve outcomes where vaccination is intended as a control measure, enabling serogroup M to be detected quickly for the first time. All of the conventional PCR serogrouping tests developed in 2002 have been upgraded to a modern real time format, which will improve sensitivity and reduce test times and costs. New recommendations for sampling lesions for diagnosis, specifically to avoid sampling dry or contaminated lesions should improve the diagnostic success rate nationally. Importantly, a full validation study of recently published virulence tests based on protease genes that have been touted as diagnostic solutions revealed that these tests are not accurate and if applied in Australia would result in a very high rate of false positive diagnoses. In contrast, the elastase test is suitable for routine use and is more accurate than the gelatin gel test. Investigation of the bacterial community in footrot lesions has uncovered great diversity that merits more detailed investigation. Future research should focus on understanding the true virulence mechanisms in footrot, which might be detectable in non-protease genes of *D. nodosus* and the other flora of the foot lesion. This will lead to better diagnostic strategies and control measures.

A National Workshop was successfully conducted and there was overwhelming support and an identified need for a national approach to footrot management and control. The Proceedings of this workshop were published and made available to all participants. These proceedings are already being used by field veterinarians and animal health officers as a resource and reference for footrot research and extension in the field, and should be distributed more widely.

During this project one PhD scholar and several honours students received specialist training in footrot, both on farm and in the laboratory. On-going training opportunities are required to meet future need for footrot expertise to service the sheep industries.

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1   Background

1.1 Diagnosis of virulent footrot

1.1.1 Background to diagnosis of footrot

Virulent footrot is an important disease of sheep in southern Australia. As well as production impacts there are very serious animal welfare considerations. Diagnosis of footrot is cumbersome, expensive and slow and this hinders disease control. Modern technologies exist which might be able to be applied to improve diagnosis. There is lack of agreement between the State jurisdictions on how to apply laboratory tests for the diagnosis of footrot. While this is partly due to different states being in different phases of control or eradication, it is also because tests have not been standardised between the states and the accuracy of current tests has been questioned. Thus NSW places most emphasis on clinical examination of feet, while Western Australia relies on several lab tests to provide a definitive diagnosis. Regardless, all current lab tests for diagnosis of footrot rely on microbiological culture of the causative bacterium *Dichelobacter nodosus*. The procedure is as follows:

1. Collect swabs from the lesions on the feet of affected sheep

2. Place swabs in transport medium and send to a laboratory immediately

3. Swabs are used to inoculate 4% hoof agar plates, which are incubated anaerobically for 3-4 days

4. Pure cultures of *D. nodosus* are obtained then subcultured into broth medium and incubated anaerobically for 3-4 days

5. Broth cultures are used to perform the Gelatin Gel test which takes at least 24 hours

6. Pure cultures are used to inoculate Elastin agar plates. Dissolution of elastin particles is assessed visually every few days for up to 28 days. Plates are incubated anaerobically throughout this period

7. Other tests for protease may be applied, for example the zymogram test is used in Western Australia. An *intA* PCR test is used in NSW to test for another putative virulence factor

8. Colonies of *D. nodosus* from the subculture plates are tested using 3 multiplex PCR assays to determine the pilus serogroup of *D. nodosus*

9. Slide agglutination tests can be used to check PCR results, but always need to used if serogroup M is suspected because there is not yet a PCR test available for this serogroup

10. The time for reporting of all results is up to 35 days

1.1.2 Diagnosis of footrot is expensive

The actual costs of testing have been calculated to be more than $3000 per flock based on the testing of up to 20 isolates of *D. nodosus* obtained from up to 10 affected sheep. Historically, fewer sheep/isolates have been tested, and subsidies have been applied by state jurisdictions to reduce

the costs to producers. However, recent epidemiological research and modelling has shown that testing fewer isolates/sheep reduces diagnostic accuracy (Hill et al., 2010).

1.1.3 Diagnosis of footrot depends on specialised laboratories

The diagnostic procedure outlined above is highly specialised. Currently there are only a few laboratories in Australia with the capacity to perform the entire procedure, and only the University of Sydney has the capacity to perform the serogroup test. The ovine footrot reference laboratory in Western Australia is no longer operating. There is a trend internationally for animal health diagnostic tests to be developed and provided in kit form to relatively non-specialised laboratories in the public and private sectors. This is impossible for footrot diagnosis without modernising the diagnostic procedures.

1.1.4 Accuracy of tests for footrot

None of the current diagnostic tests are completely accurate. Historically there are many examples where laboratory results suggest a diagnosis of virulent footrot, but examination of sheep on farm suggests otherwise. This could be due to lack of test standardisation and laboratory error. However, there are other possible reasons. None of the diagnostic tests have been properly validated, and it is assumed that all cases of virulent footrot are due to the presence of certain "virulent" strains of *D. nodosus.* The possibility that other bacterial species contribute to the development of severe footrot lesions has not been investigated due to lack of suitable methodologies. While it has been known for over 40 years that *D. nodosus* is essential, the contribution of the many other bacteria that are present on the feet of affected sheep is unknown. It is possible that some of these may complement so called low-virulent strains of *D. nodosus* and lead to severe lesions, or alternatively, they may compete with so-called highly virulent strains of *D. nodosus* and inhibit the development of severe lesions.

2   Project objectives

2.1   Objectives and outcomes

2.1.1   Develop new modern diagnostic tests for virulent ovine footrot that determine whether the disease outbreak is virulent and which serogroups of *D. nodosus* bacteria are present in an outbreak.

2.1.2   Determine whether other bacterial species are preferentially associated with virulent footrot.

2.1.3   Conduct a national workshop on footrot diagnosis.

2.1.4   Build human capacity by training a PhD student and honours students.

3 Methodology

3.1 Evaluation of genotypic and phenotypic virulence tests

Clinical diagnosis of virulent footrot, which is a notifiable disease in some states of Australia, is not always straightforward and reliable. *In vitro* virulence tests like the gelatin gel and elastase tests for protease activity, and the *intA* PCR test for an inserted genetic element in *D. nodosus* are commonly used to support or to confirm a clinical diagnosis. Comparative study of these 3 tests has shown the agreement between the results are poor and not reliable (Dhungyel et al., 2013d). There is a need for more reliable tests. After commencement of this project two real-time PCR assays were reported as being capable of differentiating virulent and benign strains of *D. nodosus* (Stauble et al, 2014; Frosth et al, 2015). Both assays are based on previous Australian studies which have shown that acidic protease 2 (AprV2, encoded by the gene *aprV2*) is a key virulence determinant of virulent *D. nodosus* strains. Benign *D. nodosus* strains possess the homologous gene *aprB2,* which encodes a less potent protease (AprB2). The new assays were developed in isolation by European researchers without the knowledge of researchers in Australia. Both assays are competitive real-time PCR assays.

As with any diagnostic test development, thorough evaluation and validation with an extensive collection of field samples was essential. These two assays were evaluated using pure cultures and lesion swab samples collected from footrot outbreaks in Tasmania, South Australia, New South Wales and Western Australia. Further details of the materials and methods used in this study are presented in Appendix: 9.1.2.

3.2 Development and evaluation of new virulence tests

In addition to the *aprV2* and *aprB2* genes as likely virulence markers, Kennan et al (2014) identified single nucleotide polymorphisms in an additional 11 genes in the *D. nodosus* genome that appeared to correspond with virulence, and proposed that one or more of these SNPs might be a suitable candidate for a new molecular virulence test. The number of corresponding SNPs in each of these genes ranged from one to 21. With the aim of developing a more reliable virulence test, we undertook an evaluation of these genes as potential virulence markers. We randomly selected one to three of the corresponding SNPs in each of these genes for preliminary evaluation using a panel of 20 well-defined *D. nodosus* isolates with virulent and benign genotypes and phenotypes. Conventional PCR assays were developed to amplify the region of interest in each these genes. Purified PCR products were then sequenced using the Sanger method. A comparative study of these sequences revealed that the level of correlation between the SNPs and virulence ranged from moderate to negligible. These results indicated that the SNPs evaluated in this study were not suitable candidates for the development of a new molecular virulence test. Furthermore, these genes bore no obvious relationship with any known virulence factors, and their relationship to virulence is currently unknown. Further details of this study are provided in Appendix 9.5. At the same time these 11 genes were being evaluated, the publication of new diagnostic tests by Swiss and Swedish researchers was reported in the Australian rural media, which prompted a thorough validation study of those tests, as presented in Appendix 9.5. These two studies provide further evidence that our baseline knowledge of the molecular basis of virulence is inadequate, which has hindered the project objective of developing a new molecular virulence test.

3.3   Serogroup M-specific PCR designed and evaluated

Traditionally, bacteriological culture and purification of *D. nodosus* from footrot lesion material is required before the infecting strain(s) can be serotyped by slide or tube agglutination tests. With the development of molecular diagnostic tests such as the polymerase chain reaction (PCR), and the advent of culture-independent testing methods, bacteriological culture might no longer be necessary for the identification and typing of *D. nodosus* in lesion material. Currently, serogroup-specific PCR assays are available for serogroups A, B, C, D, E, F, G, H and I. Serogroup M was identified comparatively recently in Australia and a serogroup-M specific PCR assay is not available. The prevalence of serogroup M appears to be relatively low. However, it is now routinely tested for along with serogroups A-I; this is dependent upon the slide agglutination test. Given the close relationship between serogroup M and F, misclassification of serogroup M has often occurred using this test. Therefore a serogroup M-specific PCR assay is required.

The fimbrial gene sequences of 31 *D. nodosus* strains, representing all 10 serogroups (A-I, and M), were aligned in Clustal Omega. Thereafter, a number of serogroup M-specific forward and reverse primers were manually designed. All serogroup M-specific reverse primers were evaluated in combination with the pan-serogroup forward primer used in current serogroup-specific multiplex PCR assays (Dhungyel et al., 2002b). Further details of the materials and methods used in this study are presented in Appendix: 9.3.2.

3.4   Extraction of *D. nodosus* DNA from lesion swabs optimised

Vaccination has been used successfully to control and eradicate virulent footrot from individual flocks and entire geographic regions. However, the complex nature of the disease can make vaccination challenging: immunity is known to be serogroup-specific, with little or no cross-protection offered between serogroups, and multiple serogroups may be present in a flock. In Australia up to seven serogroups have been reported in a single flock. Multivalent vaccines containing fimbrial antigens representing all 10 serogroups (A-I, and M), such as the commercial Footvax® vaccine, have been trialled but provide only partial protection for a brief period, due to the phenomenon of antigenic competition (Raadsma et al., 1994a).

To target the appropriate *D. nodosus* strains with vaccines, in each outbreak of virulent footrot the infecting *D. nodosus* strain(s) must be cultured anaerobically in the laboratory and serotyped, a specialised undertaking that is expensive and slow to generate results. To avoid laborious agglutination tests, Dhungyel et al. (2002b) developed three triplex conventional PCR assays for typing serogroups A to I, but historically these assays have been applied only to pure cultures of the infecting *D. nodosus* strain(s).

Direct (culture-independent) serotyping based on PCR amplification of variable regions of the *fimA* gene is an alternative to culture-dependent typing. However, culture-independent methods present additional challenges, and an evaluation of culture-dependent and direct (culture-independent) identification and serotyping of *D. nodosus* has not been reported. Consequently swab extraction methods were optimised and then the two approaches were compared using lesion swabs collected from sheep from Australian sheep flocks with clinical footrot. This is the first step towards

comprehensive, culture-independent diagnosis and assessment of footrot. Further details of the materials and methods used in this study are presented in Appendix: 9.2.2.

3.5 Multiplex serogroup PCR developed and evaluated in sheep from pen and field trial using cultured bacteria and direct lesion swabs

Serogrouping of *D. nodosus* is currently done by a slide agglutination test and serogroup specific conventional multiplex PCR in groups of 3 serogroups. With advances in technology and to improve the efficiency of the tests, the possibility of adapting current serogroup-specific PCR assays to a real-time PCR platform was evaluated. Current conventional multiplex PCR amplicons vary from 189 bp to 415 bp which are not ideal for the real time PCR platform for which ideally sized amplicons are only 100- 150 bp. Therefore new sets of primers needed to be designed and evaluated.

D. nodosus fimbrial gene (*fimA*) sequence data were available for all the representative prototype strains. Multiple sequence alignment was performed using the ClustalW algorithm in MEGA v6.06 software.   A different combination of primers and TaqMan probe were designed to be specific for each serogroup using Primer Expressv3.0.1 (ThermoFisher Scientific). The specificity of each primer and probe was evaluated using the BLAST algorithm (NCBI, USA). All primers and TaqMan probes were purchased from ThermoFisher Scientific, USA.

Purified genomic DNA from a panel of reference Australian prototype strains of *D. nodosus*, representing serogroups A to I and M was used for the development and evaluation of the assay. Amplification of each target was analysed separately using serial dilutions of genomic DNA prepared from each prototype strain. DNA template concentrations ranged from 10 ng to 0.01 pg per reaction. Analytical sensitivity and specificity was conducted using the purified DNA samples from the prototype strains as the reference strains. The limit of detection (LOD) for each of primer and probe sets was conducted by testing on 10 fold serial dilutions of 10ng of purified DNA of the respective prototype strains.

Clinical samples were collected from target populations of Merino sheep in Australia. The flock level infection status was determined by clinical evaluation, bacterial culture and conventional PCR. The test status for presence/absence of *D. nodosus* and the serogroup classification of positive samples were determined by conventional PCR. All clinical samples were evaluated using singleplex conventional PCR assays for each serogroup (A to I and M) and multiplex serogroup assays (ABC, DEF, GHI and singleplex M). Further details on materials and methods used in this study are presented in Appendix 9.3.2.

3.6 Metagenomic analysis to identify other bacterial species associated with footrot

Footrot is a mixed bacterial disease, and microbial species other than *D. nodosus* present on the foot and in the environment are critical to the disease process. Some of these species are thought to be essential to the disease process. *Fusobacterium necrophorum*, which is a constituent of the normal gastrointestinal flora, has been shown to initiate the disease process by enabling *D. nodosus* to invade the epidermis, and to enhance the severity of lesions as the disease progresses. Upon invading the epidermis, *F. necrophorum* expresses a leukocytic exotoxin that prevents leukocytes

from accessing the active lesion, protecting *D. nodosus* from phagocytosis. Prior to the identification of *D. nodosus* as the essential causative agent, *F. necrophorum* was thought to be the primary causative agent due to the organisms ubiquity in footrot lesions and its role in other mixed bacterial diseases. However, unable to induce severe foot lesions by application of pure cultures of *F. necrophorum* to the feet of Merino sheep predisposed by water maceration, or the application of material from mild interdigital lesions. Spirochates are also thought to contribute to the disease process, although the means by which they contribute to the disease process is uncertain. Other species, such as *Corynebacterium pyogenes*, are thought to contribute to the disease process by enhancing the infectivity of *F. necrophorum*.

Much of what is currently known about the aetiology of ovine footrot was determined using classical microbiological techniques and examination of histological sections. These studies were limited to a small number of microbial species that could be cultured in the laboratory, or those with distinct cell morphologies that were readily identifiable in smears or histological sections. Further insights enabled by molecular techniques such as the polymerase chain reaction (PCR), have furthered our understanding of the aetiology and disease process. These studies targeted microbial species that were known to contribute to the disease process, or were suspected of contributing to the disease process based on their role in other mixed bacterial diseases of the epidermis.

The advent of new technologies such as next-generation sequencing (NGS) and amplicon-based metagenomics have enabled new insights into the aetiology of ovine footrot and the identification of other potentially significant bacterial species. The aim of this study was to characterise the microbial population of the footrot lesion using amplicon-based metagenomics, to determine which genera are most abundant in active lesions and might therefore contribute to the disease process, and to determine how the microbial population of the footrot lesion shifts as the disease progresses.

Two mobs of Merino sheep (*n* = 10 sheep per mob) were sourced from target populations in New South Wales, Australia. The sheep were selected from a proportion of the parent flock on the basis that they all had two or more feet with active lesions. The sheep were transported to the University Farms at Camden, NSW. Three clinically healthy sheep were also sourced from a flock of sheep at the university farms at Camden, to be included as negative controls. Each group of sheep was maintained in separate paddocks approximately 500 m^2 in area. Active footrot lesions that were suitable for sampling were identified and lesion material was collected from the same feet on a monthly basis for a period of 10 months. DNA was extracted from each swab and *D. nodosus* was identified via conventional PCR or real-time PCR amplification of a variable region of the *D. nodosus* 16S rRNA gene. Barcoded amplications of the 16S ribosomal (rRNA) gene were generated and sequenced on the Illumina MiSeq by the Australian Genome Research Facility Ltd (Brisbane node). Sequence data were imported into the CLC Genomics Workbench v.10.0.1 (CLC Bio, Aarhus, DK). The data were quality filtered and reads with ≥97% similarity were clustered into operational taxonomic units (OTUs). Taxonomy was assigned to the OTUs. Further details of the methods are presented in Appendix: 9.4.2

3.7 Samples collected from farms in Tasmania, South Australia and other states evaluated and results matched to field data

Footrot lesion samples were collected from 40 flocks of sheep from different parts of Australia: 24 flocks with clinically virulent footrot and 16 flocks with clinically benign footrot. Samples were collected from flocks 1-32 and 34-40 between June 2014 and August 2016.

Clinical examination and diagnosis. Three methods of clinical diagnosis were used during this study. The approach depended on whether sheep were examined by the authors or an animal health officer (AHO), the number of sheep or mobs examined, and on prior diagnostic investigations. A score was assigned to each foot according to a scoring system (Egerton and Roberts, 1971a). For Methods 1 and 2, diagnosis of clinically virulent and benign footrot was based on the prevalence of score 4 lesions observed in ≥100 randomly selected sheep, as described by Egerton (1989a, b). To align with the dichotomous classification system used by Australian regulatory authorities, outbreaks were classified as clinically benign where score 4 lesions were observed in <10% of the flock or mob, or clinically virulent where score 4 lesions were observed in ≥10% of the flock or mob. Note that most benign flocks had few sheep with severe lesions.

Method 1: A sample of ≥100 sheep was randomly selected from one or two mobs and examined by the authors. The producer presented only one mob for examination because it was either the only mob with clinical footrot on the farm, or because it was the mob with the most severe clinical signs of lameness. The producer presented two mobs for examination (50 inspected per mob) because clinical signs of lameness were previously observed in both mobs or foot lesions were previously observed in both mobs during routine husbandry procedures. A flock history was provided by the producer at the time of inspection. Lesion swabs were collected at the time of examination by the authors.

In all flocks that appeared to have clinically benign footrot, additional criteria were used to support the diagnosis:

    i.   The flock/mob must have been examined previously on two or more occasions by the authors or an experienced animal officer, according to the system described by Stewart and Claxton (1993). The disease must have been classified as clinically benign on each occasion, according to the system of Egerton and Roberts (1971). The retrospective foot score data were inspected by the authors.

    ii.   Environmental conditions must have been favourable for transmission and expression of the disease in the two weeks prior to each of the examinations (average daily air temperature ≥10°C, consistent rainfall) (Graham and Egerton, 1968). Climatic data were obtained from the nearest Bureau of Meteorology weather station.

    iii.   The flock history was obtained from the producer and did not suggest clinically virulent footrot. There was no clinical evidence of virulent footrot having been present in the flock previously, i.e. old lesions (such as damage to the abaxial hoof wall indicative of underrun lesions having been present) were not observed.

    iv.   Topical treatments that may suppress or mask the severity of disease, such as antiseptic foot bathing, had not been used in the four weeks preceding each examination.

    v.   The sheep were all Merino, which are naturally susceptible to footrot.

Method 2: A small number of animals were examined by an experienced AHO for the purpose of collecting lesion swabs. The flock had been examined by the same AHO on two or more previous occasions, and a clinical diagnosis made using Method 1. As such, there was an interval between the time at which the clinical diagnosis was made and the time at which the lesion swabs were collected. The AHO informed the authors of his or her clinical diagnosis but did not provide the retrospective foot score data.

Method 3: Sheep in a "hospital mob" were examined by the authors or an experienced AHO. Between 10 and 60 sheep from each hospital mob were examined on each farm, as indicated in Table 1. The sheep were separated from the parent flock(s) by the producer because they had the most severe clinical signs of lameness or because they were the only sheep in the parent flock(s) with foot lesions. The sheep had not been examined previously by the authors or an experienced AHO, and retrospective foot scores were not available. However, a flock history was obtained from the producer describing the progression of the disease since it was first introduced to the flock. Clinical diagnosis was based on the severity of clinical disease observed in the hospital mob, the number of sheep with score 4 lesions separated from the parent flocks(s), the size of the parent flock (and therefore a rough estimate of apparent prevalence of sheep with severe lesions was possible) and the flock history. Lesion swabs were collected at the time of examination by the authors or an AHO.

Table 3.6.1: Details of the sheep flocks sampled for the evaluation study. Field diagnosis was made using one of three approaches, as described above. NA = not available. AHO = animal health officer.

Farm	State	Operator	Diagnostic approach	Season at time of inspection	No. mobs inspected	No. sheep inspected	No. sheep with score 4 lesions	Clinical diagnosis	No. sheep with lesions sampled	No. swabs tested directly	No. isolates collected
1	SA	AHO	2	Winter	1	NA	≥10%	Virulent	11	12	12
2	NSW	Author	3	Winter	NA	54	23	Virulent	14	4	12
3	TAS	Author	3	Winter	NA	51	11	Virulent	24	11	11
4	TAS	Author	3	Winter	NA	52	40	Virulent	50	10	14
5	TAS	AHO	3	Winter	NA	42	10	Virulent	20	23	23
6	TAS	AHO	2	Winter	1	NA	≥10%	Virulent	20	23	23
7	TAS	AHO	3	Winter	NA	33	26	Virulent	20	20	20
8	TAS	AHO	3	Winter	NA	28	18	Virulent	20	19	19
9	TAS	AHO	3	Winter	NA	13	7	Virulent	13	13	13
10	TAS	AHO	3	Spring	NA	26	11	Virulent	20	15	15
11	TAS	AHO	3	Spring	NA	20	5	Virulent	20	8	8
12	SA	AHO	2	Spring	1	NA	≥10%	Virulent	10	10	10
13	SA	AHO	2	Spring	1	NA	≥10%	Virulent	6	9	9
14	SA	AHO	2	Spring	1	NA	≥10%	Virulent	7	8	7
15	SA	AHO	2	Spring	1	NA	≥10%	Virulent	10	13	13
16	SA	AHO	3	Spring	NA	16	3	Virulent	16	16	16
17	TAS	Author	3	Spring	NA	25	14	Virulent	25	15	15
18	TAS	Author	3	Spring	NA	50	19	Virulent	50	29	29
19	NSW	Author	3	Summer	NA	51	15	Virulent	50	15	15
20	NSW	AHO	3	Summer	NA	50	4	Benign	14	14	14
21	TAS	AHO	2	Summer	1	NA	≥10%	Virulent	11	11	11
22	TAS	AHO	2	Summer	1	NA	≥10%	Virulent	17	17	17
23	SA	AHO	2	Summer	1	NA	≥10%	Virulent	4	4	4
24	NSW	Author	3	Autumn	NA	20	0	Benign	20	20	21
25	NSW	Author	3	Winter	NA	NA	≥10%	Virulent	10	14	14
26	TAS	AHO	2	Winter	1	NA	≥10%	Virulent	12	5	5
27	SA	Author	1	Spring	1	100	4	Benign	40	40	20
28	SA	Author	1	Spring	1	100	0	Benign	40	40	11
29	SA	Author	1	Spring	1	100	2	Benign	40	40	12
30	SA	Author	1	Spring	1	100	0	Benign	40	40	6
31	SA	Author	1	Spring	1	170	0	Benign	40	40	3
32	SA	Author	1	Spring	1	100	0	Benign	40	40	7
33	SA	AHO	2	Spring	1	1716	42	Benign	50	0	15
34	TAS	Author	1	Winter	2	100	0	Benign	30	30	6
35	NSW	Author	1	Winter	2	120	0	Benign	21	21	10
36	NSW	Author	1	Winter	1	100	0	Benign	28	20	6
37	NSW	Author	1	Winter	1	100	0	Benign	22	20	1
38	NSW	Author	1	Winter	1	100	0	Benign	20	20	2
39	NSW	Author	1	Winter	1	100	0	Benign	25	23	0
40	WA	AHO	2	Winter	1	NA	0	Benign	30	27	0
								Total		758	469

3.8 National workshop on footrot diagnosis

A National Workshop on Footrot was conducted at the University of Sydney, Camden campus on the 9th and 10th of February 2017. The program covered diagnosis as a focus but a broad range of agenda items were presented and discussed. The list of participants and program details is included in the attached proceedings.

4 Results

4.1 Evaluation of genotypic and phenotypic protease virulence tests for *Dichelobacter nodosus* infection in sheep

Forty Australian sheep flocks were selected for this study, including 24 flocks with clinically virulent footrot and 16 flocks with clinically benign footrot. Lesion swabs were collected for direct testing from 40 flocks, but lesion swabs for microbiological culture were collected from only 38 flocks. The number of swabs collected from each flock for direct testing ranged from four to 40. The number of *D. nodosus* isolates obtained from each flock ranged from one to 29.

Amplification efficiencies and the limit of detection (LOD) of the qPCR tests developed by Stäuble et al. (2014) and Frosth et al. (2015) were calculated. Amplification efficiencies for *aprV2* and *aprB2* were 90.14 and 88.4, respectively, using the assay developed by Frosth et al. (2015). Amplification efficiencies for *aprV2* and *aprB2* were 87.8 and 91.4, respectively, using the assay developed by Stäuble et al. (2014). The LOD for *aprV2* and *aprB2* was 0.005 and 0.05 pg, respectively, for both qPCR tests.

The analytical specificity of the two qPCR assays was determined using genomic DNA extracted from pure cultures of 15 bacterial strains, along with genomic DNA extracted from pure cultures of virulent *D. nodosus* type strain A1001 and benign *D. nodosus* field strain JIR3528. Both assays were 100% specific for the *aprV2* and *aprB2* alleles, respectively, and no amplification occurred for the 15 other bacterial species.

The two qPCR tests were compared using 430 lesion swabs collected from 18 flocks. The qPCR test developed by Frosth et al. (2015) detected the *aprV2* allele in 48 lesion swabs and the *aprB2* allele in 26 lesion swabs that the test developed by Stäuble et al. (2014) did not. Consequently, a decision was made to proceed with the assay of Frosth et al. (2015) when undertaking a larger test evaluation.

The repeatability of the *aprV2/B2* qPCR test developed by Frosth et al. (2015) was evaluated for the *aprV2* and *aprB2* alleles with three concentrations of genomic DNA per reaction. The CV was similar for each of the three DNA concentrations for both the *aprV2* and *aprB2* alleles, and was <5%.

The *aprV2/B2* qPCR test was compared with clinical diagnosis using lesion swabs collected from 40 Australian sheep flocks with clinically diagnosed footrot. DNA was extracted directly from lesion swabs for analysis. An outbreak was more likely to be classified as virulent with the qPCR test than clinically ($P < 0.0009$). The level of agreement between clinical diagnosis and the *aprV2/B2* qPCR test beyond that expected by chance alone was fair (kappa statistic = 0.353). Clinical diagnosis and the qPCR test were also compared at the foot swab level. The level of agreement was considerably lower

at the foot swab level (kappa statistic = 0.096) as the *aprV2* allele was detected for 87% (363/417) of lesions swabs collected from clinically benign outbreaks. At the flock level, the DSe of the qPCR test was 100%, and the DSp was 31.3%. At the foot swab level, the DSe was 98.1%, and the DSp was 18.8%.

The elastase test was used to evaluate 469 *D. nodosus* isolates collected from 38 Australian sheep flocks. There was no significant difference (*P* = 0.0833) between the proportion of outbreaks classified as virulent by clinical diagnosis or by the elastase test. The level of agreement between clinical diagnosis and the elastase test beyond that expected by chance alone was almost perfect (kappa statistic = 0.822). At the flock-level, the DSe of the elastase test was 100%. Three outbreaks that were clinically benign were classified as virulent by the elastase test, thus DSp = 78.6%.

Clinical diagnosis and the elastase test were also compared at the isolate-level. There was a significant difference (*P* < 0.0001) between the proportion of isolates from clinically virulent outbreaks that were elastase-negative and those from clinically benign outbreaks that were elastase-positive. At the isolate-level, the level of agreement between clinical diagnosis and the elastase test beyond that expected by chance alone was moderate (kappa statistic = 0.822). DSe was 69.9% and DSp was 80.5%.

Isolates cultured from lesion swabs obtained from 38 Australian sheep flocks were subjected to the elastase test and the *aprV2/B2* qPCR for laboratory diagnosis. Isolates collected from six flocks were also subjected to the gelatin gel test. For qPCR analyses, DNA was extracted from pure cultures of *D. nodosus* to ensure that the tests were being compared on the same isolates. There was a significant difference (P <0.0001) between the results of the elastase test and the *aprV2/B2* qPCR: the qPCR was more likely to classify an isolate as virulent than was the elastase test. Only 52.7% (213/404) of *D. nodosus* isolates were classified as virulent by both tests. There was a considerable discrepancy between the elastase test and the *aprV2/B2* qPCR for isolates classified as benign by the elastase test, as 73.2% (139/190) of isolates classified as benign by the elastase test were classified as virulent by the *aprV2/B2* qPCR. The level of agreement between the elastase test and the *aprV2/B2* qPCR beyond that expected by chance alone was only fair (kappa statistic = 0.275). Of the 139 isolates that were classified as benign by the elastase test and virulent by the qPCR test, 54.0% (75/139) were obtained from sheep in flocks with clinically virulent footrot, and 46.0% (64/139) were obtained from sheep in flocks with clinically benign footrot. A large proportion of these isolates (112/139) were elastase-positive after 16-28 days of incubation.

There was a significant difference (*P* <0.0001) between the results of the gelatin gel test and the *aprV2/B2* qPCR test, with 86.2% (25/29) of isolates classified as benign (unstable) by the gelatin gel test classified as virulent by the qPCR test. The level of agreement between the gelatin gel test and the *aprV2/B2* qPCR test beyond that expected by chance alone was poor (kappa statistic = 0.101, 95% CI -0.024 – 0.244).

Paired elastase and gelatin gel test results were available for 56 *D. nodosus* isolates. There was a significant difference between the results of the two tests (P < 0.0001), with 42.9% (21/49) of isolates classified as benign by the elastase test classified as virulent by the gelatin gel test. The level of agreement between the elastase test and the gelatin gel test beyond that expected by chance alone was slight (kappa statistic = 0.193). Further details of the results are presented in Appendix: 9.1.3.

4.2 Sequence for serogroup M and serogroup-specific PCR designed and evaluated

All new primer combinations that included the pan-serogroup forward primer resulted in the detection of serogroup M; however, serogroups E and F were also detected using these primer sets, suggesting that the degree of specificity obtained when using a pan-serogroup forward primer was inadequate. Therefore the sensitivity and specificity of several alternative forward and reverse primer combinations were tested and evaluated in conventional PCR assays using DNA extracted from prototype strains representing all 10 serogroups. One set of primers produced a 76-bp amplicon and detected serogroup M only (Fig. 4.2.1). The sensitivity and specificity of these primers was evaluated with pure cultures and was found to be adequate with a conventional PCR assay.

This test can be run as a stand-alone conventional PCR test, in addition to the existing serogroup-specific multiplex PCR assays. Along with the other serogroups this test was further developed into a real time PCR assay and validated with direct swab samples; this research work is presented in section 4.4 (below).
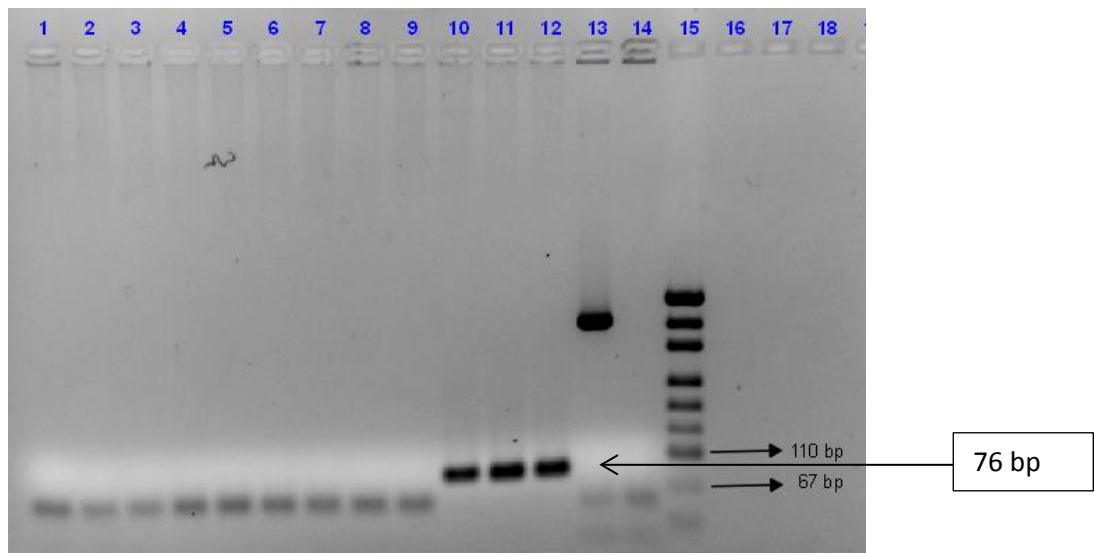

Fig. 4.2.1: PCR product run on 2% agarose gel, visualised under UV light. Lanes 1-9 serogroups A-I (all negative); Lane 10: Nepalese serogroup M isolate; Lanes 11-12: King Island serogroup M isolates; Lane 13: positive control (serogroup A), Lane 14: negative control.

4.3 Extraction of *D. nodosus* DNA from lesion swabs optimised

Twelve Australian sheep flocks with clinically virulent footrot were included in this study. Ten flocks were located in Tasmania, and two flocks in New South Wales. Between 12 and 60 sheep were inspected in each flock. Each flock was examined between August 1 and December 15, 2014.

Microbiological culture and direct PCR testing for the 16S rRNA gene of *D. nodosus* from the same swab were compared. The swab was first used to inoculate a hoof agar plate, before being placed into LB for DNA extraction and direct PCR testing. Detection of *D. nodosus* via PCR was significantly

more sensitive than detection by culture (McNemar's X^2 = 7.04, *P* = 0.0082). There were 32 swabs for which *D. nodosus* was detected by direct PCR testing but not by culture. There were 16 swabs for which *D. nodosus* was detected by culture but not by direct PCR testing. The level of agreement between the two methods was only fair (kappa = 0.282).

Microbiological culture and direct PCR for the 16S rRNA gene of *D. nodosus* were compared using lesion material collected into LB with sterile cotton-tipped swabs. Detection of *D. nodosus* via PCR amplification of the 16S rRNA gene using the swab-LB-PCR method was significantly more sensitive than detection by culture (McNemar's X^2 = 34.32, *P* < 0.0001). The level of agreement between the two methods was poor (kappa = 0.118). There were 52 swabs for which *D. nodosus* was detected by direct PCR testing but not by microbiological culture. There were seven instances in which *D. nodosus* was detected by culture but not by direct PCR testing.

Detection of *D. nodosus* via direct PCR amplification of the 16S rRNA gene was compared using the swab-mSTM-PCR and swab-LB-PCR methods. The swab-LB-PCR method was more sensitive than the swab-mSTM-PCR method (McNemar's X^2 = 10.67, *P* < 0.0011). The level of agreement between the two methods was only fair (kappa = 0.399). There were 20 lesions in which *D. nodosus* was detected via the swab-LB-PCR method but not by swab-mSTM-PCR method. There were four lesions in which *D. nodosus* was detected via the swab-mSTM-PCR method but not the swab-LB-PCR method.

The slide agglutination test was compared with PCR serotyping using genomic DNA extracted from pure cultures of each *D. nodosus* isolate obtained during this study and genomic DNA extracted directly from lesion swabs. There were both conclusive and inconclusive slide agglutination test results for isolates obtained from 9/12 flocks. The slide agglutination test results were compared with PCR serotyping of each *D. nodosus* isolate, which revealed that most of the inconclusive slide agglutination test results were cross-reactions. We failed to identify some serogroups via PCR testing of pure cultures, despite those serogroups having been identified by the slide agglutination test and direct PCR testing of lesion swabs. There were also two instances (Farms 5 and 9) where serogroups were identified via PCR typing of pure cultures but not by the slide agglutination test. Fewer serogroups were identified via direct PCR typing when the same swab was used for both microbiological culture and direct PCR testing (swab-mSTM-PCR) compared to direct PCR testing of swabs collected directly into lysis buffer (swab-LB-PCR).

The slide agglutination test and PCR serotyping were compared at the isolate-level for seven *D. nodosus* serogroups. Serogroups C and F were excluded as no *D. nodosus* isolates belonging to these serogroups were detected with both tests. Conclusive and inconclusive slide agglutination test outcomes were included in the analyses. There were no significant differences in sensitivity between the slide agglutination test and serogroup-specific PCR for any of the serogroups evaluated (*P* > 0.05). However, cross-reactions, as indicated by a positive slide agglutination test outcome and a negative PCR outcome, were observed for serogroups B, D, E, G, and I.

Effect of lesion characteristics on the detection of D. nodosus via microbiological culture

The significance of three foot lesion characteristics (foot score, lesion type, and lesion contamination) to the detection of *D. nodosus* via microbiological culture was evaluated using GLMM. The odds of detecting *D. nodosus* in a score 2, score 3 and score 4 lesion were 1.72, 7.32 and 1.90 that of a score 1 lesion, respectively, with the significant difference (P=0.021) being due to the

comparison of score 3 and the reference category score 1. The odds of detecting *D. nodosus* in a dry lesion was 0.26 that of an active lesion (P=0.027). The odds of detecting *D. nodosus* in a lesion with moderate and low degree of contamination were 4.99 and 11.21 that of a lesion with a high degree of contamination, respectively (P<0.001).

Effect of lesion characteristics on the detection of D. nodosus via swab-mSTM-PCR

The significance of three foot lesion characteristics (foot score, lesion type, and lesion contamination) to the detection of *D. nodosus* via swab-mSTM-PCR was evaluated using GLMM. The odds of detecting *D.nodosus* in a score 2, score 3 and score 4 lesion were 40.13, 25.25 and 12.74 that of a score 1 lesion, respectively. The odds of detecting *D. nodosus* in a dry lesion was 0.09 that of an active lesion. The odds of detecting *D. nodosus* in a lesion with a moderate and low degree of contamination were 14.32 and 25.15 that of a lesion with a high degree of contamination, respectively. All these results were statistically significant (P<0.001).

Effect of lesion characteristics on the detection of D. nodosus via swab-LB-PCR

The significance of three foot lesion characteristics (foot score, lesion type, and lesion contamination) to the detection of D. *nodosus* via swab-LB-PCR were evaluated using GLMM. Prior to undertaking GLMM, it was necessary to collapse categories in which there were fewer than five observations, as indicated in Table 12. Lesion score was not significant (P = 0.422) to the detection of D. *nodosus* via swab-LB-PCR. The odds of detecting D. *nodosus* in a dry lesion was 0.10 that of an active lesion (P=XX). The odds of detecting D. *nodosus* in a lesion with low/moderate soil and faecal contamination was 8.50 that of a lesion with a high degree of contamination.

4.4 Multiplex serogroup PCR developed and evaluated in sheep from pen and field trial using cultured bacteria and direct lesion swabs

Serogroup specific real time (RT) PCRs have been developed and evaluated for all 10 serogroups (A to I, and M) of *Dichelobacter nodosus.* These serogroup-specific PCRs have been further developed into multiplex RT PCRs. In accordance with the technical capacity of the ABI 7500 real time PCR platform, which was chosen because it is the current industry standard and because it is used in all of the relevant animal health diagnostic laboratories in Australia, up to 3 targets can be multiplexed in one reaction. Accordingly, multiplex PCRs have been developed in groups of 3. Our strategy has been to combine serogroups ABC, DEF, and GHI into three reactions, and then include serogroup M with a candidate putative virulence PCR detecting putative virulence genes *aprV2* and *aprB2* (Frosth et al. 2015). The practical application will be to run all 4 multiplex reactions against each test sample. This strategy confirms: the presence of each serogroup in the sample and the presence of putative virulence genes in the sample. When validation of virulence gene tests has been completed in this project it is envisaged that the most appropriate virulence gene will be included, but for the moment one of the published, non-validated genes from Europe is being used for proof of concept. We are also working on screening other putative virulence genes with the aim of modification and development of more reliable and accurate multiplex virulence tests.

These new multiplex real time PCR tests have been developed, tested and evaluated for specificity with prototype serogroup specific DNA samples (Table 4.4.1). These were further tested and evaluated with samples from pen trials and also with direct lesion swabs collected on-farm. The

results have been equally good with DNA extracted from pure cultures of *D. nodosus* and with direct testing of lesion swab samples. These new RT PCR tests have been found to be more sensitive than the conventional serogroup specific PCRs on which they were based. Examples of the results of output from the ABI machine for these new RT PCRs are presented in Figures 4.4.1, 4.4.2 and 4.4.3.

Table 4.4.1: Analytical specificity of new primers and probes in RT PCR

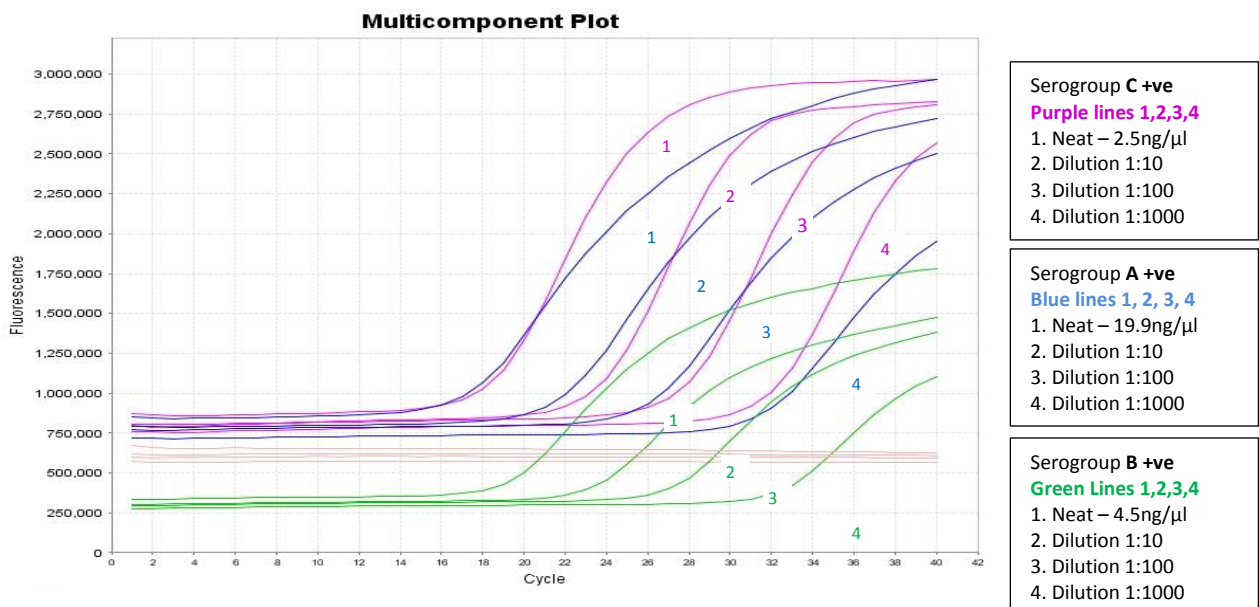| Primers & Probes	DNA Templates tested													
	A+B+C	A	B	C	D+E+F	D	E	F	G+H+I	G	H	I	M+aprV2+aprB2	M
A+B+C	All +	+	+	+	-	-	-	-	-	-	-	-	-	-
D+E+F	-	-	-	-	All +	+	+	+	-	-	-	-	-	-
G+H+I	-	-	-	-	-	-	-	-	All +	+	+	+	-	-
M	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Fig 4.4.1: Real time PCR results of multiplex serogroup ABC
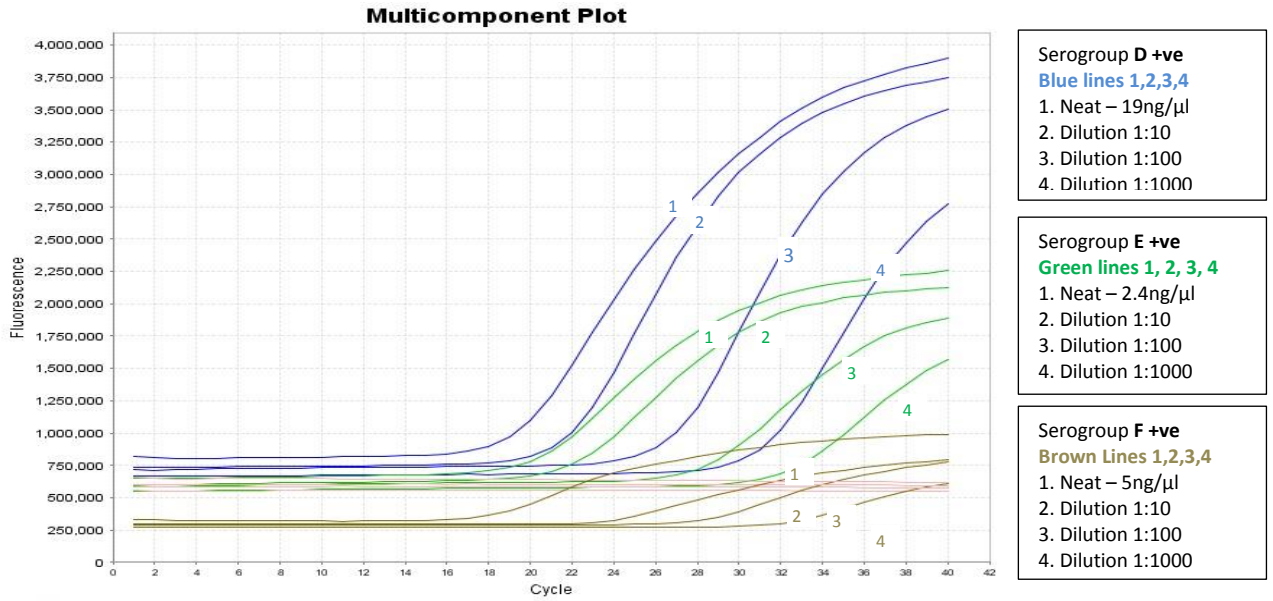
Fig 4.4.2: Real time PCR results of multiplex serogroups DEF
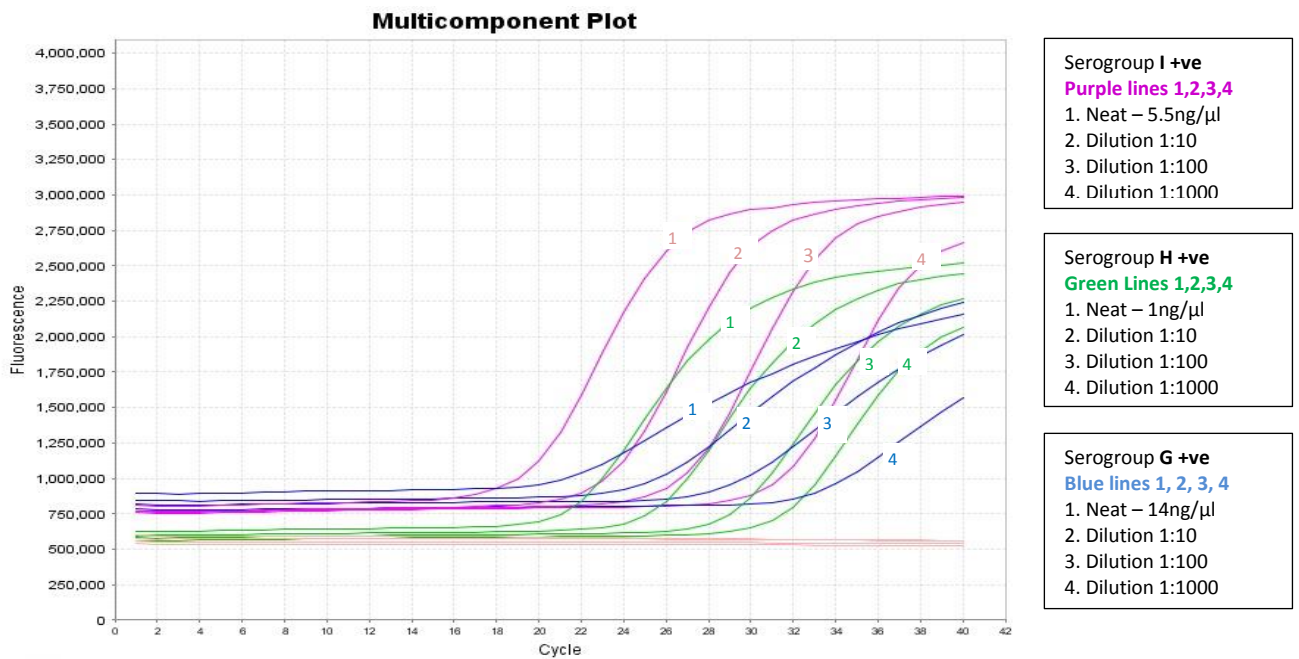
Fig 4.4.3: Real time PCR results of multiplex serogroups GHI

Note: DNA starting concentration has an impact on the results as expected. However, the assay appears to be very sensitive (detection of 1:1000 dilution in each case). The signal for different fluorescent dyes at equivalent DNA starting concentrations is variable because the individual dyes have individual emission characteristics but are presented on a standard y axis scale. However, this does not impact categorisation of the positive results.

4.5 Metagenomic analysis to identify other bacterial species associated with footrot

4.5.1 Sample selection

Ten feet/lesions were chosen retrospectively for inclusion in the longitudinal analyses, including 8 feet from the mobs with footrot and 2 from the clinically healthy mob. Swab samples were collected at monthly intervals across a period of 7 to 10-months (March to December, 2016).

4.5.2 Sequencing and bacterial diversity

D. nodosus was detected via qPCR amplification of the 16S rRNA gene in each of the specimens collected from foot lesions, and was not detected in any of the specimens collected from clinically healthy feet. A total of more than 6 million reads were obtained from the samples collected from sheep with footrot, and ~ 1 million reads from the feet of clinically healthy sheep. The bacterial community present on the feet of clinically healthy sheep was more phylogenetically diverse than that of sheep with clinical footrot, with 3 to 4 thousands OTUs identified in the specimens collected from the feet of clinically healthy sheep, and 2 to 3.5 thousands identified in the specimens collected from the feet of sheep with footrot. The dominant phyla in specimens from the feet of clinically healthy sheep were Actinobacteria, Firmicutes and Proteobacteria. The dominant phyla in specimens collected from the feet of sheep with virulent footrot were Firmicutes, Bacteroides, and Actinobacteria and Tenericutes. Examples of the diversity profiles of the three groups of the sample are presented in Figure 4.5.1.
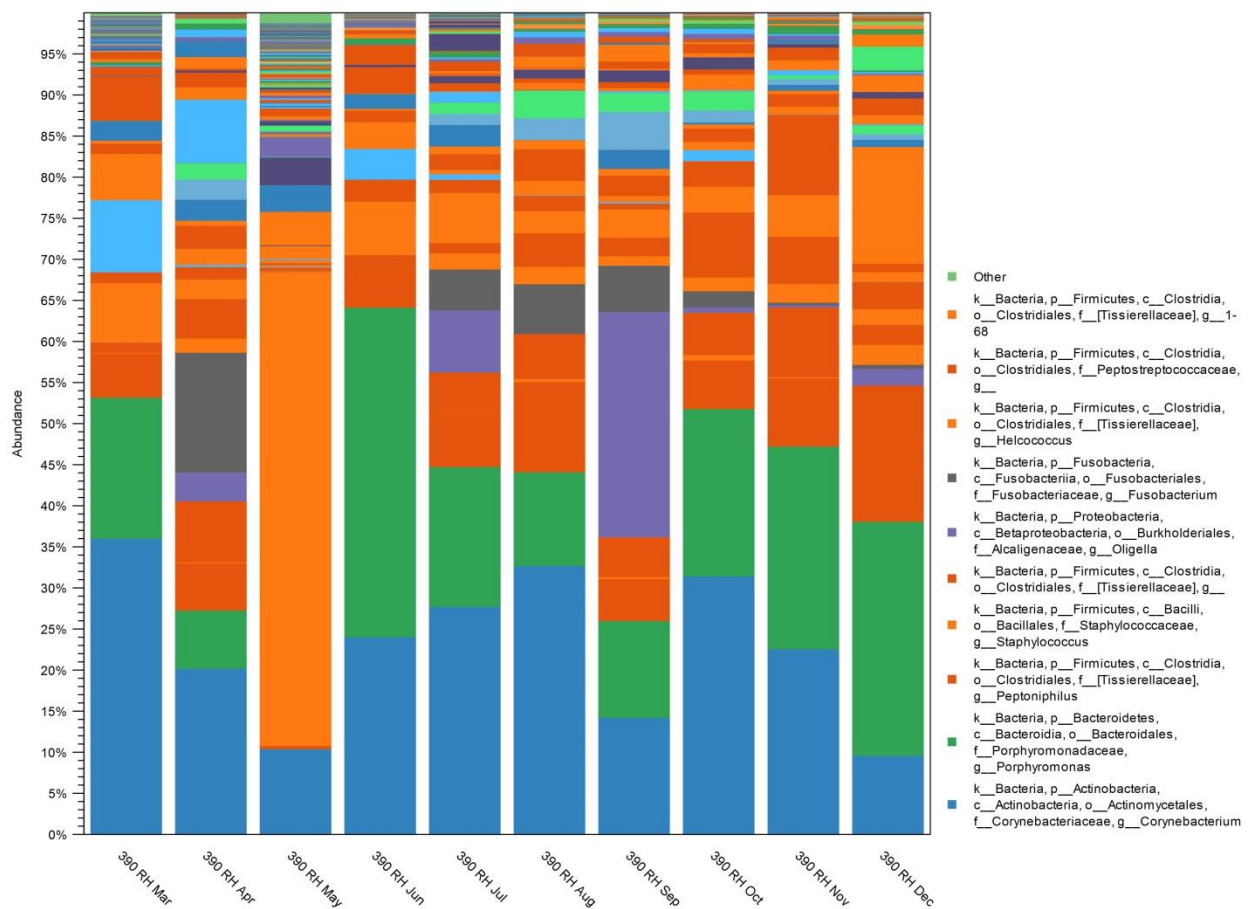
Figure 4.5.1: 16S rRNA-based bacterial diversity profile of the foot lesion of a Merino sheep with clinically virulent footrot. The same lesion was sampled on a monthly basis for a period of 10 months (March to December, 2016). Each colour represents a different taxonomic group of bacteria.

4.6 Samples collected from farms in Tasmania, South Australia and other states evaluated and results matched to field data

Forty Australian sheep flocks were selected for the present study, including 24 flocks with clinically virulent footrot and 16 flocks with clinically benign footrot (Table 9.1.1). A summary of lesion scores was available for 28 flocks. The distribution of lesion scores observed in each of these flocks is illustrated in Appendix Fig 9.1.4. The number of flocks with clinically virulent and benign footrot inspected in each State is indicated in Table 9.1.1. Twenty flocks were inspected by the authors and 20 were inspected by experienced animal health officers. Sixteen flocks were inspected during spring, 18 flocks during winter, five flocks during summer, and one flock during autumn. Lesion swabs were collected for direct testing from 40 flocks, but lesion swabs for microbiological culture were collected from only 38 flocks. The number of swabs collected from each flock for direct testing ranged from four to 40. The number of *D. nodosus* isolates obtained from each flock ranged from one to 29.

Table 9.1.1: Summary of the number of flocks sampled in each Australian State, and the number of flocks in each State that were classified as clinically virulent or benign.

	Clinical Diagnosis		
State	**Virulent**	**Benign**	**Total**
Tasmania	14	1	15
New South Wales	3	7	10
South Australia	7	7	14
Western Australia	0	1	1
Total	24	16	40

4.7   National workshop on footrot diagnosis

A total of 70 participants from across Australia with a wide representation from field vets, researchers, peak industry bodies and farmers were involved in the presentations and the discussions. Published proceedings of the workshop are attached.

5   Discussion

5.1   Evaluation of genotypic and phenotypic virulence tests

Comparing the so-called virulence qPCR test and clinical diagnosis, there was moderate to poor agreement at the flock and individual swab sample levels and the diagnostic specificity of the qPCR was low (up to ~30%). In contrast, there was much better agreement between clinical diagnoses and the elastase test at the flock and individual sample levels with diagnostic specificity ~80%. Isolate-level comparison of the qPCR test and elastase test revealed that 73.2% (139/190) of isolates that were deemed benign by the elastase test were deemed virulent by the qPCR test (*aprV2*-positive). The elastase test depends on visual assessment of digestion of elastin particles in an agar matrix, and a cut-point based on incubation time is applied, generally 10-12 days beyond which an isolate is deemed benign. Thus the amount of elastase activity and the rate at which it is elaborated influences the outcome of the test. Elastase activity was observed for 80.6% of the benign isolates after further incubation, confirming temporal and quantitative variations in the expression of the AprV2 enzyme between strains. Regardless, the results indicate that some strains that possess *aprV2* did not express detectable elastase activity and may not be capable of inducing severe disease. Consequently, identification of the *aprV2* allele may not be a reliable indicator of virulence.

Clinical diagnoses in this study were made using objective criteria that have been applied successfully in a state-wide eradication program in NSW. However, these criteria ignore the true spectrum of severity that is possible in ovine footrot, which ranges from inapparent, through mild to severe. Forcing a dichotomous clinical classification was pragmatic from the perspective of disease control, and in the present study enabled a comparison with dichotomous laboratory test outcomes. The authors acknowledge that this may lead to some inaccurate classifications of both clinical and laboratory results given that the latter also could be continuous variables. Nevertheless, the trends

are very obvious, and the discrepancies between clinical and laboratory diagnoses are substantial. It was clear that laboratory diagnoses of virulent footrot do not match the clinical pattern in flocks in which there were no sheep with severe lesions, and where there was sufficient history to be very confident that virulent footrot was not present. Test outcomes like this undermine confidence and may lead to farmers disengaging from programs to control the disease.

These findings elaborate those of Stäuble et al. (2014) and Frosth et al., (2015) who reported a high level of agreement between the qPCR test and clinical diagnosis. The high diagnostic sensitivity and specificity reported by the authors may be due in part to the case definitions used by the authors, which differ to those used in Australia. Stäuble et al. (2014) did not classify outbreaks as clinically virulent or benign; rather, flocks were classified as 'non-affected' (all feet assigned a score of 0) or 'affected' (one or more feet assigned a score ≥1). The authors report that all lesion swabs from 'affected' flocks were positive for the *aprV2* allele and negative for the *aprB2* allele, and that >80% of samples from the 'non-affected' flocks were positive for the *aprB2* allele. However, if these flocks were to be reclassified using the case definitions applied in Australia, at least two of the 'affected' flocks would be regarded as having clinically benign footrot, as no score 4 lesions were observed in these flocks. Frosth et al. (2015) assigned each of the flocks included in their study to one of four categories: (i) predominantly score 0 with some score 1 lesions, (ii) many score 1 lesions but no scores >1, (iii) at least one animal with a score 2 lesion, and (iv) at least one animal with a score 3 lesion. It is evident that under the Australian classification system, categories one and two could describe a flock with ovine interdigital dermatitis (OID), benign footrot, or the early stages of virulent footrot. Similarly, categories three and four could describe an outbreak of benign footrot or the early stages of an outbreak of virulent footrot. None of these categories describes virulent footrot exclusively. This does not suggest that the case definitions employed by Stäuble et al. (2014) and Frosth et al. (2012) were inappropriate for the European context; rather, it is important to note that validation data can be interpreted differently depending on the diagnostic criteria used to classify target flocks.

The clinical observations reported in this study support the use of a classification system that is based on the prevalence of score 4 lesions, rather than the presence or absence of foot lesions of any grade. We observed that *D. nodosus* strains classified as benign by the qPCR test (*aprB2*-positive) are capable of inducing severe, underrun lesions in a small proportion of susceptible sheep, a finding in keeping with prior knowledge of phenotypic protease tests. However, at the flock-level the impact of foot disease in these flocks was minor. In Flock 33, for example, 42/1716 sheep presented with score 4 lesions, but the overall prevalence of sheep with foot lesions of any grade in this flock was low, despite all sheep being exposed to the same strain under the same environmental conditions. It would thus be inappropriate to subject these flocks to the same regulatory activity (quarantine, compulsory disease control) as those deemed to have virulent footrot, as the *D. nodosus* strains present in these outbreaks would most likely be incapable of inducing severe disease in a large proportion of sheep. In general benign footrot is considered to not be amenable to control, and attempts to do so would expose farmers to ongoing costly measures. Culling susceptible sheep that develop severe lesions when infected with benign strains is the most practical course of action. The experience in Australia using conventional phenotypic virulence tests is that after a control program directed at virulent footrot, benign strains of *D. nodosus* persist in flocks.

The identification of *aprV2*-positive strains of *D. nodosus* in clinically healthy flocks provides further evidence that the *aprV2* may be an unreliable virulence marker. Stäuble et al. (2014) reported that seven samples collected from 'non-affected' (clinically healthy) flocks were positive for the *aprV2* allele, alone or in combination with the *aprB2* allele; in Switzerland, Locher et al. (2015) evaluated the qPCR test of Stäuble et al. (2015) as a potential screening tool for identifying virulent *D. nodosus* isolates in clinically healthy flocks, and reported that *aprV2*-positive isolates were identified in four flocks on one or more occasions, despite the flocks remaining clinically healthy for the duration of the study. This finding may also reflect differences in breed susceptibility, as the other European breeds are inherently more resistant to footrot than the Merino breed.

5.2   Serogroup M-specific PCR designed and evaluated

Serogroup M is not very common among the isolates of *D. nodosus* in Australian sheep flocks but has been reported from some parts like Tasmania. This new test for PCR typing of serogroup M is the first such test to be made available. Unlike other serogroups, the development of a serogroup M specific PCR test was complicated by the very close genetic similarity in the fimbrial gene, *fim A* of serogroup M and serogroup F. Several sets of test primers had to be designed and tested to develop and evaluate serogroup M specific primer pairs.

For a successful outbreak specific vaccination it is essential that all the serogroups prevalent in a flock be identified so as to design appropriate vaccines. Development and validation of this new serogroup M specific test has made it possible to add this test as a routine diagnostic tool for typing of isolates of *D. nodosus*.

5.3   Extraction of *D. nodosus* DNA directly from foot lesion swabs

Direct (culture-independent) PCR-based detection and serotyping of *D. nodosus* may be an alternative to traditional culture-dependent methods. PCR assays have been developed for detecting and serotyping *D. nodosus* serogroups A-I, but historically these assays have been applied predominantly to pure cultures of *D. nodosus*, and a comparison of culture-dependent and direct testing methods has not been reported. Thus, the aim of this study was to compare traditional culture-dependent and direct methods for detecting and serotyping *D. nodosus*.

First, microbiological culture and direct 16S rRNA PCR testing were compared using one swab collected from each foot lesion. Direct PCR testing was more sensitive than microbiological culture (McNemar's = 7.02, *P* = 0.0082), as *D. nodosus* was detected in 76.8% (159/207) and 85.5% (177/207) of lesions via microbiological culture and swab-mSTM-PCR, respectively. Microbiological culture and direct PCR testing were then compared using two separate swabs collected from the same lesion, the first collected in mSTM for microbiological culture and the second collected in LB for direct PCR testing (swab-LB-PCR). The swab-LB-PCR testing was more sensitive than microbiological culture (McNemar's = 34.32, *P* < 0.0001). Notably, *D. nodosus* was detected via direct PCR in 66.0 to 88.1% of lesions that were culture-negative. There were instances, however, in which *D. nodosus* was detected by microbiological culture and not by direct PCR testing. This occurred more frequently when using the swab-mSTM-PCR method than the swab-LB-PCR method (8.81% and 3.3%, respectively). This is probably a consequence of a proportion of the sample being lost during transport in mSTM and the culturing procedure, but may also be the result of PCR inhibition,

as agar is known to be a PCR inhibitor. Further optimisation of the swab-mSTM-PCR method to mitigate the effect of PCR inhibition may improve the sensitivity of the method, but this was not investigated further in the present study. Direct comparison of swab-mSTM-PCR and swab-LB-PCR confirmed that the latter method is the more sensitive, as *D. nodosus* was identified via swab-LB-PCR in 66.7% (20/30) of lesions that were negative by swab-mSTM-PCR.

Lesion characteristics had a significant effect on the detection of *D. nodosus* via both microbiological culture and direct PCR testing. Lesion score had a significant effect on the detection of *D. nodosus* via microbiological culture ($P = 0.021$) and swab-mSTM-PCR ($P < 0.001$), but not swab-LB-PCR ($P = 0.422$), possibly because the latter method is more sensitive, as discussed previously. *D. nodosus* was most likely to be detected via microbiological culture in score 3 lesions. This may be due to the physical characteristics of score 3 lesions: at the score 3 stage, the heel has only recently begun to separate, particularly at the score 3a stage (which we have not differentiated in this study), thus there is a small cavity that is relatively free of debris and necrotic material from which an unadulterated specimen can be collected. The number of viable *D. nodosus* cells is also likely to be highest here at the active margin of the advancing, underrun lesion. In contrast, the odds of detecting *D. nodosus* via the swab-mSTM-PCR method were greatest when testing material collected from score 2 lesions, possibly because a large number of living and dead *D. nodosus* cells are present in the abundant necrotic material that is typical of a score 2 lesion. *D. nodosus* was less likely to be detected in dry lesions. However, we were still able to detect *D. nodosus* in a considerable proportion of dry lesions via microbiological culture, swab-mSTM-PCR, and swab-LB-PCR (50.0%, 46.0%, and 75.5%, respectively). Contamination with soil and faecal material had the most significant deleterious effect on the detection of *D. nodosus* by microbiological culture ($P < 0.0001$) and swab-mSTM-PCR ($P < 0.0001$), but less so on swab-LB-PCR ($P = 0.007$), confirming that the latter method is more robust and potentially more tolerant of PCR inhibitors.

A comparison of culture-dependent and direct PCR serotyping at the flock-level demonstrated that more serogroups were identified using direct testing methods. Furthermore, a greater number of serogroups were identified with the swab-LB-PCR method than the swab-mSTM-PCR method, which suggests that the choice of medium may not be as important as ensuring that the entire sample is available to testing. A proportion of the sample is lost from the swab prior to DNA extraction and PCR testing when using the swab-mSTM-PCR method, namely during transport and the culturing procedure. Additional serogroups may have been present on the swabs collected in mSTM but not at a level detectable by the conventional serotyping PCR assays. The number of *D. nodosus* serogroups, and the relative abundance of each serogroup can also vary between lesion sites. Thus, the number of serogroups detected could vary between two samples collected from the same lesion depending on which part of the lesion the specimen was collected from and the volume of lesion material collected.

There were a number of discrepancies between the slide agglutination test and PCR serotyping when evaluating the same *D. nodosus* isolates, with some serogroups identified via the slide agglutination test but not by PCR. In each instance, the slide agglutination test result was deemed a false-positive, potentially a result of cross-reactivity between closely-related serogroups. This finding demonstrates that concurrent PCR typing is necessary to improve the specificity of the serotyping procedure, as the slide agglutination test is inherently subjective.

It is important to note that serogroup and virulence are independent of one another, such that virulent and benign strains of *D. nodosus* may both belong to the same serogroup. As the economic impact of benign footrot is usually negligible and does not justify the cost of vaccination, virulence testing of the infecting *D. nodosus* strain(s) must also be undertaken in parallel with diagnostic typing to identify which strain(s) should be targeted by vaccination. A number of molecular virulence tests have been developed that do not require microbiological culture of *D. nodosus*, however these tests can be unreliable. As there is no relationship between serogroup and virulence it may be difficult to determine which serogroups are virulent using culture-independent methods alone. For this reason, direct testing of lesion swabs to determine serogroup by PCR may be applied rationally when there is no doubt about the clinical presentation being virulent footrot.

5.4   Multiplex serogroup real time PCR evaluated

The analytical sensitivity of the new qPCR assays was 10 times greater than the conventional PCR assays and they were specific to *D. nodosus*. Serogroup specificity is also critical to develop effective flock specific vaccines and these primers and probes were found to be very specific between the serogroups when tested against all of the other serogroups. These new qPCR assays were tested on the direct DNA extracts from lesion swab samples and were very sensitive in that application, making them practical for diagnostic use.

Detection of multiple serogroups in a flock leads to a requirement for information about the virulence of these isolates. Vaccination needs to be targeted to include the 2 most prevalent and also the virulent serogroups. However, these qPCR assays are developed only for serotyping and are not able to distinguish virulent from benign isolates so another test would be required to address this issue. If more than 2 serogroups are detected the available phenotypic microbiological tests like Elastase and Gelatin Gel have to be used. Further molecular studies in the area of virulence associated genetic regions of *D. nodosus* may lead to PCR based tests for more reliable and efficient diagnosis.

5.5   Metagenomic analysis to identify other bacterial species associated with footrot

The aetiopathogenesis of footrot is complex, involving synergistic interactions between the essential causative agent, *D. nodosus*, and the normal microbial flora of the foot. Several bacterial species other than *D. nodosus* are thought to contribute to the disease process; however, the extent of their contribution and the nature of their interactions with *D. nodosus* have been debated. It is relatively clear that *F. necrophorum* is essential to the disease process; the bacterium colonises the interdigital skin prior to *D. nodosus*, enabling *D. nodosus* to invade the epidermis. This is due in part to the protective effect of a leukocytic toxin expressed by *F. necrophorum*, which protects both itself and *D. nodosus* from phagocytosis. Several other genera, including *Corynebacterium*, are also thought to contribute to the disease process directly and indirectly due to their relative abundance in footrot lesions and their role in other mixed bacterial diseases of the epidermis, but their role has not been clearly defined. Given the complexity of the microbial population of the hoof, it is likely that there are other important aetiological agents that are yet to be identified.

Studies based on classical microbiological techniques and examinations of histological sections are inherently limited to microbial species that can be cultured in the laboratory, or those with distinct morphologies that are readily identifiable. For this reason, our understanding of the complex aetiology of footrot has advanced slowly. The advent of new technologies such as next-generation sequencing (NGS) and metagenomics has enabled examination of the entire microbial community of the footrot lesion, and new insights into the aetiopathogenesis of footrot are beginning to emerge. Recent studies employing amplicon-based metagenomics have indicated that the composition of the microbial community varies between disease status, but the extent to which this variation drives the disease process and influences disease outcomes is unknown. The aim of this study was to characterise the microbial community of the footrot lesion across a period of 7 to 10-months, and to examine how the composition of the microbial community varies as the disease progresses.

The bacterial community present on the feet of clinically healthy sheep was more phylogenetically diverse than that of sheep with clinical footrot. Actinobacteria was the most dominant phylum on healthy feet, whilst Firmicutes was the most dominant phylum present on diseased feet. These findings suggest that particular phyla proliferate as the disease progresses, however it is uncertain whether the proliferation of these phyla is driving the disease process, or if the lesion provides an ideal environment for the proliferation of these phyla.

An objective of this study was to characterise how the microbial population of the footrot lesion shifted over time. To this end, we collected specimens from the same lesions across a 10-month period. There were variations in the relative abundance of the dominant genera at each sampling period, however it is difficult to determine if this is related to the disease process, or the sampling technique. With the advent of spring, the lesions became more active and there was a corresponding increase in the abundance of some phyla in lesions. It is possible that variation between samples collected from the same lesion is due to the sampling methodology. The microbial population of the footrot lesion is heterogeneous, thus the relative abundance of particular genera is likely to vary depending on which part of the lesion is sampled.

There are limitations to amplicon-based metagenomics. An increase in the abundance of a particular phylum does not necessarily prove causation. It is difficult to determine whether the disease process is being driven by the proliferation of particular bacteria or if the foot lesion provides an ideal environment for these to proliferate. It may also be possible that both processes are occurring simultaneously. As such, this study provides preliminary data on which to base future studies. For example vaccines targeting bacterial species other than *D. nodosus* that are essential to the development of clinical footrot may complement existing strain-specific vaccines targeting *D. nodosus*. Further details of this study are presented in Appendix 9.4.

5.6 Samples collected from farms in Tasmania, South Australia and other states evaluated and results matched to field data

A total of 1,516 samples collected from 960 sheep from 40 flocks were used to conduct a systematic evaluation of virulence tests. A systematic and thorough evaluation of these tests was possible only because of these samples. They also formed a part of the studies to compare microbial culture and direct testing of samples, and the development and evaluation of the serogroup M PCR test and the qPCR serogrouping tests.

5.7   National workshop on footrot diagnosis

Among the many issues presented and discussed at the workshop, consensus was reached on the need for harmonisation of diagnostic protocols and procedures at the national level so that there was a common understanding about the disease. The feedback received from the participants was very positive. Proceedings of the workshop have been prepared and published, and copies distributed to all the participants.

6   Conclusions/recommendations

This project has delivered new diagnostic approaches that are fully validated. A new PCR for serogroup M will improve outcomes where vaccination is intended as a control measure, enabling serogroup M to be detected quickly for the first time. These new tests are now part of the routine testing procedure for serogrouping *D. nodosus* isolates in Australia.

All of the conventional PCR serogrouping tests developed in 2002 have been upgraded to a modern real time format, and multiplexed, which will improve sensitivity and reduce test times and costs. These new real time PCR assays are much faster, more sensitive, accurate and reliable with the added advantage of revealing the intensity of each serogroup in the samples, which will inform footrot control and management based on specific vaccination.

Direct PCR detection of *D. nodosus* and its serogroups from lesion swabs was more sensitive than microbiological culture, particularly when the entire sample collected in a swab was available for direct testing. *D. nodosus* was more likely to be detected via microbiological culture and direct PCR testing in active lesions with a low amount of soil/faecal contamination than other lesion types and contaminated lesions. These findings will lead to new recommendations for sampling lesions for diagnosis, and this should improve the diagnostic success rate nationally, because the *D. nodous* isolation rate is currently quite variable between laboratories.

Importantly, a full validation study of recently published qPCR virulence tests based on protease genes that have been touted as diagnostic solutions in press releases from State jurisdictions revealed that these tests are not accurate and if applied in Australia would result in a very high rate of false positive diagnoses. In contrast, the elastase test is suitable for routine use and is more accurate than the gelatin gel test. The qPCR test was reported to be capable of identifying *D. nodosus* and differentiating virulent and benign strains according to the presence or absence of the *aprV2* and *aprB2* alleles (Frosth et al., 2015; Stäuble et al., 2014). In the present study, we demonstrated that *aprV2*-positive *D. nodosus* isolates are frequently isolated from outbreaks of clinically benign footrot. Because of its poor diagnostic specificity, we have concluded that the qPCR test is not fit for purpose, as there is a considerable risk of subjecting producers to unnecessary regulatory activity. We have also demonstrated that many *D. nodosus* isolates that possessed the *aprV2* allele did not appear to express a mature AprV2 protease in a form detectable by the elastase test, and therefore may not be capable of inducing virulent footrot *in vivo*. Further investigation of the molecular basis of virulence is clearly required. This study also highlights the risk of relying exclusively on laboratory virulence tests for the diagnosis of virulent footrot. Future research should focus on understanding the true virulence mechanisms in footrot, which might be detectable in non-

protease genes of *D. nodosus* and the other flora of the foot lesion. This will lead to better diagnostic strategies and control measures.

Investigation of the bacterial community in footrot lesions has uncovered great diversity that merits more detailed investigation because it is uncertain whether the proliferation of these phyla is driving the disease process, or if the lesion provides an ideal environment for the proliferation of these phyla.

During this project one PhD scholar and several honours students received specialist training in footrot, both on farm and in the laboratory. On-going training opportunities are required to meet future need for footrot expertise to service the sheep industries.

A National Workshop was successfully conducted and there was overwhelming support and an identified need for a national approach to footrot management and control. The Proceedings of this workshop were published and made available to all the participants. These proceedings are already being used by field veterinarians and animal health officers as a resource and reference for footrot research and extension in the field and should be distributed more widely.

7   Key messages

This project has delivered new diagnostic approaches that are fully validated, and identified pitfalls with new technologies. Producers should demand application of validated diagnostic procedures by all of the state jurisdictions. Where new approaches are recommended, producers should be provided with the validation data in an easily understood form to enable meaningful assessment and extension activities supported by peak bodies. There are considerable risks to producers in application of novel technologies where the implications of false positive diagnoses have not been fully considered. Producers should also require greater dialogue between state jurisdictions to resolve disagreements about diagnostic approaches to footrot.

Footrot is a disease that does not disappear at regional level without concerted effort by groups of like-minded producers supported by trained professionals. The costs of the disease are real in terms of lost production and poor animal welfare outcomes. The benefits of control and eradication are undoubted. However, the risk that the disease is driven underground and subsequently spread through lack of reporting by producers (due to stigma, risk of quarantine being applied) will be magnified unless the quality of diagnosis is improved nationally and the means of diagnosis harmonised.

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