Comparative study of the elastase test (bacterial virulence) with clinical diagnosis of footrot outbreaks in NSW.

Final Report

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Prepared by: Dr. Alison Collins,
Assoc. Prof. Om Dhungyel and
Dr Damian Collins (Biometrician),
with the technical support of Janet Eamens,
Elise Beggs and Karen Smith.

Executive Summary

The prevalence of virulent footrot increased significantly in NSW sheep flocks in the 16 months following the end of a prolonged drought and the introduction of new breeding animals into NSW from across Australia. In this spring 2020 to summer 2021 period, 41% of the footrot disease inspections resulted in virulent footrot diagnosis and 59% of flocks were diagnosed with benign footrot. The majority of flocks examined were from the same five LLS regions that produce more than 75% of the NSW annual wool clip. This study of 184 flocks compared clinical diagnosis of footrot with the 'elastase' bacterial *in-vitro* virulence test used by the veterinary diagnostic laboratory at Elizabeth Macarthur Agricultural Institute, DPI, NSW.

Case histories were collected from all flocks including the percentage of score 4 and 5 foot lesions, the percentage of lame sheep, the age and breed of sheep, the environmental conditions and the timing of the most recent foot bathing or antibiotic treatment of sheep. *Dichelobacter nodosus* was isolated from the five worst affected sheep in each flock and bacterial virulence was measured by 3 methods; the mean elastase activity rate, the percentage of *D. nodosus* isolates elastase positive at 12 days, and the first day that elastase activity is detected in any isolate. All three measures of bacterial virulence were significantly correlated, as were the two clinical disease measures (5 score 4 and 5 lesions and % lame sheep).

Logistic regression models were fitted to the data to identify the minimum number of disease predictors that best discriminate between benign and virulent footrot diagnosis. Combining the five predictors Log_{10} % score 4 and 5 lesions, environmental score, Log_{10} mean elastase rate, the percentage of *D. nodosus* isolates elastase positive at 12 days and the first day that elastase activity was detected produced the model that best fitted the data collected from the 184 NSW flocks (ie. the lowest AIC value of the models examined). The inclusion of all three elastase measurements in the model suggests that the elastase test currently used to support clinical diagnosis of footrot needs some refining. Future work is aimed at developing a more rapid and sensitive molecular test to measure the rate of elastase activity by quantifying the messenger ribonucleic acid concentration (mRNA) that dictates the production of the hoof degrading enzyme/s by *D. nodosus*.

To illustrate the modelling, we plotted the predicted regression curves showing the probability of virulent footrot diagnosis with incremental changes in each individual predictor according to the model. For example, the probability of virulent footrot diagnosis increased to 68% when more than 10% score 4 and 5 foot lesions were observed, compared with a baseline of about 20% when there were no score 4 and 5 lesions. This study also better defined the relationship between *D.nodosus* virulence and clinical disease. Currently, the elastase test focusses on the number of days before elastase activity is detected in all ten isolates from a flock. In many cases, the time needed to detect elastase activity varies between isolates from the same flock, making it hard to interpret bacterial virulence across the whole flock. Our modelling shows that if elastase activity isn't detected by 12 days, the probability of virulent footrot was below 70%, but in some flocks only a portion of the isolates are elastase positive at 12 days. By adding the elastase activity rate and the proportion of isolates elastase positive at 12 days, the lab will be able to provide submitting vets with a better estimate of bacterial virulence in the flock. For example, when the mean elastase rate is greater than 0.2 (ie. 4.2mm diameter of elastin cleared over 21 days), the probability of virulent footrot diagnosis is greater than 60%. Likewise, when 25% of all *D. nodosus* isolates are elastase positive by 12 days, the probability of virulent diagnosis increases to 62%. The elastase enzyme activity test can continue to be used to support clinical diagnosis of footrot, but additional measures identified in this project will help in the interpretation of bacterial virulence. The ultimate aim of future research is to

measure bacterial virulence directly from the hoof swab so that we are measuring virulence in all *D.nodosus* isolates, not just two isolates from each hoof.

Background and objectives:

Field strains of the footrot bacterium *Dichelobacter nodosus* range from highly virulent through to benign. Within this range there is a point above which a strain causes virulent footrot (which is subject to regulatory quarantine and control measures), and below which strains cause benign footrot (with no regulatory consequences). Highly virulent footrot is easy to recognise clinically and relatively easy to eradicate because infected sheep can be readily identified. Despite the success of the NSW Strategic footrot plan in eliminating classically virulent footrot from much of the State, lower-virulent footrot has persisted and increased in prevalence, due to the difficulty in recognising lower-virulent (intermediate) footrot if the environmental conditions are not ideal and if the disease has not been fully expressed. The failure of eradication programmes for lower-virulent footrot may result in prolonged quarantine, causing economic hardship for affected farmers. Lack of experience with diagnosing footrot and lack of validation in our diagnostic tools (elastase test at NSW DPI) makes diagnosis and quarantine decisions difficult for field veterinarians required to meet the State's regulatory guidelines and requirements.

NSW DPI footrot diagnostic policy requires that diagnosis is based on clinical signs, but laboratory bacterial virulence tests can be used to support diagnosis in cases where disease expression is sub-clinical. NSW DPI currently use the elastase virulence test which measures the production of the thermostable acidic protease enzyme responsible for severe lesions and degrading the hoof. While this test has been validated with field studies in South Eastern Australia and Western Australia (Dhungyel et al., 2013; McPherson et al., 2017), it is yet to be validated against NSW clinical diagnosis guidelines. NSW guidelines state that *under environmental conditions suitable for the expression of footrot, D. nodosus isolates that are elastase positive between 4 and 11 days are usually capable of causing virulent footrot; whereas isolates that have not produced elastase by 28 days are usually benign* (NSW DPI PRO-2006/024/2. 2016). Interpretation is difficult for isolates that produce elastase between 11 and 28 days, and in these cases (76% of all submitted cases in 2019/2020 financial year in NSW) the elastase results can't clearly support veterinarians in the clinical diagnosis of footrot (Table 1).

Table 1. Interpretation of elastase results between July 2019 and June 2020

	Elastase positive ≤ 11d	Elastase positive 12-28d	Elastase negative ≥ 28d
No. accessions	1	13	3
% accessions	6%	76%	18%

This project was aimed at monitoring the rate of elastase clearing (digestion of elastin particles) in *D. nodosus* isolate cultures from flocks across NSW to get a better idea of how useful the elastase test was in supporting footrot diagnosis. The elastase activity was combined with other data collected from these flocks including the breed and age of affected sheep, environmental conditions on farm, the incidence and severity of foot lesions, treatments used and biosecurity procedures on farm to identify factors that aid in the diagnosis of footrot.

Methods:

Case histories were collected from each of 184 footrot investigations between August 2020 and December 2021 to help identify factors that impact on clinical disease and therefore aid in the diagnosis of footrot. The breed and age of affected sheep was recorded, along with scores for optimal moisture (50 -200mm rain over the previous 3 months), pasture (active growth and high % clover) and temperature (mean minimum temperature greater than 10°C for preceding 2-3 weeks). The prevalence of lameness was recorded, as well as the prevalence and severity of lesions (score 0 to 5) in the hoofs of 100 randomly selected sheep per flock. Any antimicrobial treatments as well as recent antiseptic foot bathing was recorded. *Dichelobacter nodosus* were isolated from the samples collected from 5 worst affected lesions from each accession (lab submission). Bacterial virulence was measured by growing pure isolates of *D. nodosus* on agar media containing elastin particles and recording clearing of elastin by the bacterial enzyme elastase every 3-4 days over a 28 day period. Virulence was measured by the following means:

1. % score 4 and 5 lesions in flocks where ≥ 100 sheep were scored randomly
2. Elastase rate = the mean rate of elastase activity (diameter of elastin cleared/day over 21 days) averaged over all *D. nodosus* isolates from that flock.
3. Elastase pos @ 12d = % of *D. nodosus* isolates that cleared elastin at or before 12 day.
4. Positive elastase mob = at least one *D. nodosus* isolate cleared elastin by 28 days
5. Negative elastase mob = no *D. nodosus* isolates cleared elastin by 28 days
6. First day elastase positive = the minimum number of days before at least one *D. nodosus* isolate per flock cleared elastin in agar.

The frequency distribution of data for the 184 flocks was graphed and transformed if needed to reduce the overt skewness patterns for further analysis. The reason for doing this was to reduce the chance of regression being dominated by the extreme end of the distribution, and so improve predictive ability. The distribution of mean elastase activity rate and % isolates elastase positive at 12 days were skewed left, towards lower activity rates and less isolates elastase positive at 12 days and were transformed by $\log_{10}$ and Logit functions to reduce skewness (Figure 1). Data from the first day that *D. nodosus* isolates were elastase positive showed no apparent skewness between 7 and 28 days, so no transformation was applied.

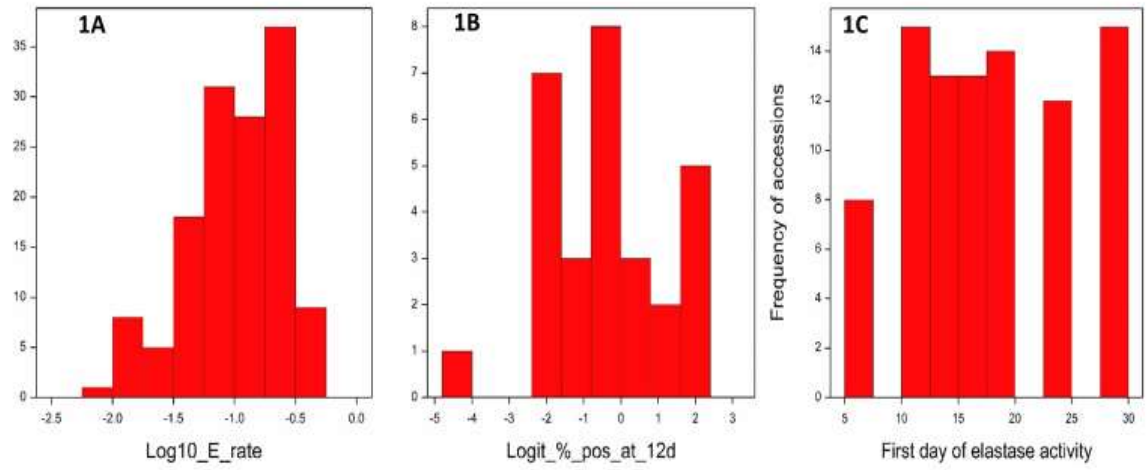

Figure 1. Frequency distribution of measures of elastin clearing by isolated *D. nodosus* bacteria; Log_{10} mean elastase rate (a), percentage of isolates per mob elastase positive at 12 days (b), and the first day that *D. nodosus* isolates were elastase positive (c).

The percentage of score 4 and 5 lesions per flock and the % lame sheep were also skewed left so were Log_{10} transformed to reduce this skewness (Figure 2).

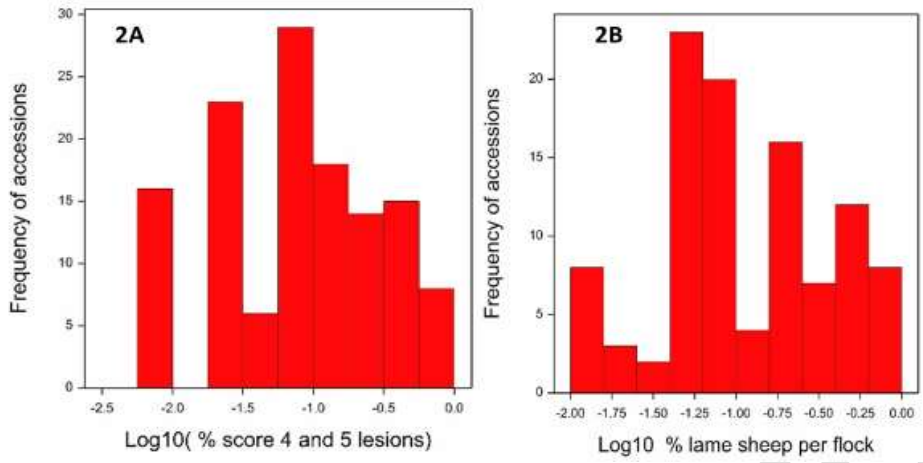

Figure 2. Frequency distribution of measures of disease severity; percentage of score 4 and 5 lesions per mob (a), and the proportion of lame sheep per mob (b).

Results:

Factors that influence footrot diagnosis

1. *Regional influence:*

Between August 2020 and December 2021, the NSW LLS performed 438 footrot investigations on 233 NSW sheep and goat flocks (Figure 3), with 46.8% of investigations being repeat clinical disease inspections on the same 233 properties. Laboratory diagnostics (elastase test) were only submitted for 223 of the 438 investigations (51%), with all submissions from unique mobs except for two mobs which were tested twice. Investigations where either elastase testing or foot scoring were not completed were omitted from the dataset. We therefore enrolled 184 sheep flocks in this comparative study of bacterial virulence (elastase activity) with clinical diagnosis of footrot on farm.

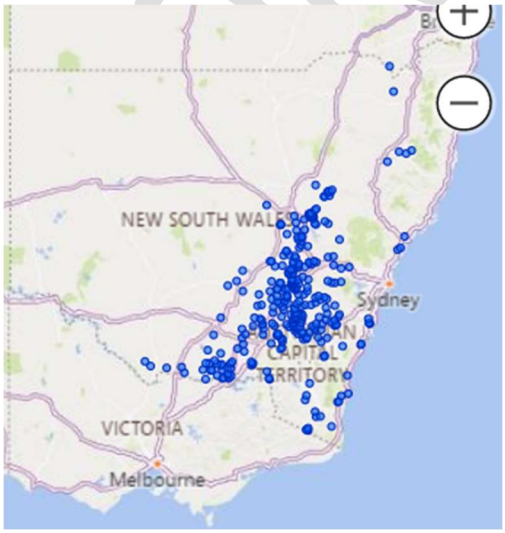

Figure 3. Map of footrot investigations by NSW LLS staff between August 2020 and December 2021 (Courtesy of Dr Scott Ison, Business Partner Animal Biosecurity and Welfare, NSW LLS)

Most laboratory accessions were submitted from the Riverina, South East, Central West, Murray and Central Tablelands Local Land Services (LLS) regions (Table 2). This coincides with the same five LLS regions that produce more than 75% of the annual wool clip by weight in NSW (AWTA Wool Testing - Volume and Trends). The percentage of lab submissions from each region was similar to the percentage of footrot investigations per region, indicating that all regions were submitting samples for elastase testing from field investigations. The number of laboratory submissions in this 16 month period (184 flocks) increased significantly relative to the previous 12 months (July 2019 to June 2020) where only 17 accessions were submitted for elastase testing due to drought conditions over 86.9% of NSW (NSW State Seasonal Update – June 2020).

Table 2. The percentage of footrot sample submissions for elastase testing between August 2020 and December 2021 in each NSW Local land Service region.

LLS Region	# Flocks	% total flocks
Central Tablelands	37	20.1%
Central West	37	20.1%
Hunter	3	1.6%
Murray	27	14.7%
Northern Tablelands	3	1.6%
North West	1	0.5%
Riverina	36	19.6%
South East	40	21.7%
Total	184	

In the 2020/21 study period, 41% of the 438 footrot disease inspections by NSW LLS resulted in a virulent diagnosis and 59% of flock inspections were diagnosed with benign footrot. Of the subset of 184 flocks where samples were submitted for elastase activity, 42.9% were diagnosed with virulent footrot, 46.7% were diagnosed with benign footrot, 8.2% had an open diagnosis and 2.2% showed no disease. In the 16 months since the drought broke in NSW, we've observed a seven-fold increase in the percentage of flocks diagnosed with virulent footrot from submitted accessions. In this post drought period, NSW flocks were restocked with animals from all over Australia, potentially introducing virulent footrot back into regions where it had previously been eradicated. In addition, much of NSW experienced optimal conditions for the expression and transmission of footrot. In the main wool producing regions, higher than average rainfall and warm temperatures persisted throughout spring 2020 (except November 2020), autumn 2021 and spring 2021 (NSW state seasonal update | DPI Climate Branch).

NSW LLS regions with flocks expressing greater than 10% score 4 and 5 footrot lesions included Riverina (32.1%), Murray (62.5%), South East (16.6%), Central West (15.4%) and Central Tablelands (26.3%) (Figure 4). Of the 165 flocks with diagnoses, virulent footrot diagnosis was more frequent than benign footrot in the Murray LLS, whereas the small number of submitted accessions from flocks in the Northern Tablelands and North West LLS were diagnosed with benign footrot (Figure 5). In the Central Tablelands, Central West and Riverina, benign and virulent footrot diagnoses were equally common. Benign diagnosis was more common than virulent disease in the South East region over this same 16-month period.

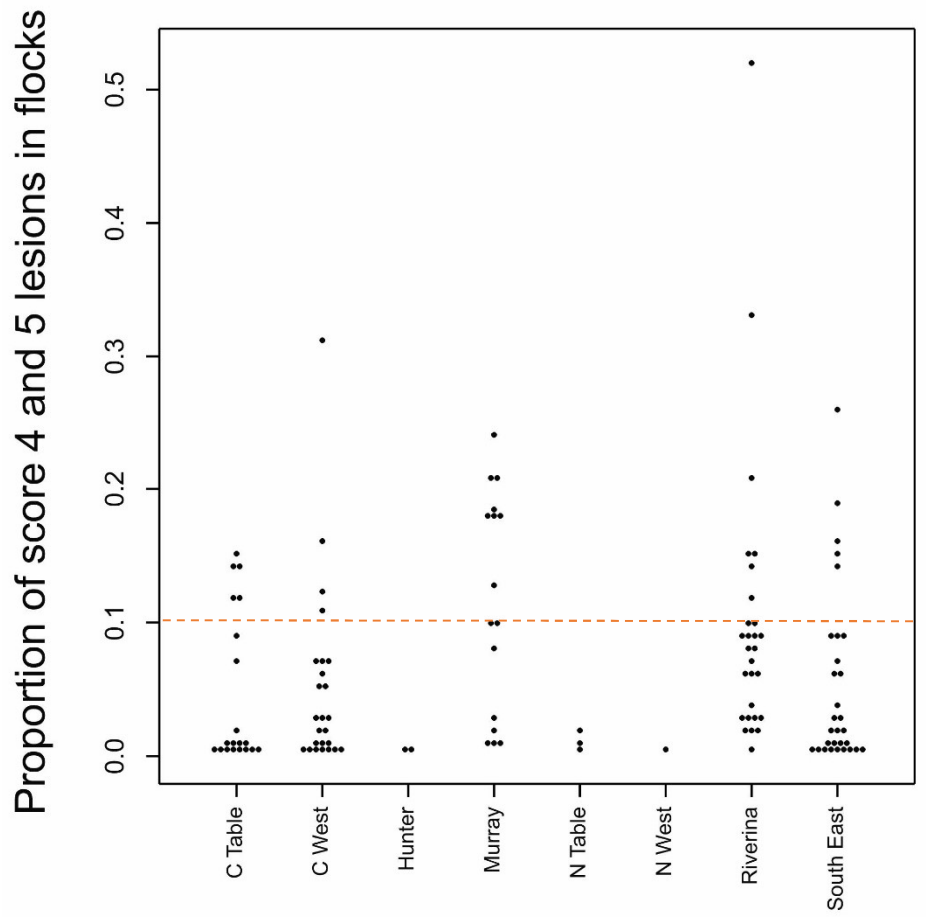

Figure 4. Proportion of severe score 4 or 5 lesions in sheep flocks across all NSW LLS regions.

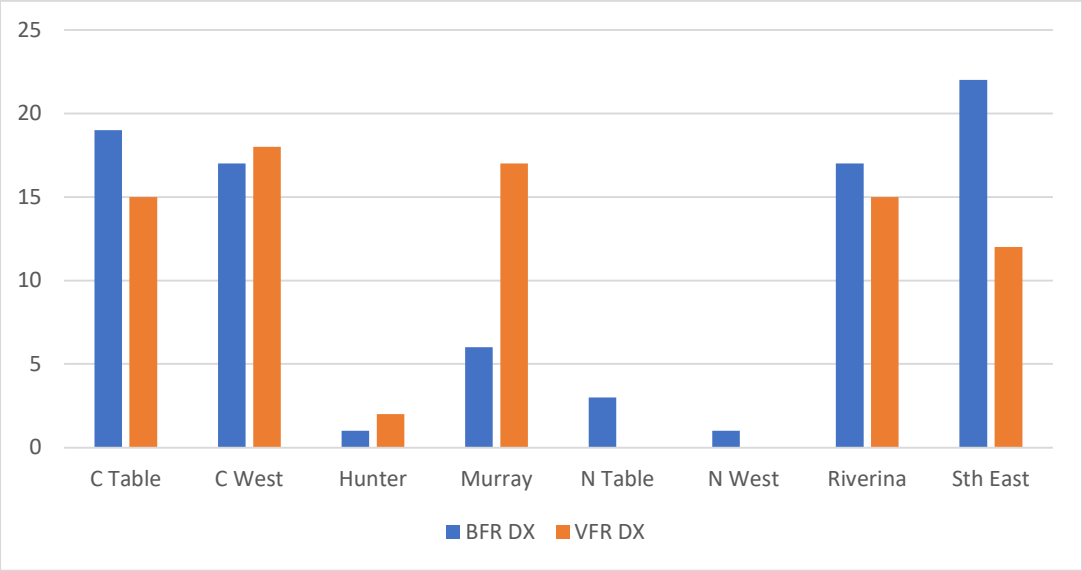

Figure 5. Number of flocks with virulent and benign diagnosis of ovine footrot across the NSW LLS regions from August 2020 to December 2021. Flocks without disease (3) or open diagnosis (16) are excluded.

2. *Effect of sheep breed on clinical footrot:*

The majority of samples submitted for footrot virulence testing were from merino flocks (68.5%), followed by crossbred flocks (17.1%) and a small number of Dohne, Dorper, White Suffolk and Australian White flocks (Table 3). There was no difference in the proportion of merino flocks with either benign or virulent footrot diagnosis, and flock numbers for other breeds were too small to compare. A higher proportion of severe footrot lesions (score 4 and 5) were observed in merino flocks relative to other breeds, but a small proportion of Australian White, crossbred, and mixed breed flocks had severe lesions in more than 10% of inspected sheep (Figure 6).

Table 3. Proportion of breeds within sample population and percentage of each breed diagnosed with benign (BFR) or virulent footrot (VFR).

Breed	# flocks	% flocks	% BRF	% VRF
Aussi White	7	3.9%	66.6%	33.3%
Cross bred	31	17.1%	41.9%	58.1%
Dohne or Dorper	7	3.9%	42.9%	57.1%
Merino	124	68.5%	47.9%	40.3%
Mixed	9	5.0%	55.6%	44.4%
White suffolk	3	1.7%	66.6%	33.3%

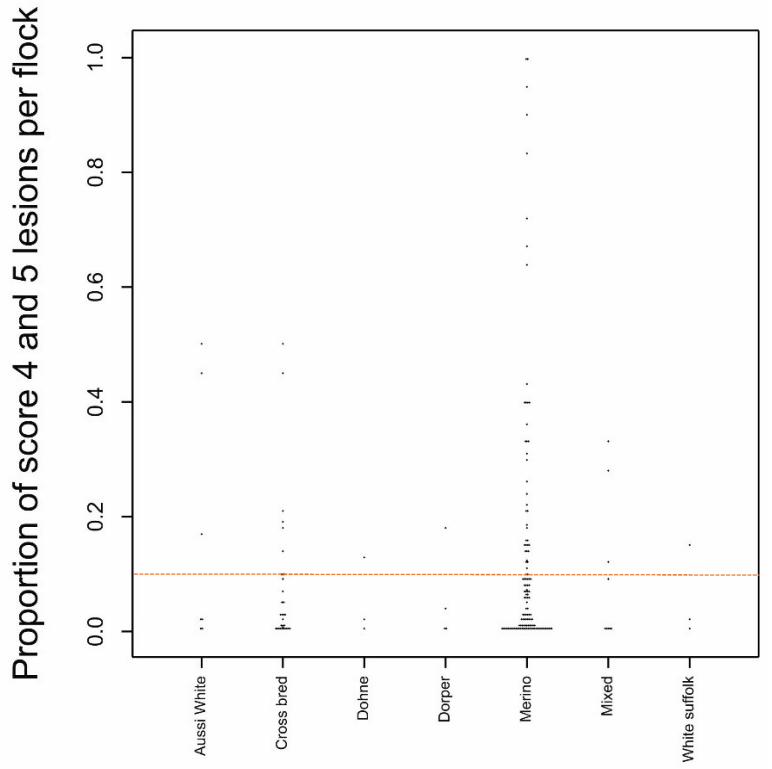

Figure 6. Proportion of score 4 and 5 lesions in different sheep breeds from 184 flocks.

3. *Age effect on clinical footrot:*

The majority of samples submitted for footrot virulence testing were from ewe mobs (60%), with a smaller proportion of lamb mobs (7%), wethers (4%) or mixed age groups (6%). The proportion with virulent footrot did not vary markedly between age, except for mixed ewes and rams, of which there were only 3 flocks (Table 4).

Table 4. Proportion of mob ages within sample population and percentage of each age group diagnosed with benign (BFR) or virulent footrot (VFR).

	# flocks	% ages	% BFR	% VFR
Ewes	124	60%	51.4%	48.6%
Weaners	4	2%	50.0%	50.0%
Rams	5	3%	60.0%	40.0%
Ewes and lambs	7	4%	42.9%	57.1%
Ewes and Rams	3	2%	100.0%	0.0%
Lambs	13	7%	53.8%	46.2%
Hoggetts	5	3%	50.0%	50.0%
Mixed	11	6%	45.5%	54.5%
Wethers	8	4%	62.5%	37.5%

4. *Effect of environment on clinical footrot*

Previous studies have demonstrated that footrot is more fully expressed and more likely to spread between sheep when overnight minimum temperatures are above 10°C and rainfall is above 50mm per month over 3 months. This has led to a combined environmental scoring system for temperature, moisture and pasture length. Optimal footrot spread conditions require a maximum score of 3 for each factor and a total score of 9 for all three factors. However, no clear relationship between higher environmental scores and more severe disease (≥ 10% score 4 or 5 lesions) was observed in this study period (Table 5 and Figure 7). No significant correlation was observed between more severely affected flocks and environmental scores greater than 7 to 9 (Spearman's rank correlation coefficient = - 0.042, P = 0.38). While wet and warm environmental conditions are important to disease expression and transmission of infection, virulent strains of *D. nodosus* must be present to cause underrunning of the hoof and virulent disease. Temperature, moisture (rainfall) and pasture scores from our study were skewed towards higher environmental scores, equating to warmer and wetter conditions and more active pasture growth (Figure 6). Not surprisingly these wet and warm conditions coincide with when vets are most frequently called out to inspect lame sheep in spring and autumn.

Table 5. The percentage of flocks with greater than 10% score 4 and 5 lesions for each environmental score.

Total environmental score	3	4	5	6	7	8	9
% flocks ≥ 10% score 4 or 5 lesions	62.5%	20%	16.6%	45.5%	21.7%	7.1%	21.9%
# flocks ≥ 10% score 4 or 5 lesions	5	1	1	5	5	1	9
Total # flocks	8	5	6	11	23	14	41

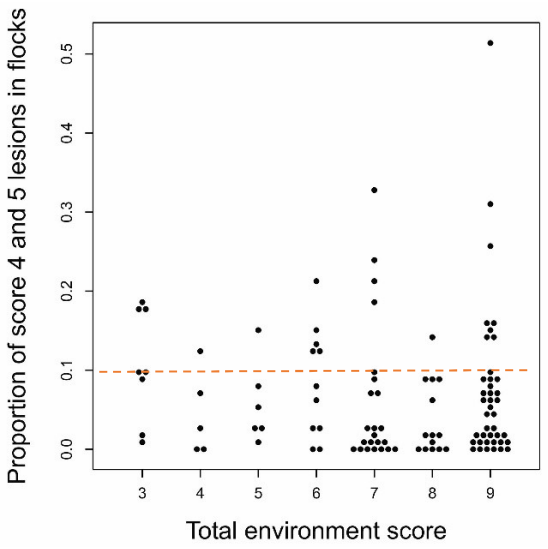

Figure 7. The relationship between increased footrot severity (> 10% score 4 and 5 lesions) and environmental score (temperature, rainfall and pasture growth).

5. *Effect of recent treatment on clinical footrot*

In some flocks, sheep were treated for footrot in the weeks preceding the collection of lesion samples for *D. nodosus* culture and elastase testing. LLS submitters provided the type and timing of treatment (disinfectant foot bath or antibiotic). In flocks where no treatment was given, an arbitrary number of 26 weeks was recorded, and 11 flocks had to be excluded because submitters were not able to learn whether treatment had been provided. Flocks were separated into 'recent treatment' if antibiotics were given in the preceding week or sheep were foot bathed less than 8 weeks previously. Of the recently treated flocks, 60% were diagnosed with virulent footrot and 40% were diagnosed as benign (Figure 8A). Of the non-treated flocks, 57.4% were diagnosed with virulent footrot and 42.6% were diagnosed as benign. A slightly higher proportion of non-treated sheep (29.4%) had early elastase activity (< 12 days) compared to recently treated sheep (20.5%) (Fig 8B).

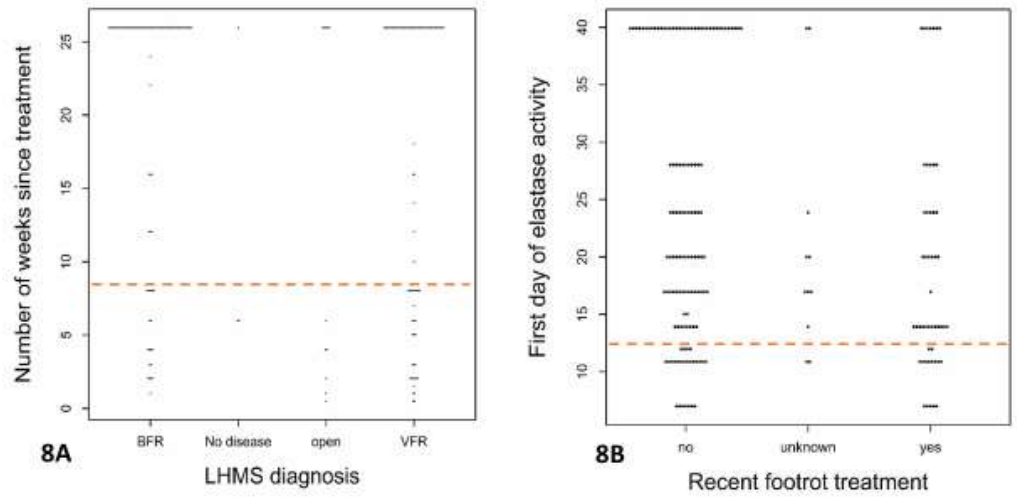

Figure 8. LHMS footrot diagnosis (benign, virulent, open or no disease) and the number of weeks since the last treatment (foot bathing or antibiotics) (a) and the relationship between early elastase activity (before 12 days) and recent footrot treatment of flocks (b).

6. Correlations between flock level disease and bacterial virulence

Measures of higher bacterial virulence including increased mean elastase rate, earlier elastase activity and an increased percentage of isolates elastase positive at 12 days and 28 days were highly correlated with each other (0.74, Table 6). Likewise, disease severity measures at the flock level correlated with each other (% score 4 & 5 lesions and % lame sheep, 0.48). Significant negative correlations were observed between the first day that *D. nodosus* isolates were elastase positive and all other bacterial virulence and disease parameters, indicating that increased numbers of virulent bacteria and a higher incidence of hoof underrunning occurred in flocks where elastase activity was detected early. The mean elastase activity of *D. nodosus* was the only other bacterial virulence measure that correlated with disease severity at the flock level, measured as the percentage of score 4 and 5 foot lesions on a random mob of 100 sheep (0.29, Table 6).

Table 6. Spearman's rank correlation coefficients between bacterial virulence and disease severity

	% E pos@12d	% E pos @28d	Mean elastase rate	% score 4 & 5 lesions	% lame	First day Elast pos
% E pos @12d	1					
% E pos @28d	0.74*	1				
Mean elastase rate	0.5*	0.73*	1			
% score 4 & 5 lesions	0.09	0.08	0.29*	1		
% lame	0.2	0.14	0.18	0.48*	1	
First day Elast pos	-0.60*	-0.45*	-0.77*	-0.33*	-0.23*	1

*Asterisk denotes significant probability ($P < 0.05$) that two virulence measures are correlated.

7. Factors that impact on footrot diagnosis

Virulent diagnosis appears to be associated with a higher mean proportion of lame sheep per flock (Figure 9a), with 45% of benign flocks and 74.4% of virulent flocks showing more than 10% lameness ($Log_{10} \leq -1$). Virulent flock diagnosis also appears to be associated with a higher percentage of score 4- and 5-foot lesions per mob, with 53.7% of virulent and 17% of benign flocks experiencing more than 10% score 4 and 5 lesions ($Log_{10} \leq -1$) per flock (Figure 9b).

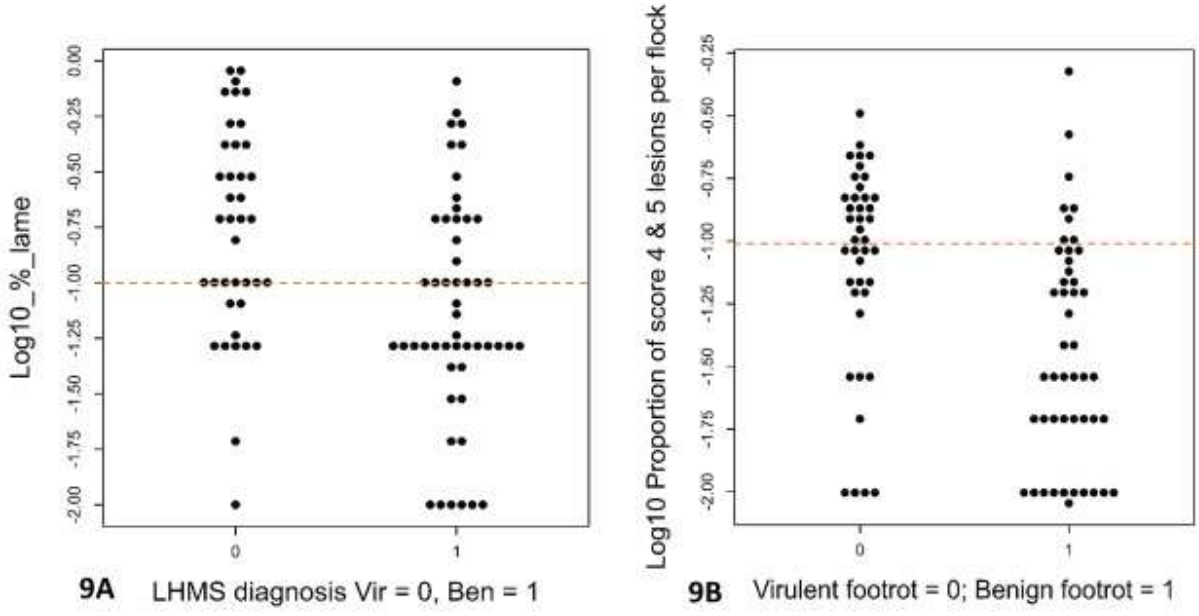

Figure 9. Association between flock footrot diagnosis (benign or virulent) with (a) the proportion of lame sheep per flock (log_{10} transformed) and (b) the percentage of score 5 and 5 lesions per flock (log_{10} transformed).

Diagnosis of virulent footrot also appears to be associated with a rapid rate of elastase activity and early detection of elastase activity in the *D. nodosus* isolates cultured from the 5 worst affected sheep per mob (Figure 10a and 10b).

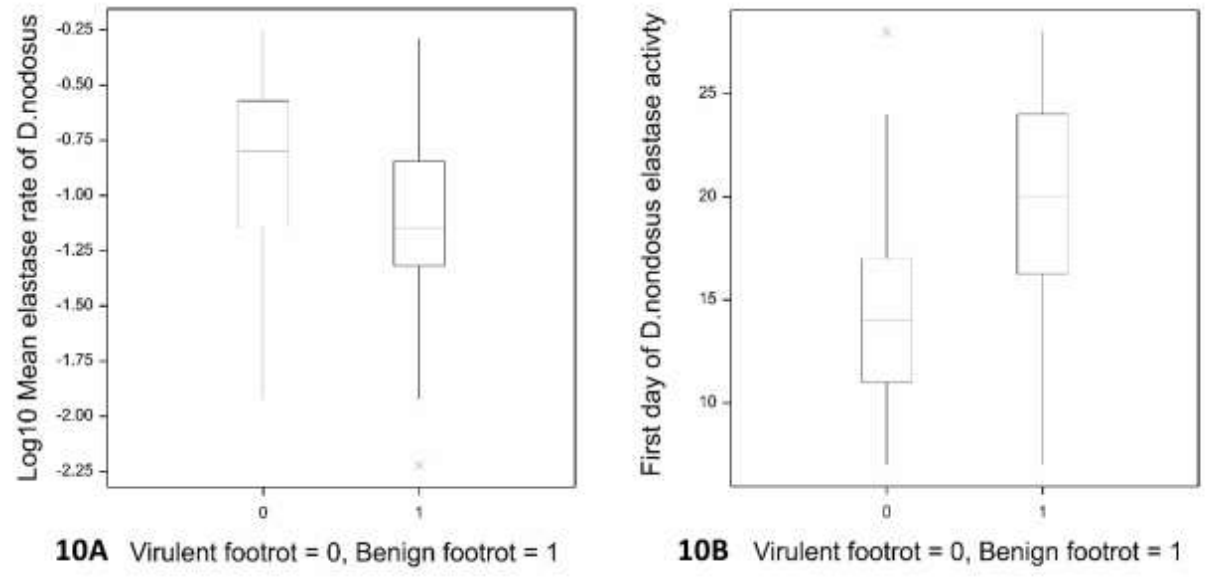

Figure 10. Box plots showing association between flock footrot diagnosis (benign or virulent) with the mean elastase rate (log_{10} transformed) (a) and with the first day that elastase activity was detected in *D. nodosus* isolates (b).

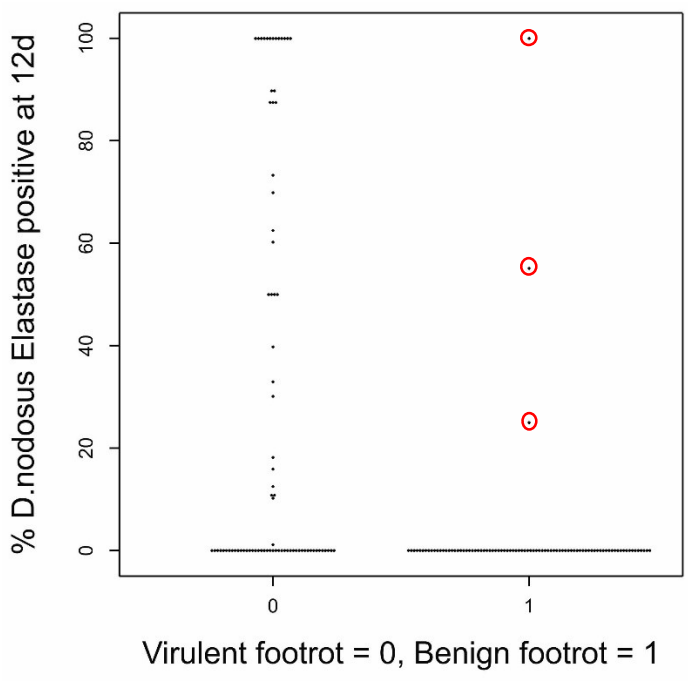

Figure 11. Dot histogram showing association between flock level footrot diagnosis (benign versus virulent) with the percentage of *D. nodosus* isolates elastase positive at 12 days showing 3 outlier results circled in red.

The percentage of *D. nodosus* isolates elastase positive before 12 days also appears to be associated with virulent footrot diagnosis of flocks (Figure 11), with the exclusion of 3 outliers diagnosed as benign footrot (circled in red). The elastase activity test was repeated on these samples, and the bacterial virulence was repeatable. This highlights the complexity of footrot diagnosis, as virulent bacteria were isolated from these 3 flocks, but the LLS vet did not observe clinical evidence of footrot maybe because of host immunity or sub-optimal environmental conditions.

Using the DPI footrot diagnosis guidelines, virulent *D. nodosus* bacteria (elastase detected before 12 days) were isolated from 22.8% of the flocks monitored and benign *D. nodosus* bacteria (elastase not detected by 28 days) were isolated from 25% of flocks. In the remaining 52.2% of flocks, the *D. nodosus* isolates produced elastase between 11 and 28 days, not clearly supporting either benign or virulent footrot diagnosis (Table 7). It is therefore clear that we need to find a way to distinguish benign from virulent footrot in a significant proportion of cases where the current elastase test and measurements can't support diagnosis. Modelling all the factors important in virulent footrot diagnosis and determining their relationship to clinical disease may help support vets trying to make a diagnosis in the field.

Table 7. Interpretation of elastase results between August 2020 and December 2021

	Elastase positive ≤ 11d	Elastase positive 12-28d	Elastase negative ≥ 28d
No. accessions	42	96	46
% accessions	22.8%	52.2%	25%

Generalized linear modelling of factors important in virulent footrot diagnosis

Nine predictors of disease were included in the generalized linear model used to identify the most important predictors in the clinical diagnosis of virulent footrot at the flock level. Predictors included the percentage of score 4 and 5 lesions, the mean rate of elastase activity, the percentage of *D. nodosus* isolates that are elastase positive at 12 days, the first day that elastase activity is observed in *D. nodosus* isolates, the combined environment score (temperature + rainfall + pasture growth), the percentage of lame sheep per mob, the age of sampled animals, the breed of sheep and whether the sheep had recently been treated with either a disinfection footbath or antibiotics. Clinical diagnosis was separated into binary alternatives of either virulent footrot (VRF = 0) or benign footrot (BFR = 1). Flocks with an open diagnosis or no disease were removed from the analysis, leaving 165 flocks in the data set. The model couldn't tolerate zero values for predictors that were $\log_{10}$ transformed (equates to negative infinity), so a value of 50% of the lowest value for that predictor (0.005) was used to replace zero values in the data for % of lame sheep per flock and the % score 4 and 5 lesions per flock prior to applying the $\log_{10}$ transformation. Many benign flocks were missing data about the first day that *D. nodosus* isolates were elastase positive, because their isolates didn't produce elastase before the 28-day cut off. Rather than removing these flocks from the analysis, we set an arbitrary value of 40 days to clear elastase for all isolates where elastase activity wasn't detected by 28 days.

Logistic regression was used for the binary response (virulent or benign). A range of models were examined to find the smallest number of predictors that best explained the factors important in the flock diagnosis of virulent footrot. The quality of each statistical models tested for our data set was evaluated by calculating the Akaike information criterion (AIC), which estimates prediction error. Using our data set, the AIC was calculated by adding the residual deviance from each model to two-times the number of predictors in the model, ie. AIC = deviance + 2*{number of predictors}
The model that best fits our data will therefore have the lowest residual deviance with the fewest predictors, equating to the lowest AIC. The initial models included only one predictor to test the goodness of fit. From this analysis, the predictors Log_{10} (% score 4 and 5 lesions per mob) had the lowest AIC and was therefore considered the single predictor that explains virulent footrot with our data set (Table 8).

Table 8. Best fit analysis (Akaike information criterion) for each logistic regression using single predictors in the generalized linear model of virulent footrot diagnosis

Single predictor models	AIC	Best fit predictor ranking
% *D. nodosus* isolates Elastase positive @ 12days	185.4	3
First day that *D. nodosus* express elastase	184.6	2
Log_{10} Mean elastase rate	199.1	4
Log_{10} % score 4 & 5 lesions	179.9	1
Log_{10} % lame	220.4	9
Total environment score	207.1	5
Animal age	210.2	6
Breed - Merino (Crossbred)	220.2	8
Weeks since last treatment or recent treatment	212.5	7

The logistic regression analysis provided an estimate of each predictor (equivalent to the gradient of the regression on the underlying logit scale), along with the y intercept so that the goodness of fit for each model could be compared with the data we collected. A negative gradient was observed in the regression between benign diagnosis and % *D. nodosus* isolates elastase positive at 12days (Table 9), indicating that as the percentage of elastase positive isolates increased, the probability of benign diagnosis decreased, and consequently the probability of virulent diagnosis increased (Figure 12).

Table 9. Predicted values for the gradient of each regression model.

Single predictor models	estimate of predictor	t Probability
% *D. nodosus* isolates Elastase positive @ 12days	-0.0422	<0.001
First day that *D. nodosus* express elastase	0.0991	<0.001
Log_{10} Mean elastase rate	-0.786	<0.001
Log_{10} % score 4 & 5 lesions	-1.642	<0.001
Log_{10} % lame	-0.209	0.323
Total environment score	0.373	<0.001
Animal age	-0.4	0.68
Breed - Merino (Crossbred)	0.363	0.282
Weeks since last treatment	-0.499	0.155

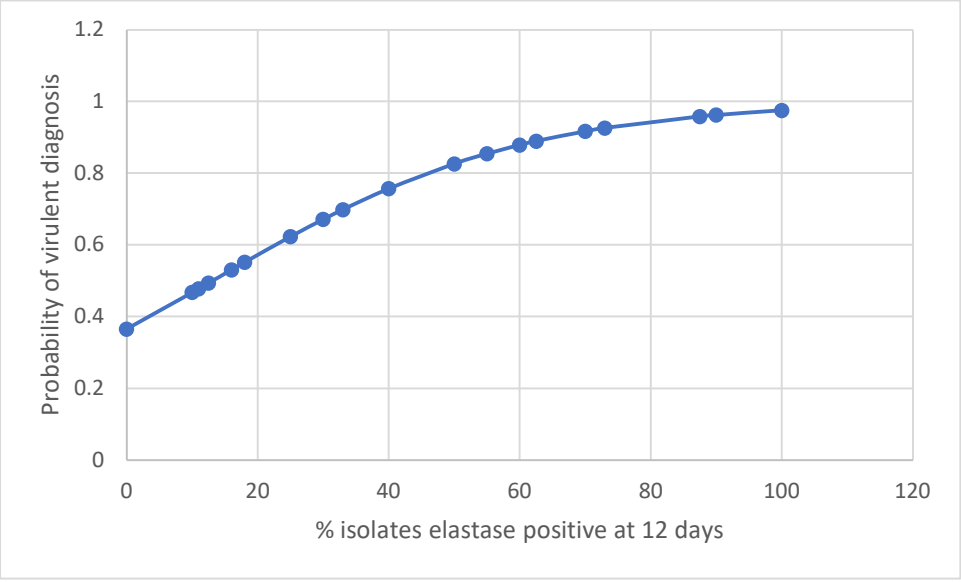

Figure 12. Predicted probability of virulent footrot versus the percentage of *D. nodosus* isolates elastase positive at 12 days (from the predictor model with only % *D. nodosus* isolates elastase positive at 12 days).

The predictors Log_{10} mean elastase rate and Log_{10} % score 4 and 5 lesions were also negatively correlated with benign footrot diagnosis (Table 9), ie. as these predictors increased, the likelihood of benign footrot decreased and consequently, the probability of virulent footrot increased (Figure 13 and 14). Once the *D. nodosus* mean elastase rate was greater than 0.2 (ie. 4.2mm diameter of elastin

cleared over 21 days) for our NSW flocks, the probability of virulent footrot was greater than 60% (Fig. 12). Likewise, once the % score 4 and 5 lesions exceeded 10%, the probability of virulent footrot was more than 70% (Fig. 13).

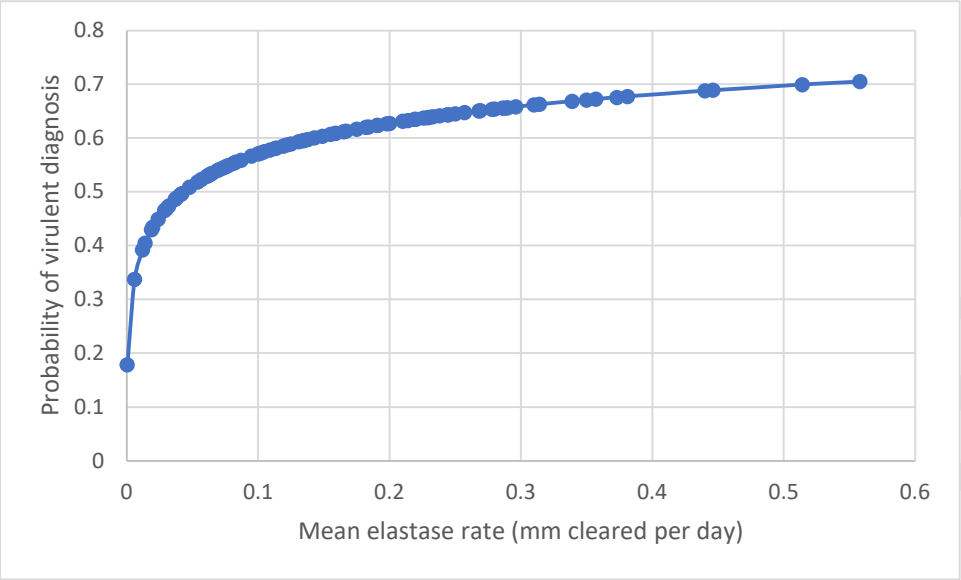

Figure 13. Predicted probability of virulent footrot versus the mean elastase rate (from the predictor model with only mean elastase rate).

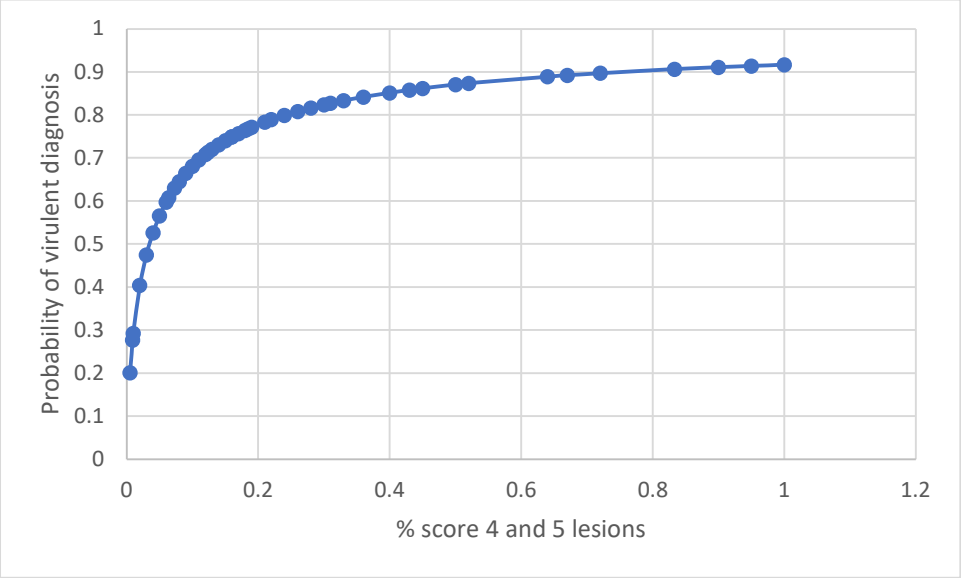

Figure 14. Predicted probability of virulent footrot versus the % of score 4 and 5 lesions (from the predictor model with only % of score 4 and 5 lesions).

In contrast, the predictors environment score and the first day that elastase activity was detected were positively associated with benign diagnosis (Table 9), ie. as the number of days increased before elastase activity was detected, the probability of benign footrot increased and consequently the probability of virulent footrot decreased (Figure 15).

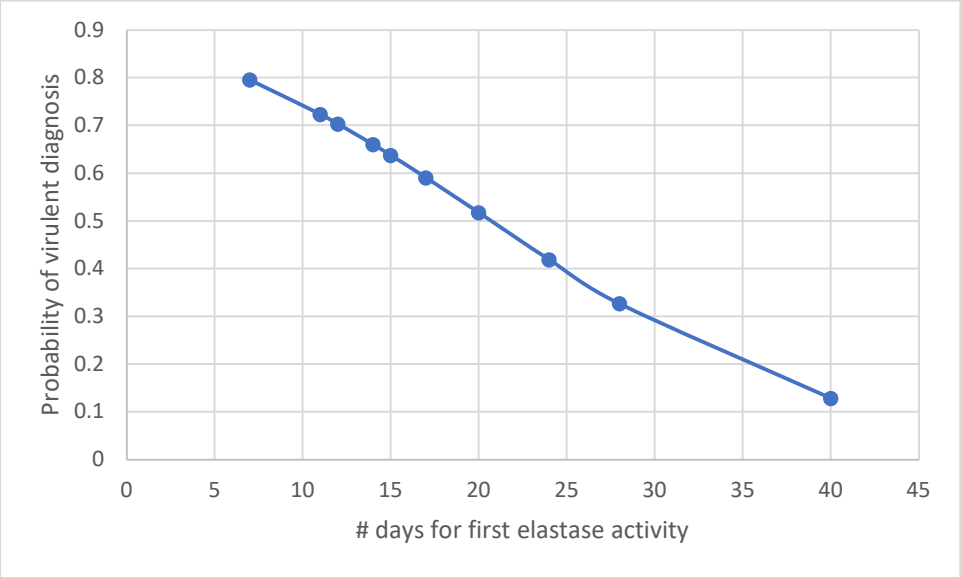

Figure 15. Predicted probability of virulent footrot versus the first day that elastase was detected (from the predictor model with only first day that elastase was detected).

The positive association between increasing environment score and benign diagnosis was significant (probability < 0.001), but hard to explain. Increasing environmental scores (wet and warm conditions with good pasture growth) aid in the expression and transmission of virulent footrot, but by themselves can't cause virulent footrot. The virulent strain of *D. nodosus* needs to be present and active in the hoof to cause virulent footrot.

Adding a second predictor to the highest ranked single predictor model (Log_{10} % score 4 & 5 lesions) reduced the AIC and thus increased the goodness of fit for the model. The best fit regression including the two predictors Log_{10} % score 4 and 5 lesions and % *D. nodosus* isolates elastase positive at 12 days (Table 10). Adding the predictors % lame animals per flock and recent treatment into the model reduced the predictive value of the model.

Table 10. Best fit analysis (Akaike information criterion) for each logistic regression using two predictors in the generalized linear model of virulent footrot diagnosis

Two predictor models	AIC	Best fit ranking
Log_{10} % score 4 & 5 lesions + % elastase positive @ 12d	162.2	1
Log_{10} % score 4 & 5 lesions + Log_{10} Mean elastase rate	172	4
Log_{10} % score 4 & 5 lesions + First day elastase positive	162.6	2
Log_{10} % score 4 & 5 lesions + Breed Merino	176.9	5
Log_{10} % score 4 & 5 lesions + Environment score	168.8	3
Log_{10} % score 4 & 5 lesions + Recent treatment	181.4	6
Log_{10} % score 4 & 5 lesions + Log_{10} % lame	181.8	7

Adding the predictor 'environment score' to the two highest ranked predictors (Log_{10} % score 4 & 5 lesions and % *D. nodosus* isolates elastase positive @ 12d) reduced the AIC further and therefore increased the model's predictive value (Table 11). Other models containing different combinations of 3 predictors also increased the goodness of fit relative to the best 2 predictors model. Adding recent treatment to the best 2 predictors model reduced the predictive value of the model.

Table 11. Highest ranked 'Best fit' analysis (Akaike information criterion) for the best logistic regressions using three predictors in the generalized linear model of virulent footrot diagnosis.

Three predictor models	AIC	Best fit Rank
Log_{10} % score 4 & 5 lesions + % elastase pos @ 12d + Environment score	154.0	1
Log_{10} % score 4 & 5 lesions + Enviro score + first day elastase positive	155.5	2
Log_{10} % score 4 & 5 lesions + First day elastase pos + Log_{10} Mean elastase rate	157.2	3
Log_{10} % score 4 & 5 lesions + % elastase pos @ 12d + first day elastase pos	158.0	4
Log_{10} % score 4 & 5 lesions + % elastase pos @ 12d + Mean elastase rate	161.8	5
Log_{10} % score 4 & 5 lesions + % elastase pos @ 12d + Breed Merino	162.0	6
Log_{10} % score 4 & 5 lesions + First day elastase pos + Breed Merino	163.0	7
Log_{10} % score 4 & 5 lesions + % elastase pos @ 12d + Recent treatment	163.2	8
Log_{10} % score 4 & 5 lesions + Enviro score + Log10 mean elastase rate	163.3	9
Log_{10} % score 4 & 5 lesions + First day elastase pos + Recent treatment	164.4	10

The four predictors models that best fitted our data contained Log_{10} % score 4 & 5 lesions, environment score and at least 2 different elastase activity parameters (% *D. nodosus* isolates elastase positive @ 12d, Log_{10} mean elastase rate and first day that *D. nodosus* isolates are elastase positive) (Table 12). While these predictors are significantly correlated to each other (Table 5), they each measure a different aspect of the *D. nodosus* elastin degradation activity. It appears that the individual elastase predictors are imperfect but adding 2 or 3 of the elastase predictors into the same model increases the goodness of fit of the model to our data. The 4 predictor best fit regression (containing Log_{10} % score 4 and 5 lesions, environment score, Log_{10} mean elastase rate + first day that *D. nodosus* isolates were elastase positive) is a better fit than the highest ranked 3 predictor model.

Table 12. Highest ranked 'Best fit' analysis for the four-predictor logistic regression models that best fit our virulent footrot diagnosis data with their AIC (Akaike information criterion) values.

Four predictor models	AIC	Best fit Rank
Log_{10} % score 4 & 5 lesions + Enviro score + Log_{10} elastase rate + first day elastase positive	150.4	1
Log_{10} % score 4 & 5 lesions + Enviro score + Log_{10} mean elastase rate + % elastase positive at 12d	154.5	2
Log_{10} % score 4 & 5 lesions + Log_{10} mean elastase rate + % elastase positive at 12 days + first day elastase positive	155.2	3

The regression model containing the following 5 predictors was the best fit in modelling the predictors important in virulent footrot using our data set; Log_{10} % score 4 and 5 lesions + Enviro score + Log_{10} mean elastase rate + % isolates elastase positive @12d + first day elastase positive (Table 13). Adding the predictors recent treatment, merino breed, log_{10} % lame sheep or any combination of these additional predictors increased the AIC and therefore reduced the goodness of fit of the model to our data.

Table 13. Highest ranked 'Best fit' analysis (Akaike information criterion) for the highest-ranking logistic regressions using five or six predictors in the generalized linear model of virulent footrot diagnosis.

Five and six predictor models	AIC	Best fit Rank
Log_{10} % score 4 & 5 lesions + Enviro score + Log_{10} mean elastase rate + % isolates elastase positive @12d + first day elastase pos	149.2	1
Log_{10} % score 4 & 5 lesions + Enviro score + Log_{10} mean elastase rate + % isolates elastase positive @12d + first day elastase pos + Recent treatment	150.2	2
Log_{10} % score 4 & 5 lesions + Enviro score + Log_{10} mean elastase rate + % isolates elastase positive @12d + first day elastase pos + Breed -Merino	150.9	3
Log_{10} % score 4 & 5 lesions + Enviro score + Log_{10} mean elastase rate + % isolates elastase positive @12d + first day elastase pos + log_{10} % lame	151.1	4
Log_{10} % score 4 & 5 lesions + Enviro score + Log_{10} mean elastase rate + % isolates elastase positive @12d + first day elastase pos + breed-Merino + Recent treatment	152	5
Log_{10} % score 4 & 5 lesions + Enviro score + Log_{10} mean elastase rate + % isolates elastase positive @12d + first day elastase pos + log_{10} % lame + breed-Mo	152.8	6

Discussion:

In diagnosing virulent footrot, vets have to rely on clinical signs of underrunning hoof lesions (score 4 and 5), recording the prevalence and severity of lesions in a random inspection of 100 sheep per flock. NSW DPI footrot diagnostic guidelines suggest that once the prevalence of score 4 and 5 lesions reaches 5% of the inspected mob, a virulent diagnosis is more likely. Our modelling of factors important in virulent footrot diagnosis certainly supported these guidelines, with the probability of virulent footrot diagnosis elevated to 68% when more than 10% score 4 and 5 foot lesions were observed, compared with a baseline of about 20% probaility of virulent diagnosis when no score 4 and 5 lesions were observed.

However, other factors need to be considered in diagnosing footrot, including the age and breed of sheep, and recent wet and warm conditions to encourage good pasture growth. Clinical signs of virulent footrot may be subdued in cold winters and dry summers and may also be masked by recent treatment with antibiotics or foot bathing in disinfectants. Lower-virulent footrot (intermediate) is more difficult to recognise if the environmental conditions are not ideal and if the disease has not been fully expressed.

Under these conditions, vets can submit samples from the affected foot lesions for isolation of *D. nodosus* and characterisation of the virulence of isolated bacteria. Many attempts have been made to develop a laboratory test that supports virulent footrot diagnosis by measuring the expression of

bacterial virulence genes. Five swabs are collected from the worst affected sheep and up to 10 different *D. nodosus* isolates are purified and tested for elastase activity. In virulent *D. nodosus* strains, the aprV2 acidic protease gene which encodes for a thermostable protease enzyme and expression of this enzyme is thought to be involved in hoof tissue damage. The elastase test measures the enzyme activity of this protease in a specialised agar containing elastin particles. Virulent isolates of *D. nodosus* can digest elastin particles, producing clear zones along the bacterial streak lines within 10 to 12 days of incubation, whereas benign *D. nodosus* isolates show no evidence of elastin clearing when incubated for up to 28 days. However, a significant proportion of *D. nodosus* isolates from affected hooves clear elastin between 12 and 24 days (76% of flocks in drought affected 2019-2020 and 52.2% flocks in 2020-2021), and currently NSW DPI has no guidelines on how to interpret these in-between bacterial virulence results. In addition, it is relatively common that these 10 isolates differ in the time needed for elastin clearing to be observed, and up to now there has been no way to interpret variable elastase results from the same flock.

In this study, we evaluated three different ways to measure elastase activity from *D. nodosus* isolates including the mean elastase rate, the first day that any *D. nodosus* isolate from the mob cleared elastin, and the percentage of isolates that clear elastin by 12 days incubation. The mean elastase rate measured the diameter of cleared elastin between 7- and 28-days incubation and divided that diameter by 21 days. Values varied from zero to a maximum of 0.558 and the mean elastase rate correlated highly with other measures of elastase activity (the percentage of isolates elastase positive at 12 days and the first day that any isolate cleared elastin) and with measures of disease severity (the percentage of score 4 and 5 lesions per mob). However, by itself the mean elastase activity was only a moderately good predictor of virulent footrot diagnosis (60% probability when elastase rate was ≥ 0.2mm/day) and testing still required about 6 weeks to produce results.

The predictor 'first day that any *D. nodosus* isolate from a mob cleared elastin' was correlated highly (but negatively) with other measures of elastase activity (the percentage of isolates elastase positive at 12 days and the mean elastase rate) and with measures of disease severity (% score 4 and 5 lesions per mob). The first day that any isolate cleared elastin was a good predictor of virulent footrot diagnosis (the probability of virulent diagnosis was 70% if any isolate cleared elastin by 12 days), and this earlier measure may reduce the time needed to produce results.

The percentage of isolates that clear elastin by 12 days incubation was also highly correlated with other measures of elastase activity (the first day that any isolate cleared elastin and the mean elastase rate) and with measures of disease severity (% score 4 and 5 lesions per mob). The probability of virulent diagnosis increased to 75% in outbreaks where greater than 40% of isolates cleared elastin by 12 days relative to 36.6% probability of virulent diagnosis when no isolates from a mob cleared elastin by 12 days.

While each of these three elastase measures increased the probability of correctly predicting virulent footrot diagnosis, the best model included all three elastase measures instead of any single elastase predictor of virulent diagnosis. In addition, the best model to predict virulent footrot diagnosis included the predictors % score 4 and 5 lesions and the environmental score. It is possible that studies performed under different conditions (different sheep breeds, ages and environment) might highlight other factors important in the diagnosis of virulent footrot, and to that end we plan to continue monitoring the relationship between bacterial virulence (elastase activity) and virulent diagnosis (% score 4 and 5 lesions) in diagnostic submissions to the EMAI laboratory. However, we've also commenced a new project to develop a more rapid and sensitive molecular test to measure the rate of elastase activity by quantifying the messenger ribonucleic acid concentration

(mRNA) that dictates the production of the hoof degrading enzyme by *D. nodosus*. This molecular test could reduce testing times down to two weeks instead of six if *D. nodosus* isolates need to be purified from hoof swab samples. The ultimate aim is to extract and quantify mRNA for the acidic protease gene aprV2 directly from hoof swabs so that we can calculate the virulence profile of all *D. nodosus* isolates in an affected hoof. In the meantime, the elastase enzyme activity test can continue to be used to support clinical diagnosis of footrot, but additional measures identified in this project will help in the interpretation of bacterial virulence.

References

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McPherson A, Dhungyel OP, Whittington RJ Evaluation of genotypic and phenotypic protease virulence tests for Dichelobacter nodosus infection in sheep. J Clin Microbiol 2017;55:1313–1326.

NSW Department of Primary Industries. Procedure for diagnosis of footrot.PRO-2006/024/2. 2016.

NSW Department of Primary Industries. NSW State Seasonal Update - June 2020

NSW Department of Primary Industries. NSW state seasonal update | DPI Climate Branch

Industry presentations and workshops to date:

1. Footrot – the role of elastase testing in diagnosis. Local Land Services webinar, September 2020
2. Status of virulent footrot in South and Western NSW, and diagnostic efficiency, NSW District Veterinarians conference. March 2021.
3. Footrot case discussion. LLS on-line workshop, May 2021.
4. Evaluation of *D. nodosus* elastase test in NSW flocks August 2020 to December 2021. NSW District Veterinarians Conference (due April 2022).
5. Preparing a scientific publication for the Australian Veterinary Journal.

Expenditure:

Heads of expenditure	Value
TO Salary	$17,700
Travel	$100
Consumables	$19,649
Total Budget	$37,449