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## **Comparative study of the commonly used virulence tests for laboratory diagnosis of ovine footrot caused by *Dichelobacter nodosus* in Australia**

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**Abstract**

Footrot in sheep and goats is expressed as a spectrum of clinical entities ranging from benign, which is a self limiting interdigital dermatitis to highly virulent, in which severe under running of the horn of the hoof occurs. Interactions between the host, the virulence of the causative strain of *Dichelobacter nodosus* and environmental conditions determine the severity of the disease. Clinical diagnosis of virulent footrot, which a notifiable disease in some states of Australia, is not always straightforward. Therefore, the Gelatin Gel and Elastase tests for protease activity, and the *intA* PCR test for an inserted genetic element in *D. nodosus* are commonly used to support or to confirm a clinical diagnosis. A comparative study of these laboratory tests with a large number of samples collected from 12 flocks of sheep with clinically virulent footrot was conducted. Based on the elastase test, 64% of the isolates tested were classified as virulent compared to 91% on the gelatin gel test and 41% according to the *intA* test. The agreement between the elastase and the gelatin gel test was low ( $\kappa = 0.12$ ) as were the agreements between other tests. Only about 21% of the isolates were virulent in all 3 tests. Therefore these tests on their own may not provide standard and reliable results and are likely to remain as supplementary tests for clinical diagnosis of the disease.

**Keywords:**

Footrot, virulence, elastase test, gelatin gel test, intA PCR test.

**Introduction**

Virulent footrot is an economically significant disease in most sheep rearing countries. Its clinical manifestation in sheep and goats is determined by the virulence of the strain of *Dichelobacter nodosus* involved (Egerton and Parsonson 1969; Stewart, Clark et al. 1984), environmental conditions (Graham and Egerton 1968) and the innate and acquired resistance of the host (Bulgin, Lincoln et al. 1988; Skerman, Johnson et al. 1988; Raadsma, Attard et al. 1995). Implicit in the environment is the microbial community of the hoof, but the dependencies between *D. nodosus* and other microflora are not understood. Interactions between all of these factors determine the severity of the disease which can be expressed as a spectrum of clinical entities ranging from benign, which is a self limiting interdigital dermatitis, to highly virulent in which severe underrunning of the horn of the hoof occurs (Egerton 1986; Stewart, Peterson et al. 1986). Three clinical categories of footrot have been described for convenience: virulent, intermediate and benign (Egerton 1986; Stewart 1989). Despite the complex host-pathogen-environment interaction, the terms virulent and benign are also used to describe strains of *D. nodosus*, implying their potential to cause the respective clinical forms of footrot.

Sometimes the clinical diagnosis of virulent footrot is not easy, particularly in the early stages of an outbreak, or during hot dry conditions when footrot cannot express fully. Therefore laboratory tests have been developed to confirm the clinical diagnosis. The elastase test measures the temporal and quantitative activity of proteases (Egerton and Parsonson 1969) (Stewart 1979) while the gelatin gel test measures a difference in protease thermostability between benign and virulent strains of *D. nodosus* (Liu and Yong 1993; Palmer 1993). However, some false positive results have been reported and have led to unnecessary quarantine procedures. More recently, the *intA* PCR test (Cheetham 2006) has been used in New South Wales as a supplementary test to the gelatin gel test in situations where gelatin gel test results are at odds with a field diagnosis of benign footrot. Surprisingly a large scale comparison of these virulence tests has never been conducted.

The aim of this study was to compare the elastase, gelatin gel test and *intA* PCR tests on a large number of *D. nodosus* isolates from 12 flocks of sheep with virulent footrot.

**Materials and methods*****Dichelobacter nodosus* culture samples**

Foot lesion swabs were collected from sheep in 12 flocks enrolled in a footrot vaccination trial in three states of southern Australia (South Australia, Tasmania and Victoria) and were cultured by the standard method of isolation of *D. nodosus* (Stewart and Claxton 1993). Each flock was visited 2 to 6 times prior to vaccination by a veterinarian who was instructed to randomly select sheep from each mob in the flock and sample sheep with foot lesions, identified by obvious

lameness and/or clinical signs of footrot by examination of each foot. Up to 40 sheep with lesions from each flock were sampled on each visit. If fewer than 40 sheep were found with foot lesions at the time of the visit all sheep with lesions were sampled. In most cases the foot with the most severe clinical lesion was sampled by inserting a swab into the most active part of the lesion, usually at the active margin of the underrun lesion or interdigital skin lesion if no underrunning was present. Between 1 and 12 colonies of *D. nodosus* of varying morphology were selected and subcultured from each primary culture and each of these was treated as a separate isolate. Each isolate was tested for serogroup and virulence by the in-vitro virulence tests: elastase, gelatin gel and *intA* PCR test.

#### **Elastase test**

A loopful of a pure culture of *D. nodosus* test sample was streaked onto a quarter of an elastin agar plate (Stewart 1979) as a linear streak. On each plate, isolates with known elastase activity, positive at 4-8 days and negative at 28 days, were streaked on to other quarters as controls. The plates were incubated anaerobically using gas generating kits (Gas Pak, BBL, Becton Dickinson and Company, Cockeysville, USA). Plates were examined after 4, 8, 12, 16, 20, 24 and 28 days incubation at 37°C. After each examination, the plates were re-incubated anaerobically. An isolate was regarded as virulent if growth occurred and the elastin particles around the inoculum were digested and a clear zone was visible by day 12, and if clearing did not occur by this time the isolate was regarded as benign. The test was repeated if the results of the control samples did not match the expected clearing patterns.

#### **Gelatin gel test**

The principle of the gelatin gel test developed by Palmer (1993) is that extracellular proteases produced by virulent strains of *D. nodosus* are more heat stable than those produced by benign strains. Briefly, a pure culture of the sample of *D. nodosus* was grown in TAS broth for 2 to 4 days to achieve a concentration of  $10^8$  cells per ml, measured by spectrophotometry. These broth cultures (1 ml) were diluted with 1ml of Hepes test buffer in glass test tubes, mixed well and an aliquot of 20 µl from each of the samples was placed into the top well of the gelatin gel. The test dilutions were placed in a water bath at 68°C. After 8 minutes of incubation at 68°C another 20 µl of the samples were placed into the middle well. The samples were further incubated for 8 minutes at 68°C and 20 µl of each were placed into the lower well. Known positive and negative samples were included as controls. The gels were incubated overnight (18 hrs) in a moist chamber at 37°C, after which undigested gelatin was precipitated by flooding with hot (60°C to 70°C) saturated ammonium sulphate solution. The zone of proteolysis indicated by clearing around the wells of the sample inoculums was measured using calipers. Isolates with a ratio of more than 50% of the zone of clearance of the preheated compared to 16 minutes heated sample were defined as stable (virulent) and those with less than 50% clearing were taken as unstable (benign).

### **DNA extraction from *D. nodosus* cultures on plates for *intA***

*D. nodosus* cells were collected from pure cultures on 2% hoof agar plates using sterile cotton swabs and placed into 300 µl of sterile PBS in 1.5 ml screw capped microfuge tubes. The cells were extracted by vortexing the tubes briefly and swabs were discarded. The samples were boiled by placing the tubes in heating blocks at 100°C for 10 mins, cooled and centrifuged at 3,000 xg for 1 min. The supernatant was transferred into a clean microfuge tube and DNA was pelleted by centrifugation at 12,000 x g for 10 mins. DNA pellets were dissolved into 100 µl of sterile water. 2 ul of these DNA suspensions were used as templates for PCR.

### ***IntA* PCR analysis**

PCR was used to test for the presence of *intA* genes by amplification of part of the gene using primers IntA1- ACATCATGCGACTCACTGAC and IntA2- TCTCTGGTCGGTCGTACAAT (Cheetham 2006). The 20 µl amplifications were performed in 0.2 ml thin walled PCR tubes (MJ Research, Inc. Massachusetts, USA). The PCR mixture contained a final concentration of 20 mM Tris-HCl, 50mM KCl, 3mM MgCl<sub>2</sub> and 200 µm each of the dNTPs. The concentration of the primers was 0.5 µM each. One unit of Taq polymerase (GIBCO-BRL) was added to the reaction mix and approximately 50-100 ng of DNA was added as template. The amplification cycles consisted of 94°C for 4 mins followed by 94 °C for 30s, 60 °C for 30s and 72 °C for 30s for 5 cycles, 94 °C for 30s, 58 °C for 30s and 72 °C for 30s for 25 cycles, and final extension at 72 °C for 2 mins. The PCR products were electrophoresed in 1% agarose gels, stained with ethidium bromide and visualized under UV illumination. Isolates with positive 530 bp PCR products were defined as virulent and those with negative results were defined as benign.

### **Statistical analysis**

Frequencies and relative frequencies of virulent and benign isolates detected by various tests were calculated. McNemar's test was conducted to verify if there were significant differences between the proportions of virulent isolates detected by each test. The level of agreement between each pair of tests was assessed using Cohen's kappa statistic. As Cohen's kappa has been previously shown to give biased results in many situations (Rao 2008) prevalence adjusted and bias-adjusted kappa (PABAK) was also calculated using a SAS macro (Cunningham 2009). An overall estimate of kappa was also obtained to determine agreement between all diagnostic tests. Finally, intra-class correlation coefficient (ICC) was calculated using the latent variable method employing the generalized linear mixed model approach.

### **Results**

In this study a total of 608 animals were sampled from 12 flocks, with a median of 51 animals per flock (range 36-64). Similar numbers of *D. nodosus* isolates per sheep were obtained from swabs collected from the active margin of underrun lesions or the interdigital skin lesions.

Different proportions of isolates were classified as virulent in each test (Table 1<sup>1</sup>). The majority of isolates (91%) were virulent according to the results of the gelatin gel test, an intermediate proportion in the elastase test (64%) and the minority in the *IntA* PCR test (41%). As the latter test had least agreement with the clinical diagnosis on each farm after testing 1601 isolates and was the most expensive it was not used further.

#### **Agreement between Elastase and Gelatin gel tests**

The proportion of isolates testing virulent were significantly different between Elastase and Gelatin gel tests (McNemar test  $p < 0.001$ ). Agreement between the two tests beyond that expected by chance alone was slight (Kappa = 0.12; 95% confidence interval of kappa: 0.094, 0.15). Prevalence and bias adjusted kappa (PABAK) was calculated to be 0.33. Using both tests in parallel (i.e. virulent on either test is considered virulent), 94.4% of the isolates were virulent but using both tests in series (i.e. virulent on both tests is considered virulent) only 60.8% of the isolates tested were virulent (Table 2).

#### **Agreement between Elastase and IntA PCR tests**

The proportions of isolates testing virulent were significantly different between the Elastase and the *IntA* PCR tests (McNemar  $p < 0.001$ ). Agreement between the two tests beyond that expected by chance alone was slight (Kappa = 0.15; 95% confidence interval of kappa: 0.11, 0.19; PABAK: 0.11). Using both tests in parallel (i.e. virulent on either test is considered virulent), 77.9% of the isolates were virulent but using both tests in series (i.e. virulent on both tests is considered virulent) only 33.6% isolates tested were virulent (Table 3).

#### **Agreement between Gelatin gel and IntA PCR tests**

The proportions of isolates testing virulent were significantly different between the Gelatin gel and the *intA* PCR tests (McNemar  $p < 0.001$ ). Agreement between the two tests beyond that expected by chance alone was negligible (Kappa = 0.05; 95% confidence interval of kappa: 0.03, 0.07). Using both tests in parallel (i.e. virulent on either test is considered virulent), 92.9% of the isolates were virulent but using both tests in series (i.e. virulent on both tests is considered virulent) only 40.8% isolates tested were virulent (Table 4).

Overall, about 32.4% of the isolates tested were virulent using all 3 tests, and 4.6% were negative (benign) on all 3 tests. Overall agreement between the three tests was calculated to be 0.048 (95% CI: 0.023, 0.072). The intra-class correlation coefficient (ICC) was estimated to be 0.043.

#### **Discussion**

The *in-vitro* virulence tests used in this study are based on different virulence factors. The elastase and gelatin gel tests are phenotypic tests based on protease activities while the *intA* test is based on an inserted genetic element that is unrelated to protease. Given prior

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<sup>1</sup> All tables are located at the end of this document.

knowledge of the utility of these tests in supporting clinical diagnosis, the level of agreement between the three tests was surprisingly low with only about 21% of the isolates tested being classified as virulent by all three tests. It was particularly noticeable that the agreement between the elastase and the gelatin gel tests was also low, given that both tests measure aspects of protease activity of the same gene product, AprV2 (Kennan, Wong et al. 2010). This subtilisin-like protease was proven to be required for virulence and uses a novel disulphide-tethered exosite to bind substrates (Kennan, Wong et al. 2010). Deletion of the AprV2 protease and its replacement with a benign equivalent AprB2 resulted in a strain that was classified as benign in both the elastase and gelatin gel tests (Kennan, Wong et al. 2010).

The 12 flocks in the trial from which these isolates came were selected based on the presence of clinically virulent footrot, therefore a higher proportion of isolates were expected to be virulent compared to random samples. The high proportion of individual elastase and gelatin gel test virulent results could be explained by this bias in flock selection. However, the low proportion with *intA* test positive samples could not be explained. This is a relatively new test which has not been validated in the field with such a large number of isolates. The original rationale for use of the *intA* test was in situations where a clinical diagnosis of benign footrot was at odds with gelatin gel test results (stable) suggesting virulent footrot. In such flocks, negative *intA* test results tended to match the clinical diagnosis of benign footrot (Cheetham, Tanjung et al. 2006), while 116/154 gelatin gel stable (virulent) isolates from 51 farms with clinically virulent footrot were positive for *intA*. In the present study less than 40% of *intA* results were in agreement with the gelatin gel test results of stable. Similar results have been reported from a study in Norway (Gilhuus et al., 2012, personal communication). Eventhough the *intA* test was suggested as a supplementary test to the gelatin gel test, poor agreement in these studies makes the use of this test doubtful even as a supplementary test.

Given the poor agreement between the gelatin gel and the elastase test results in particular, how is it that both tests have been used successfully for several decades in footrot diagnosis? The explanation probably lies in the fact that usually about 5 *D. nodosus* isolates are characterized during a flock investigation. If only one yields a virulent test outcome, and the clinical diagnosis is in concurrence, appropriate disease control is implemented. That is, flock level diagnosis is relatively accurate. The issue of how many isolates must be examined to achieve accurate diagnosis at flock level has been studied in the context of serogroup identification (Hill, Dhungyel et al. 2010). Another explanation for poor agreement is the possibility that the positive-negative cut-off points for one or both tests are inappropriate. The conventional cut-off point for the elastase test is 10 days, compared to 12 days used in this study. If 10 days had been used, presumably fewer isolates would have been classified as virulent in that test. Using a later time for elastase activity would lead to a greater number of isolates being classified as virulent, leading to better agreement with the gelatin gel test. Alternatively, increasing the stringency of the gelatin gel test would bring it into better agreement with the elastase test.

Other tests for virulence of *D. nodosus* have been used routinely or trialed in Australia. A zymogram test which measures the electrophoretic activity of the proteases (Every 1982; Kortt, Burns et al. 1983; Gordon, Yong et al. 1985) is no longer commonly used because of the technical difficulties. A monoclonal antibody-based ELISA to detect proteases was able to differentiate strains of *D. nodosus* causing different clinical forms of footrot (Stewart, Kortt et al. 1990) but this test has not been assessed extensively against other tests and has not been applied in disease control programs.

As all of the tests based on phenotypic expression of proteases are likely to be influenced by factors that affect the growth and thus the physiology of the organism, genetic tests for protease genes or other genes may have advantages. Thus virulence-associated gene probes have been developed (Katz, Howarth et al. 1991) (Liu and Yong 1993; Liu 1994). An assessment of these probes in dot-blot hybridization assays using a collection of 96 *D. nodosus* isolates showed general agreement with elastase and gelatin gel test results and correlation with clinical manifestations of footrot (Liu 1994). PCR assays were developed based on the nucleotide sequence of the gene probes developed for dot-blot hybridization tests (Liu and Webber 1995). However, there were discrepancies between the phenotypic and genotypic classification of the isolates by this method. Subsequently a gene probe test based on recombinant plasmids containing *vap* or *vrl* genomic regions was developed into a PCR test (Rood, Howarth et al. 1996). Concurrently a series of genetic elements (*intA*, *intB*, *intC* and *intD*) were identified on the chromosome of *D. nodosus* (Cheetham, Tattersall et al. 1995; Bloomfield, Whittle et al. 1997), which led to the *intA* test (Cheetham, Tanjung et al. 2006) that was evaluated in this study. In addition, the virulence of strains of *D. nodosus* was shown to be dependent on the fimbrial subunit gene (*fimA*) and the phenomenon of twitching motility which depends on fimbrial gene expression (Kennan, Dhungyel et al. 2001).

The results of this study highlight the need for a test for virulent footrot which offers more consistent results, or at least results that closely match the clinical potential of isolates of *D. nodosus* to cause disease. Genetic tests for all of the known virulence factors have not yet been developed, but they may overcome some of the limitations of the phenotypic tests, the repeatability of which is not fully understood. Furthermore, genetic tests may be more stable, are not likely to be influenced by the physiological state of the organism, and may be quicker and cheaper, particularly if tests can be devised that do not require prior microbial culture. However, it is likely that the virulence of *D. nodosus* is polygenic, and individual strains may differ in the complement of genes responsible for their potential to cause clinical disease.

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## TABLES

**Table 1:** Elastase, Gelatin gel and *IntA* PCR test results of the isolates tested

Test	Frequency (Percentage)		Total
	Virulent	Benign	
Elastase	1834 (63.9%)	1037 (36.1%)	2871
Gelatin Gel	2591 (90.9%)	260 (9.1%)	2851
<i>IntA</i> PCR	658 (41.1%)	943 (58.9%)	1601

**Table 2:** Agreement between Elastase and Gelatin Gel tests

Elastase test	Gelatin Gel test		Total
	Virulent	Benign	
Virulent	1734	100	1834
Benign	857	160	1017
Total	2591	260	2851

**Table 3:** Agreement between Elastase and *IntA* PCR tests

Elastase test	<i>IntA</i> PCR test		Total
	Virulent	Benign	
Virulent	738	746	1484
Benign	228	487	715
Total	966	1233	2199

**Table 4:** Agreement between Gelatin gel and *IntA* PCR tests

Gelatin gel test	<i>IntA</i> PCR test		Total
	Virulent	Benign	
Virulent	944	1128	2072
Benign	75	164	239
Total	1019	1292	2311