Assessment of a rtPCR for the detection of virulent and benign *Dichelobacter nodosus*, the causative agent of ovine footrot, in Australia

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**Abstract**

**Background:** Ovine footrot is a highly contagious bacterial disease of sheep, costing the Australian sheep industry millions of dollars annually. *Dichelobacter nodosus*, the causative agent of footrot, is a gram-negative anaerobe classed into virulent and benign strains as determined by thermostability of their respective proteases. Current methods for detection of *D. nodosus* are difficult and time-consuming, however new molecular techniques capable of rapidly detecting and typing *D. nodosus* have been reported.

**Results:** A competitive real-time PCR (rtPCR) method, based on the ability to detect a 2 nucleotide difference in the *aprV2* (virulent) and *aprB2* (benign) extracellular protease gene has been tested on Australian samples for determining detection rates, along with clinically relevant cut-off values and performance in comparison to the traditional culturing methods. The rtPCR assay was found to have a specificity of 98.3% for virulent and 98.7% for benign detection from samples collected. Sheep with clinical signs of footrot showed a detection rate for virulent strains of 81.1% and for benign strains of 18.9%. A cut-off value of a Ct of 35 was found to be the most appropriate for use in Victoria for detection of sheep carrying virulent *D. nodosus*.

**Conclusions:** In summary, the rtPCR assay is significantly more capable of detecting *D. nodosus* than culturing, while there is no significant difference seen in virotyping between the two methods.

**Keywords:** Australia, Benign, Footrot, Real-time polymerase chain reaction, Sensitivity, Sheep, Specificity, Victoria, Virulent

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**Background**

Ovine footrot is a highly contagious bacterial disease of sheep, causing lesions in the hoof and lameness [1]. The primary aetiologic agent of ovine footrot is *Dichelobacter nodosus*, a gram-negative anaerobe [2]. Many strains of *D. nodosus* exist consisting of multiple serogroups that are classified in Australia into virulent or benign based on extracellular protease activity. Infections with benign strains may appear as inflammation of the interdigital skin (interdigital dermatitis), while infections with virulent strains may vary from interdigital dermatitis to severe lesions with extensive necrosis and separation of the horn from the soft tissue [3, 4]. Footrot lesions are graded using a simple scoring system ranging from 0 (clinically healthy) to 5 (severe underrunning of the hard horn of the hoof) [3, 4]. The severity of lesions produced by virulent strains is reliant on environmental conditions, with temperatures of above 10 °C and consistent rainfall required for the full expression of virulence factors [5]. Subsequently, when environmental conditions are not optimal, infection with virulent strains may not be apparent clinically or it may present itself as mild infection imitating benign footrot [6]. The virulence potential of the *D. nodosus* strains may be determined by measuring the
thermostability of serine proteases of isolates using the gelatin gel (gelatinase) test [7]. However, culture-based assays have been reported to have modest diagnostic power [8]. Furthermore, culture-based tests are labour-intensive and requires several weeks for the results to become available [9]. It has been reported that the acidic protease 2 (AprV2) plays a key role in virulence of D. nodosus [10]. Virulent strains have the aprV2 gene encoding a thermostable protease. Benign strains have the gene aprB2 encoding a thermolabile protease. The aprV2 and aprB2 alleles vary by a two-base pair substitution. This difference has been exploited in the probe design of a real-time polymerase chain reaction (rtPCR) assay [11]. Using this rtPCR assay, the presence of D. nodosus and its virulence can be determined within 1 day. The test is also capable of detecting both benign and virulent in the same clinical sample. There are limited data on the diagnostic performance of the rtPCR in sheep in Europe [12]. The objective of this paper was to evaluate the rtPCR assay with clinical samples collected from sheep in Victoria, Australia, with confirmed or suspected virulent footrot and sheep considered to be free of infection with D. nodosus.

Methods
Sample collection
The clinical samples were obtained retrospective from Victoria Government Veterinary Diagnostic unit collection. The samples were submitted by Victorian District Veterinary Officers, Animal Health Officers and private veterinary practitioners from the interdigital skin of lame sheep for routine diagnostic testing. Flocks were selected for this study if individual foot scores corresponding to sample labelling from individual sheep were provided, as well as the clinical history of the flock as determined by the submitting persons. Three hundred eighteen sheep from 10 flocks (#1 to 10) considered free of footrot, 170 sheep from 13 flocks (#12 to 24) with confirmed and/or suspected virulent footrot and 27 sheep from a closed flock with the history of virulent footrot that was deemed successfully eradicated. Prior to sampling, each sheep was examined and foot lesions scored and recorded. The interdigital skin of the foot with the highest score was sampled using two sterile swabs. One swab was placed into Stuarts Transport Media for culture and the second swab was placed into 800 μL of phosphate buffered saline (PBS) (8.1 mM Na₂HPO₄, 137 mM NaCl, 1.4 mM KH₂PO₄ and 2.6 mM KCl) with 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0), for rtPCR. The origin, breed, sex, age, foot score of the sheep sampled and their flock history are presented in Tables 1 and 2. Samples were kept at 4 °C after collection and sent the following day to AgriBio, 5 Ring Rd., La Trobe, Bundoora, Victoria, 3083. Samples were submitted for disease investigations pertaining to footrot.

Culturing of D. nodosus
Swabs collected from sheep from flocks 1 to 14 were plated at AgriBio one to 2 days after collection onto 4% (w/v) agar with 3% (w/v) ground hoof media (Footrot Reference Laboratory, Department of Agriculture, Perth, Australia) and anaerobically incubated at 37 °C for 7 days. Plates were examined for D. nodosus growth and suspect colonies subcultured and gram stained as described elsewhere [9]. D. nodosus isolate A198 (aprV2) (AC: 6466), virulent control, and isolate C305 (aprB2) (AC: 6465), benign control, were obtained from the Footrot Reference Laboratory and grown concurrently with sample plates. Isolates morphologically consistent with D. nodosus were sent on ice to the Footrot Reference Laboratory for gelatinase testing. Swabs collected from sheep from flocks 15 to 24 were sent on ice by overnight courier to the Footrot Reference Laboratory, Perth, Western Australia for culture and gelatinase testing.

DNA extraction
DNA was extracted from swabs using a commercial extraction kit (MagMAX™ – 96 Viral RNA isolation kit, Ambion, Austin, USA) and purification system (Kingfisher-96 magnetic particle handling system, Thermo Fisher Scientific, Finland). Swabs from two positive culture controls were used as positive extraction controls. Swabs collected from sheep from flocks 1 to 14 were subjected to DNA extraction on two separate occasions (runs).

aprV2/B2 rtPCR
Primers, probes and cycling conditions as described by Stauble et al., 2014 were used [11]. A commercial rtPCR kit (AgPath-ID™ One Step RT-PCR Kit, Ambion, Austin, USA) was used as master mix according to manufacturer’s instructions, with final concentrations of 300 nM primers, 100 nM DnAprTM-vMGB, 250 nM DnAprTM-bMGB and 5 μL of template DNA. Primers and probes were synthesised commercially (Primers and probes, Applied Biosystems, California, USA) Reactions were carried out and analysed (7500 Fast Real-Time PCR System, Life Technologies) with a set threshold of 0.05. Two DNA extracts derived from sheep from flocks 1 to 14 were assayed by the rtPCR in two separate runs. Singular DNA extracts derived from sheep from flocks 15 to 24 were assayed by the rtPCR in one run.

Positive extraction controls from live cultures of isolate A198 and C305, and purified and isolated genomic DNA from the same culture isolates were used as rtPCR controls in each run. The rtPCR run was considered valid when results obtained in rtPCR controls were
concordant. Results are reported as cycling threshold (Ct) values, the point at which the sample signal exceeds the threshold of 0.05. Samples producing a probe-specific fluorescent signal were defined as being positive. The effect of two cut-off values; Ct < 40 and Ct < 35, on the rtPCR detection rate and specificity, was assessed.

Data analysis
Considering the culture/gelatinase method lacks adequate diagnostic accuracy and both the virulent and benign strains of D. nodosus may produce subclinical or mild, clinically unapparent infection, detection rates of the aprB2 rtPCR and aprV2 rtPCR were calculated using data obtained from 135 sheep with foot lesions and 35 healthy sheep from the 13 flocks with confirmed or suspected virulent footrot. From data collected through the clinically healthy trial, the use of a Ct of 40 showed poor discrimination between healthy and clinically affected sheep in both aprV2 and aprB2 results (p > 0.005, both, Table 4). When using a Ct

Table 1 Descriptive characteristics of 11 Australian sheep flocks considered free of virulent footrot were sampled between June '15 and August '15 for evaluation of the specificity of an rtPCR for detection of virulent (aprV2) and benign (aprB2) protease genes of D. nodosus

| Flock ID | No. animals sampled | Sampling date | Flock origin (shire or city) | Breed | Age | Sex | Comments/Flock history |
|----------|---------------------|---------------|-----------------------------|-------|-----|-----|------------------------|
| 1        | 18                  | 02.06.2015    | City of Broken Hill, NSW    | Merino Lambs Mixed | Abbatoir line. |
| 2        | 18                  | 02.06.2015    | Blayney Shire, NSW          | Mixed Mixed Mixed | Abbatoir line. |
| 3        | 19                  | 02.06.2015    | Shire of Ararat, Vic        | Mixed Rams Male | Abbatoir line. |
| 4        | 18                  | 02.06.2015    | Shire of Ararat, Vic        | Merino Mixed Female | Abbatoir line. |
| 5        | 19                  | 02.06.2015    | Shire of Ararat, Vic        | Merino Lambs Mixed | Abbatoir line. |
| 6        | 18                  | 02.06.2015    | City of Wagga Wagga, NSW    | Merino Ewes Female | Abbatoir line. |
| 7        | 18                  | 02.06.2015    | Southern Grampians, Vic     | Crossbreed Ewes Female | Abbatoir line. |
| 8        | 55                  | 16.06.2015    | Yarra Ranges Shire, Vic     | Coopworth crosses Ewes Female | Farm has had previous intermittent lameness, footrot has not been confirmed as the cause. |
| 9        | 81                  | 01.07.2015    | Strathbogie Shire, Vic      | Merino Wethers Male | Farm has no history of footrot, but lameness occasionally observed. Sheep footbathed late 2014. |
| 10       | 54                  | 17.07.2015    | Wellington Shire, Vic       | Merino Wethers Male | Well managed merino stud, no history of footrot. Wether had strayed into adjoining properties and had been caught and shorn 2 days prior to sampling. |
| 11a      | 27                  | 05.08.2015    | East Gippsland Shire, Vic   | Merino Ewes Female | Virulent footrot first introduced in the 80’s, treated by footbathing. Second footrot introduced in 1995; eradicated by footbathing, antibiotic regime and culling. A closed flock currently. No footbathing for ≥ 10 years. |

* Flock 11 has been excluded from specificity and sensitivity calculations because of its history.
Table 2: Descriptive characteristics of 11 Australian sheep flocks considered having virulent footrot were sampled between October '14 and July '15 for evaluation of the specificity of an rtPCR for detection of virulent (aprV2) and benign (aprB2) protease genes of D. nodosus.

| Flock ID | No. animals sampled | Sampling date  | Flock origin (shire or city) | Breed | Age | Sex | Comments/Flock history |
|----------|---------------------|----------------|-----------------------------|-------|-----|-----|------------------------|
| 12       | 26                  | 27.07.2015     | Wangaratta Rural City, Vic   | Dorper| Rams| Male| Farm has a history of virulent footrot. Footbathing and foot pairing done frequently. Minimal lameness and lesions currently present. Sheep previously footbathed in May 2015. |
| 13       | 10                  | 10.07.2015     | East Gippsland Shire, Vic    | Merino Cross | Mixed| Mixed| Footrot introduced December 2014 by purchased rams. Owners observed lameness of about 1 in 150 animals in March/April 2015. |
| 14       | 36                  | 23.06.2015     | Mitchell Shire, Vic          | Merino| Rams| Male| Farm suspected of virulent footrot, samples taken on a confirmatory visit. |
| 15       | 10                  | 13.10.2014     | Strathbogie Shire, Vic       | Merino| NR  | Female| History of footrot and lameness in flock |
| 16       | 10                  | 13.10.2014     | Greater Shepparton City, Vic | Merino cross | 2.5 y| Female| A mob of South African Merino X Merino yearling ewes purchased in Nov-Dec 2013. Sheep had been on agistment. A few sheep lame when they arrived; footbathed 2–4 weeks before sampling. |
| 17       | 10                  | 14.10.2014     | Shire of Newstead, Vic       | Merino| Mixed| Female| Property has a footrot history - previously treated successfully with Footrite®. This season a recurrence of lameness, some mobs reached a 20%. |
| 18       | 9                   | 20.10.2014     | Indigo Shire, Vic            | Dorper| Adult| Female| Lameness in more than one foot. Lesions suggestive of benign footrot. |
| 19       | 10                  | 03.11.2014     | Shire of Glenelg, Vic        | NR    | 3.5 years| Female| Footrot appeared in June; source not determined, appears to be clinically aggressive; high prevalence of score 4/5 (20%). These sheep last footbathed ~ 3 weeks before sampling. |
| 20       | 10                  | 04.11.2014     | Southern Grampians Shire, Vic| NR    | 2 years | Female| Footrot has probably been present for a long time. Controlled by regular footbathing. Last month have had 30% of average long term rainfall for this time of the year. These sheep last footbathed ~ February 2014 |
| 21       | 10                  | 17.11.2014     | Bass Coast Shire, Vic        | Droper| Mixed| Mixed| Many lambs, ewes and some rams reported lame with lesions very suggestive of footrot. Treated with footbath (formalin) and antibiotics. The lesions look in a process of healing but are still obvious (inflammation limited to interdigital space). |
| 22       | 10                  | 18.11.2014     | Mitchell Shire, Vic          | Merino| NR  | NR  | Virulent footrot for several years. |
| 23       | 10                  | 24.11.2014     | Southern Grampians Shire, Vic| NR    | Adult| Female| Footrot appeared in June (source not determined) with high prevalence of score 4/5 (20%). Last 3 months have had 30% of average long term rainfall for this time of the year. These sheep last footbathed ~ 3 weeks earlier. |
| 24       | 9                   | 01.12.2014     | Colac Ottway Shire, Vic      | Crossbred| Adult| Female| 10 of 15 sheep examined had feet lesions scored ≥ 2 |
(Ct ≤ 31) in the aprV2 rtPCR in both runs. Two other sheep (Flock 9) tested positive for the benign strain of *D. nodosus* by culture/gelatinase test and also by the aprB2 rtPCR in both runs (Ct ≤ 33.74). One of these two sheep gave also a weak reaction (Ct 38.39) in the aprV2 rtPCR in one run. All three sheep that yielded growth of *D. nodosus* and tested positive by the aprV2 rtPCR and/or aprB2 rtPCR were clinically healthy (Table 3).

From the 170 animals clinically affected or suspected, 135 were scored 1 or above and considered clinically affected, while 35 were scored 0. The rtPCR produced positive virulent results in 112 of the 135 clinically affected sheep, giving a detection rate of 83% when using a Ct of 35, or 81.1% overall when using all 170 animals (Table 3).

In comparison, 45 animals from the 170 in the affected group had *D. nodosus* isolates successfully obtained and 43 had the gelatinase test performed (Table 6). There was a significant difference between the two methods capabilities to detect virulent *D. nodosus* (p < 0.0001). Comparing the gelatin gel designation of virulence to

| Flock ID | No. sheep tested | Results | aprB2 rtPCR | aprV2 rtPCR |
|----------|------------------|---------|-------------|-------------|
| 40       | 35               | -       | -           | -           |
| 35       | 35               | -       | -           | -           |
| 40       | 35               | -       | -           | -           |
| 35       | 35               | -       | -           | -           |

Table 3 Clinical scores and results of *D. nodosus* culture, gelatinase test and rtPCR (aprB2/aprV2 positive, two cut offs, duplicate runs) in 318 sheep from flocks considered to be free of footrot (clinically healthy), 170 sheep from flocks considered having virulent footrot (clinically affected) and 27 sheep from a closed flock (#11) that apparently eradicated virulent footrot more than 10 years ago. Flocks were sampled from October 2014 to August 2015.

| Flock ID | No. sheep tested | Clinical Score | Culture | Gelatin test | aprB2 rtPCR | aprV2 rtPCR |
|----------|------------------|---------------|---------|-------------|-------------|-------------|
| 1        | 18               | 0             | -       | -           | -           | -           |
| 2        | 18               | 0             | -       | -           | -           | -           |
| 3        | 19               | 0             | -       | -           | -           | -           |
| 4        | 19               | 0.6           | -       | -           | -           | -           |
| 5        | 18               | 0             | -       | -           | -           | -           |
| 6        | 18               | 2.3           | -       | -           | -           | -           |
| 7        | 18               | 0             | -       | -           | -           | -           |
| 8        | 55               | 0             | -       | -           | -           | -           |
| 9        | 81               | 0             | -       | -           | -           | -           |
| 10       | 54               | 0.5           | -       | -           | -           | -           |

Subtotal 318

| Flock ID | No. sheep tested | Clinical Score | Culture | Gelatin test | aprB2 rtPCR | aprV2 rtPCR |
|----------|------------------|---------------|---------|-------------|-------------|-------------|
| 1        | 18               | 0             | -       | -           | -           | -           |
| 2        | 18               | 0             | -       | -           | -           | -           |
| 3        | 19               | 0.1           | -       | -           | -           | -           |
| 4        | 18               | 0.6           | -       | -           | -           | -           |
| 5        | 19               | 0             | -       | -           | -           | -           |
| 6        | 18               | 2.3           | -       | -           | -           | -           |
| 7        | 18               | 0             | -       | -           | -           | -           |
| 8        | 55               | 0             | -       | -           | -           | -           |
| 9        | 81               | 0             | -       | -           | -           | -           |
| 10       | 54               | 0.5           | -       | -           | -           | -           |

Subtotal 318

Flock 11 has been excluded from specificity and sensitivity calculations because of its history

Results expressed as a number of sheep in which the highest rated foot was in the particular score

Results expressed as a number of sheep from which an organism morphologically consistent with *D. nodosus* was isolated

Results expressed as a number of sheep that tested positive
Table 4 Results of duplicate runs at cut offs Ct 40 and 35 for aprV2 and aprB2 rtPCR in 297 clinically healthy sheep and 21 sheep with foot lesions from 10 flocks considered free of footrot

| Foot lesion | Cut off 40 |       |       | Cut off 35 |       |       |
|-------------|------------|-------|-------|------------|-------|-------|
|             | Run 1 +ve  | Run 1 -ve | Run 2 + ve | Run 2 -ve | Run 1 +ve  | Run 1 -ve | Run 2 + ve | Run 2 -ve |
| aprB2       |            |        |        |            |        |        |
| Positive    | 0          | 21     | 2      | 19         | 0      | 21     | 0      | 21      |
| Negative    | 18         | 279    | 10     | 287        | 4      | 293    | 4      | 293     |
| Specificity | 93.90%     | 96.60% | 96.60% | 98.70%     | 98.70% |        |        |
| McNemars two tailed p value | 0.7488 | 0.1374 | 0.0014 | 0.0014 |        |        |
| aprB2 cut off agreement | Run 2 + ve | 5      | 7      | 4          | 0      |        |        |
| Run 2 -ve   | 13         | 293    | 0      | 314        |        |        |
| Kappa       | 0.302      | *fair* |        | 1          | *perfect* |        |        |
| aprV2       |            |        |        |            |        |        |
| Positive    | 4          | 17     | 4      | 17         | 4      | 17     | 4      | 17      |
| Negative    | 10         | 287    | 15     | 282        | 5      | 292    | 5      | 292     |
| Specificity | 96.60%     | 94.90% | 98.30% | 98.30%     |        |        |
| McNemars two tailed p value | 0.2482 | 0.8597 | 0.019  | 0.019     |        |        |
| aprV2 cut off agreement | Run 2 + ve | 12     | 7      | 9          | 0      |        |        |
| Run 2 -ve   | 2          | 297    | 0      | 309        |        |        |
| Kappa       | 0.713      | *good* |        | 1          | *perfect* |        |        |

Specificity is shown along with p-value for McNemar’s test for independence between lesion score and rtPCR result, and kappa statistic for agreement between rtPCR runs

Table 5 Results from two cut offs of Ct 40 and 35 from the aprB2 rtPCR and aprV2 rtPCR obtained from two runs, in 72 sheep randomly sampled from 3 flocks considered having virulent footrot

| Foot lesion | Cut off 40 |       |       | Cut off 35 |       |       |
|-------------|------------|-------|-------|------------|-------|-------|
|             | Run 1 +ve  | Run 1 -ve | Run 2 + ve | Run 2 -ve | Run 1 +ve  | Run 1 -ve | Run 2 + ve | Run 2 -ve |
| aprB2       |            |        |        |            |        |        |
| Positive    | 9          | 28     | 8      | 29         | 7      | 30     | 7      | 30      |
| Negative    | 2          | 33     | 1      | 34         | 1      | 34     | 1      | 34      |
| Overall % positive | 15.30% | 12.50% | 11.10% | 11.10% |        |        |
| McNemars two tailed p value | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |        |        |
| aprB2 cut off agreement | Run 2 + ve | 8      | 1      | 8          | 0      |        |        |
| Run 2 -ve   | 3          | 60     | 0      | 64         |        |        |
| Kappa       | 0.768      | *good* |        | 1          | *perfect* |        |        |
| aprV2       |            |        |        |            |        |        |
| Positive    | 33         | 4      | 33     | 4          | 30     | 7      | 29     | 8       |
| Negative    | 19         | 16     | 23     | 12         | 15     | 20     | 16     | 19      |
| Overall % positive | 72.20% | 77.80% | 62.50% | 62.50% |        |        |
| McNemars two tailed p value | 0.0035 | 0.0005 | 0.1356 | 0.153    |        |        |
| aprV2 cut off agreement | Run 2 + ve | 51     | 5      | 44         | 1      |        |        |
| Run 2 -ve   | 1          | 15     | 1      | 26         |        |        |
| Kappa       | 0.779      | *good* |        | 0.941      | *very good* |        |        |

The p-value for McNemar’s test for independence between lesion score and rtPCR result is shown along with kappa statistic for agreement between rtPCR runs
the rtPCR results, there is no significant difference between the two tests when using 37 of the isolates (McNemars Test, $p = 0.479$). Samples that tested positive for $aprV2$ and $aprB2$ via rtPCR and had $D. nodosus$ successfully isolated were excluded from the above calculation as no sample had more than one isolate obtained.

None of the 135 clinically affected sheep and 35 clinically healthy sheep tested negative by the $aprV2$ rtPCR but positive for the virulent strain of $D. nodosus$ by the culture/gelatinase test. The agreement between results produced by the $aprV2$ rtPCR and that obtained by the culture/gelatinase test ranged from fair (Kappa = 0.2–0.222) to poor (kappa = 0.082–0.158) (Table 7). In total, when the affected flock samples were cultured, an overall detection rate of 25% was obtained (Table 7).

There was a significant difference ($p \leq 0.0015$) between numbers of animals tested positive for the virulent strain of $D. nodosus$ by the $aprV2$ rtPCR and culture/gelatinase test among the 35 clinically healthy sheep. In this group, the $aprV2$ rtPCR gave positive results in 15 (42.9%) and 16 (45.7%) of clinically healthy animals at a Ct of 35 cut-off value in run 1 and run 2, respectively, whereas the gelatinase test gave positive results in 3 of the healthy animals (Table 7).

The same method was applied to $aprB2$ rtPCR positive samples, resulting in a detection rate of 18.9%, with 23 of 135 clinically affected animals (17%) positive for $aprB2$ via rtPCR. Using culturing and the gelatin gel test, 9 (6.7%) of the 135 clinically affected animals tested positive for benign $D. nodosus$. Again, there was a significant difference between the two methods capabilities to detect benign $D. nodosus$ ($p = 0.0022$). Two of the 135 clinically affected sheep tested positive for benign $D. nodosus$ by the culture/gelatinase test but negative by the $aprB2$ rtPCR. The agreement between results produced by the $aprB2$ rtPCR and that obtained by the culture/gelatinase test ranged from fair (Kappa = 0.336–0.378) to poor (kappa = 0–0.163) (Table 8).

Among the 35 clinically healthy sheep, there was no significant difference ($p = 1$) between numbers of animal’s positive for the benign strain of $D. nodosus$ by the $aprB2$ rtPCR and culture/gelatinase test. In this group, the $aprB2$ rtPCR at a Ct of 35 gave a positive reaction in

| Flock ID | Score | Gelatinase gel test | rtPCR |
|---------|-------|---------------------|-------|
| 12      | 1     | S                   | aprV2 |
| 1       | S     | aprV2               |
| 0       | S     | aprV2               |
| 0       | NA    | aprV2               |
| 0       | S     | aprV2               |
| 0       | NA    | aprV2               |
| 13      | 1     | U                   | aprV2 and aprB2 |
| 1       | S     | aprV2 and aprB2     |
| 3B      | U     | aprV2               |
| 3C      | S     | aprV2               |
| 14      | 5     | S                   | aprV2 |
| 1       | S     | aprV2 and aprB2     |
| 15      | 5     | S                   | aprV2 |
| 16      | 3     | S                   | aprV2 |
| 2       | S     | aprV2               |
| 2       | S     | aprV2               |
| 3       | S     | aprV2               |
| 4       | S     | aprV2               |
| 17      | 4     | S                   | aprV2 |
| 2       | S     | aprV2               |
| 2       | U     | aprB2               |
| 4       | U     | aprB2               |
| 4       | U     | aprB2               |
| 3       | U     | aprB2               |
| 3       | S     | aprV2               |
| 2       | S     | aprV2               |
| 4       | S     | aprV2               |
| 2       | S     | aprV2               |
| 18      | 1     | S                   | aprV2 |
| 1       | S     | aprV2               |
| 1       | S     | aprV2               |
| 19      | 5     | S                   | aprV2 |
| 5       | S     | aprV2               |
| 5       | S     | aprV2               |
| 20      | 3A    | S                   | aprV2 and aprB2 |
| 2       | U     | aprV2 and aprB2     |
| 3B      | S     | aprV2 and aprB2     |
| 22      | 3A    | S                   | aprV2 |
| 3A      | U     | aprV2               |
| 4       | S     | aprV2               |

Foot scores are according to Stewart et al., 1983 [4]. An additional file describes the foot scoring in more detail (see Additional file 1).
Table 7 Comparisons of identification of *D. nodosus* by the *aprV2* rtPCR and culture/gelatinase gel test from subsets of presence/absence or both of foot lesions from the clinically affected flocks, consisting of 135 clinically affected sheep and 35 clinically healthy sheep

| Flocks   | Sheep clinical status (foot lesions) | No. sheep tested | *aprV2* PCR Run/Cut-off | Culture Gelatinase Thermostable (Virulent) vs *aprV2* PCR | Concordant results | Culture +ve PCR -ve | PCR + ve Culture -ve | p value | Kappa | Percentage positive results |
|----------|-------------------------------------|------------------|--------------------------|----------------------------------------------------------|-------------------|---------------------|---------------------|---------|------|---------------------------|
| 12 to 24 | +ve                                 | 135              | Run 1 / 40               |                                                           |                   |                     |                     | < 0.0001 | 0.082 | 23.0% | 87.4%                   |
| 12 to 24 | +ve                                 | 135              | Run 1 / 35               |                                                           |                   |                     |                     | < 0.0001 | 0.1   | 23.0% | 85.2%                   |
| 12 to 14 | -ve                                 | 35               | Run 1 / 40               |                                                           |                   |                     |                     | 0.0002   | 0.146 | 86%  | 54.3%                   |
| 12 to 14 | -ve                                 | 35               | Run 2 / 40               |                                                           |                   |                     |                     | < 0.0001 | 0.093 | 86%  | 65.7%                   |
| 12 to 14 | -ve                                 | 35               | Run 1 / 35               |                                                           |                   |                     |                     | 0.0015   | 0.222 | 86%  | 42.9%                   |
| 12 to 14 | -ve                                 | 35               | Run 2 / 35               |                                                           |                   |                     |                     | 0.0009   | 0.2   | 86%  | 45.7%                   |
| 12 to 14 | -ve & + ve                          | 72               | Run 1 / 40               |                                                           |                   |                     |                     | < 0.0001 | 0.104 | 12.5% | 72.20%                  |
| 12 to 14 | -ve & + ve                          | 72               | Run 2 / 40               |                                                           |                   |                     |                     | < 0.0001 | 0.078 | 12.5% | 77.80%                  |
| 12 to 14 | -ve & + ve                          | 72               | Run 1 / 35               |                                                           |                   |                     |                     | < 0.0001 | 0.158 | 12.5% | 62.50%                  |
| 12 to 24 | -ve & + ve                          | 170              | Run 1 / 40               |                                                           |                   |                     |                     | < 0.0001 | 0.114 | 200% | 80.6%                   |
| 12 to 24 | -ve & + ve                          | 170              | Run 1 / 35               |                                                           |                   |                     |                     | < 0.0001 | 0.143 | 200% | 76.5%                   |

The *p*-value for McNemar's test for independence between culture gelatinase thermostable (Virulent) and *aprV2* PCR result is shown. Detection rates of both culture and rtPCR are presented as percentage positive results.
Table 8: Comparisons of identification of *D. nodosus* by the *aprB2* rtPCR and culture/gelatinase gel test from subsets of presence/absence or both of foot lesions from the clinically affected flocks, consisting of 135 clinically affected sheep and 35 clinically healthy sheep.

| Flocks | Sheep clinical status (foot lesions) | No. sheep tested | *aprB2* PCR Run and/or Cut-off | Culture Gelatinase Thermolabile (Benign) vs *aprB2* PCR | Percentage positive results |
|--------|-------------------------------------|-----------------|--------------------------------|------------------------------------------------------|-----------------------------|
|        |                                     |                 | +ve    | -ve    | +ve PCR | -ve PCR | Culture +ve | PCR -ve | Culture -ve | p value | Kappa | Culture | *aprB2* PCR |
| 12 to 24 | +ve                                  | 135             | Run 1 / 40 | 7 | 108 | 2 | 18 | 0.0008 | 0.348 | 6.7% | 18.5% |
| 12 to 24 | +ve                                  | 135             | Run 1 / 35 | 7 | 110 | 2 | 16 | 0.0022 | 0.378 | 6.7% | 17.0% |
| 12 to 14 | -ve                                  | 35              | Run 1 / 40 | 0 | 33  | 0 | 2  | 0.4795 | 0     | 0.0% | 5.7%  |
| 12 to 14 | -ve                                  | 35              | Run 2 / 40 | 0 | 34  | 0 | 1  | 0     | 0     | 0.0% | 2.9%  |
| 12 to 14 | -ve                                  | 35              | Run 1 / 35 | 0 | 34  | 0 | 1  | 1     | 0     | 0.0% | 2.9%  |
| 12 to 14 | -ve                                  | 35              | Run 2 / 35 | 0 | 34  | 0 | 1  | 0     | 0     | 0.0% | 2.9%  |
| 12 to 14 | -ve & + ve                           | 72              | Run 1 / 40 | 1 | 60  | 1 | 10 | 0.0159 | 0.112 | 2.8% | 15.3% |
| 12 to 14 | -ve & + ve                           | 72              | Run 2 / 40 | 1 | 62  | 1 | 8  | 0.0455 | 0.143 | 2.8% | 12.5% |
| 12 to 14 | -ve & + ve                           | 72              | Run 1 / 35 | 1 | 63  | 1 | 7  | 0.0771 | 0.163 | 2.8% | 11.1% |
| 12 to 14 | -ve & + ve                           | 72              | Run 2 / 35 | 1 | 63  | 1 | 7  | 0.0771 | 0.163 | 2.8% | 11.1% |
| 12 to 24 | -ve & + ve                           | 170             | Run 1 / 40 | 7 | 141  | 2 | 20 | 0.0003 | 0.336 | 5.3% | 15.9% |
| 12 to 24 | -ve & + ve                           | 170             | Run 1 / 35 | 7 | 141  | 2 | 17 | 0.0013 | 0.376 | 5.3% | 14.1% |

The *p*-value for McNemar’s test for independence between culture gelatinase thermostable (benign) and *aprB2* PCR result is shown. Detection rates of both culture and rtPCR are presented as percentage positive results.
Discussion

New molecular techniques capable of rapidly detecting and typing *D. nodosus* are required for improved diagnostics in Australia. A rtPCR method, capable of detecting and discriminating virulent *D. nodosus* strains, has been developed under European conditions. This rtPCR was assessed on Australian samples for detection rates, along with clinically relevant cut-off values. Ct values of 40 and 35 for positive identification cut-off were investigated with regards to repeatability between runs, with signals above Ct 35 showing more discrepancies than those below 35. This is common in rtPCR assays, often with results past the Ct of 35 commonly seen as outliers [13]. In addition to higher specificities and more significant results at a Ct of 35 for positive samples (*aprV2, p* = 0.002, McNemar results at a Ct of 35 for positive samples (*aprV2* [13]. In addition to higher specificities and more significant results at a Ct of 35 for positive samples (*aprV2, p* = 0.002, McNemar’s two-tailed *p*-value), no animal scored 1 or above returned a positive result above a Ct of 35, supporting the use of a cut-off set at 35 in diagnosing animals with some form of the disease. Interestingly, 6 animals that are clinically negative were *aprV2* positive after lowering the cut-off. This could suggest that these animals be monitored for clinical signs of footrot when favourable environmental conditions occur. They may potentially be asymptomatic carriers depending on circumstance, with the ability to re-infect the flock [14, 15].

The diagnostic power of rtPCR was assessed using the current clinical scoring system to judge if an animal was diseased, or free from disease, as this is the currently accepted method of diagnosis in Victoria. The detection of *D. nodosus* on 3 animals within the healthy population, by both rtPCR and culturing, suggests the presence of infection but not the disease [15]. As the population used for the detection of *D. nodosus* on clinically affected animals was deliberately chosen for clinical virulence, a low detection rate for benign *D. nodosus* was anticipated. From the animals that had the *aprB2* gene detected in the rtPCR, 81% were in association with a co-infection, where *aprV2* was also detected. As the virulent form of footrot is the clinical disease of interest, no additional investigation into a purely benign population was conducted. The reported overall detection rate for *aprV2* at a Ct of 35 is conservative, with an increase being seen when using a Ct of 40 (89.7%), and also when only using animals scored 3–5, which are traditionally considered virulent [9]. However, as the population used had confirmed or suspected of virulent footrot, the full range of scores were used to indicate the presence of disease. As *D. nodosus* is found in the sheep hoof environment and its presence does not always result in disease [16], it was expected that a number of the clinically negative sheep, throughout the whole data set, would return a positive result due to the nature of the sheep hoof and its environment. Further research monitoring the development of disease in association with environment on these animals may provide insights into the usefulness of *D. nodosus* detection prior to lesion formation, and therefore have practical management applications.

Time taken to receive a result using the rtPCR was typically within 1 day of sample collection or receipt. This is in comparison to the average of 2 to 4 weeks taken for a result when using the culturing method, while the rtPCR also provided better detection of *D. nodosus* from the samples collected. The advantage this provides would allow for a timelier confirmation of the presence of *D. nodosus*, confirming the clinical symptom is in association with the presence of bacteria. The gelatinase test relies on the phenotypic expression of proteases and the associated thermostability of those produced, and the culturing of *D. nodosus* is difficult and requires specialist skills and media. There are also inherent disadvantages to using this method, including the chance of missing strains of *D. nodosus* in the sample, and in the instance of this study, no facility was available in Victoria for the virotyping of isolates, so transport was required. This increases the likelihood of damage to the bacteria in transport and may affect the expression of proteases, the amount made or the ability to reliably have thermostability measured [17]. This may have been the cause of the disagreements from one sample in the clinically healthy data, and two from the clinically affected data. Despite this, there was no significant difference between the two tests when it came to identifying the virulence of the isolates. Instances of results where the rtPCR has detected both virulent and benign protease from a sample, yet culturing missed one or the other strain, are shown in 6 cases from the clinically affected data.

There are many challenges with footrot and the assessment of new diagnostic testing methods due to complex interplay between *D. nodosus*, environment and the host which may result in clinical signs of disease [18]. The method of assessment here reflects the way implementation and sample collection would occur in the field, and so the analysis is appropriate for Victoria’s methods of disease investigation. The most prominent difficulty is that the development of lesions is required for a visual diagnosis, to which rtPCR detection rate has been evaluated against. Factors like sheep breed, management, weather, and timing of inspection may all contribute to the lack of lesion, yet *D. nodosus* may still be present and found by the rtPCR. This would contribute to reduced specificity due to being rtPCR positive for *D. nodosus*, yet lesion negative, or lesions that are healing and not indicative of the infecting strain [19, 20].

1 (2.9%) animal. None of the 35 clinically healthy sheep tested positive for the benign strain of *D. nodosus* by the culture/gelatinase test.
Conclusions
The improved speed and detection of virulent D. nodosus by this rtPCR assay could lead to a change of animal husbandry practices if the focus were to shift from clinical disease to the detection of virulent D. nodosus, indicating infection [15]. The ability to pool samples for this type of rtPCR has also been demonstrated [21, 22], an advantage in time and cost over culturing. Testing of this nature is also capable of detecting and quantifying the bacteria associated with the clinical disease, providing the basis to measure the success of various management practices for both treatment and prevention of footrot [23].

Additional file

Additional file 1: Definition of footrot scoring system. (DOCX 12 kb)

Abbreviations
EDTA: Ethylenediaminetetraacetic acid; rtPCR: Competitive real-time PCR

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Authors’ contributions
NB, LZ was responsible for the experimental work, data analysis, and writing of the manuscript. JG contributed to the writing of the manuscript and reviewed the drafts. EK and NB provided knowledge, training and culturing of D. nodosus. GR and TB coordinated the project, contributed to the experimental design, and reviewed the drafts. All authors read and approved the final version of the manuscript.

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