# **Ovine Footrot**

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# Part 1. Diagnostic Overview

#### **Summary**

Footrot is a contagious disease affecting the interdigital epidermis and living tissues of the digits of sheep and other cloven-hoofed animals including goats, deer and cattle. The transmitting agent is the anaerobic, Gram-negative bacterium, Dichelobacter nodosus. The disease is characterised by lameness, ulceration of the interdigital epidermis and, in advanced cases, separation of the fully keratinized hoof from the foot and degeneration of the horn. The disease has welfare and economic issues because severe footrot results in lameness, inappetence, loss of body condition, reduced wool growth and a decline in wool quality. Optimal conditions for disease expression occur in late spring, early summer, and occasionally autumn, in the temperate areas of Australia and New Zealand. The disease occurs world-wide.

The clinical disease manifests as foot lesions that range in severity, depending on the virulence of the strain present, environmental conditions at the time and breed of sheep. When host and environment factors are not limiting, virulent lesions are related to strains of D. nodosus that produce thermostable proteases, whereas benign lesions are associated with strains that produce heat-labile proteases. Other bacterial virulence factors such as twitching motility due to type IV pili, genetic elements involved in regulation, and outer membrane proteins are likely to be involved in the pathogenesis of footrot, but the mechanisms involved are not yet fully determined.

Footrot is diagnosed by evaluation of lesion characteristics and culture of the bacterium. Different definitions of virulent footrot exist in different Australian states. These are based on the use of laboratory tests and/or a flock-based clinical examination. Isolates of D. nodosus can be characterised by the gelatin gel thermostability test, the elastase test, or measured by an in-vivo pen test. Of these, the gelatin-gel test has proven to be the most sensitive for determining virulence potential of the bacterium.

# Aetiology

*Dichelobacter nodosus* is the primary aetiological agent of ovine footrot. The disease is characterised by a lesion in the foot ranging in severity from mild to severe tissue damage.<sup>1,2</sup>

*D. nodosus* is a large Gram-negative anaerobic rod, 0.6-0.8  $\mu$ m wide by 3-10  $\mu$ m in length and has rounded ends that may have terminal swellings.<sup>1</sup> *D. nodosus* belongs to the family *Cardiobacteriaceae*, which also contains *Cardiobacterium* and *Suttonella*, both of which are aerobic. Sequencing of the 16S rRNA gene demonstrates 93% homology between the three members of the family.<sup>2</sup>

*D. nodosus* cannot cause infection unless there is prior damage to the interdigital skin or skinhorn junction in the hoof.<sup>3</sup> *D. nodosus* is associated with an ulcerated stratum corneum and invades to the stratum lucidum through the avascular skin layers.<sup>3</sup> It is rarely the dominant organism in the lesion, which contains other bacteria such as *Fusobacterium necrophorum*, *Bacteroides* species, *Trueperella (Arcanobacterium) pyogenes, Staphylococcus* species and spirochaetes.<sup>1,3</sup> *F. necrophorum* is thought to have a role in pathogenesis particularly as an initiator of damage to the interdigital skin thereby allowing entry of *D. nodosus*.<sup>4</sup> Infection with *F. necrophorum* alone can result in interdigital dermatitis but does not lead to footrot.<sup>4-7</sup>

The clinical expression of *D. nodosus* infection is affected by a number of factors: the strain of *D. nodosus* and the presence of virulence factors (namely proteases and type IV pili), environmental factors (including moisture and temperature), sheep breed and age, and other, as yet undefined, factors.<sup>1,3,5-12</sup>

Lesion severity ranges from mild to severe and purportedly relates to the nature of the proteolytic enzymes produced by *D. nodosus*, although contributions from other bacteria are also likely. *D. nodosus* produces a number of extracellular proteases including gelatinase, elastase, fibrinogenase, collagenase and caseinase.<sup>1,9,13,14</sup> Crude enzyme prepared from culture supernatant causes an ulcerated lesion when injected into the hoof.<sup>9</sup>

*D. nodosus* has type IV pili that exhibit a classic twitching motility and are involved in adhesion to host cells.<sup>10-12</sup> Based on pilus antigens, *D. nodosus* has ten serogroups (serogroups A-I, M) according to the Australian serogroup classification<sup>15</sup> whereas a UK classification system identifies 17 serotypes<sup>16</sup> and a USA system identifies 21 serotypes.<sup>17</sup>

The main environmental factors affecting lesion severity are temperature and moisture<sup>1,6</sup> as under favourable conditions infection with a virulent strain is more likely to result in a severe lesion, whereas the same strain under less than ideal conditions may result in a lesion showing less damage to the foot and therefore be classified clinically as benign.<sup>6,18</sup>

A range of lesion severity occurs within a flock and this is related to host immunity and differences in response due to age of sheep and breed.<sup>19</sup> The immune response to natural and experimental infection varies among breeds of sheep and within an infected flock. Natural resistance to footrot through heritable genetic factors appears to be due to alleles in the ovine major histocompatibility complex (MHC) class II region.<sup>20,21</sup> Involvement in the SY6 and SY1b Class I ovine lymphocyte antigens may also contribute to disease resistance by producing a greater antigen response after vaccination.<sup>22</sup> However, infected or vaccinated sheep do not develop long-term immunity and can become re-infected. The greatest immune response is generated by pilus (fimbrial) antigens compared to cell envelope antigens, lipopolysaccharide or extracellular protease antigens, and antibody levels are highest when lesions are severe and infection prolonged<sup>23</sup> or vaccine doses are given at longer intervals.<sup>24</sup>

Maximum expression of virulence occurs when factors contributing to virulence and expression are optimal. These factors continue to cause debate amongst scientists and policy

makers when defining virulent and benign footrot. As a result, the classification and diagnosis of virulent and benign footrot are different between states in Australia and are based on laboratory tests, and/or on a flock assessment.

The pathogenesis of ovine footrot is still being elucidated although research suggests a number of factors associated with *D. nodosus* may be involved including type IV fimbriae, serine proteases, and genetic elements involved in regulation, such as the *intA* gene.<sup>10-14,25-28</sup>

A laboratory-based diagnostic classification of footrot is determined by the virulence potential of the isolate of *D. nodosus*, as measured by the thermostability of serine proteases using the gelatin gel test.<sup>29,-33</sup> Lesion scores in individual sheep are recorded, but the flock is defined as having virulent footrot based on the isolation of a protease thermostable (S) strain, whereas benign footrot is classified based on isolation of a protease thermolabile (unstable, U) strain.<sup>31</sup> The strains can be further classified by analysis of the protease isoenzymes through the zymogram test, which separates the isoenzymes according to molecular weight. Virulent and benign isolates can be differentiated based on their isoenzyme patterns.<sup>13,14,31</sup>

A field-based (flock-level) diagnosis of benign or virulent footrot is not defined at the individual sheep level, but is based on the prevalence and severity of infection within a flock and relies on examination of a sufficient number of sheep to establish a clinical diagnosis. In some flocks, an approved laboratory test may be used to assist the field veterinarian in confirming the diagnosis.<sup>34</sup>

# **Clinical Signs**

The first sign of footrot may be lame sheep, although footrot-affected sheep are not always lame. Usually more than one hoof, with both digits, is affected. Footrot begins with inflammation of the interdigital skin and may progress to invasion of the germinal layer of the hoof, which separates the soft tissue of the foot from the horn.<sup>1</sup> The lesion has a distinctive foetid smell and moist, white-grey, necrotic material is usually present on the surface of the soft tissue separated from the horn. Hoof lesions are graded (score 1-5) according to degree of damage to the interdigital skin<sup>35</sup> and this is discussed under 'Gross Pathology' and in Part 2 ('Scoring footrot lesions').

Sheep with severe footrot lesions show inappetence, which leads to a reduced rate of wool growth; reduced body weight and reduced wool growth are directly related to the severity of lesions and number of feet infected.<sup>36-38</sup> The annual mortality rate of sheep with severe footrot may be up to 4.5%. In contrast, mild lesions have minimal effect on production.<sup>37</sup>

Sheep with mild footrot lesions may or may not show lameness and two or more feet may be affected with lesions confined to the interdigital skin. Footrot with mild lesions tends to occur in early spring or late autumn but may persist in a small proportion of the flock as a chronic infection throughout summer. Lesions tend to self-cure rapidly, particularly as the environment dries out, but the disease may reappear in the following spring. Mild lesions apparently or temporarily respond to footbathing treatment, compared with severe lesions.<sup>39</sup>

For a flock-prevalence approach to determining benign or virulent footrot, a flock with benign footrot will have sheep with score 1 or 2 lesions and may have a small percentage with score 4 lesions; however, these will regress with treatment.<sup>34,35,39</sup> A flock with virulent footrot, under environmental conditions favourable to expression of the disease (and in the absence of recent interventions which may mask expression), typically has a proportion of sheep with score 4 and 5 lesions and a rapid progression.<sup>34</sup>

In a laboratory-based diagnosis of footrot, a flock with benign footrot is one that has sheep with mild lesions of score 1 or 2 and no detectable thermostable (S) strains, and a flock with virulent footrot is one that has sheep with severe and mild lesions and isolates producing thermostable (S) strains.<sup>31</sup>

#### Epidemiology

Sheep and goats are the primary species affected and they are susceptible at all ages. Sheep and goats may develop a severe form of the disease whereas cattle and deer develop a mild form.<sup>1,40</sup> Footrot has been reported in wild ungulates, ibex (*Capra ibex ibex*) and mouflon (*Ovis aries musimon*) in Germany and Switzerland.<sup>41</sup>

Although Merino sheep are more susceptible to infection with *D. nodosus* compared to coarse-woolled British breeds, first-cross ewes can develop severe disease under the right conditions. British breeds are generally more resistant to natural infection, with lesions more likely to be mild and of short duration. However, in experimental infections, all breeds express severe lesions when the organism is applied directly to the interdigital skin.<sup>42</sup> Natural resistance to infection varies within a flock and manifests as mild lesions, delayed infection or self-curing lesions.

Environmental factors affect footrot expression and transmission, and although footrot may occur year round, it is highly transmissible and shows peaks of clinical expression in temperate areas of southern Australia in late spring and early summer, when there is adequate moisture and ambient temperatures above 10°C.<sup>1,6</sup> Suboptimal environmental conditions are not conducive to full clinical expression of virulent footrot and lesions examined under these conditions may be confined to the interdigital skin and present as mild lesions.<sup>1,6</sup> In one study, Merino sheep artificially infected with an identical strain of D. nodosus and held under different environmental conditions presented with markedly different clinical expression and lesion scores that ranged from asymptomatic to severe.<sup>18</sup> The prevalence of lesions and persistence of infection varied between sites; dry warmer climates tended to result in lower prevalence and decreased persistence, whereas wetter and cooler climates resulted in a higher prevalence of lesions and increased persistence. Infected sheep in sub-optimal environments had increased self-cure compared to sheep in optimal climates. Upon removal of infected sheep from a sub-optimal to an optimal environment, lesions increased in severity.<sup>18</sup> Other studies, involving a mixture of benign or less virulent strains, investigated footrot expression related to environment using a flock-based assessment and found no differences in clinical expression of footrot.<sup>37,43</sup> Although the use of two different methods for diagnosis of benign or virulent footrot complicate the comparison of data in research studies, it should be noted that a change in environment that is conducive to the expression of virulent footrot may have implications for sale and movement of sheep if a misdiagnosis is made.

*D. nodosus* can survive in moist soil for up to two weeks,<sup>1,7</sup> although a recent study indicates that under experimental conditions with optimal factors for growth, survival can be as long as 24 days in the presence of hoof powder and a temperature of  $5^{\circ}C$ .<sup>44</sup>

Footrot is transmitted by contact with contaminated soil or pasture<sup>1,45</sup> and to avoid infection it is recommended that pasture be spelled for a sufficient time before introducing new sheep. In Australia the effectiveness of spelling pasture from one to two weeks may vary according to environmental conditions. Although transport vehicles are not recognised as a significant factor in transmission of ovine footrot, molecular typing of strains from one outbreak indicated that contaminated material left in a truck used to transport diseased sheep from one farm resulted in infection of naïve sheep subsequently transported from two other farms.<sup>46</sup>

Cattle may act as reservoirs of infection. In one Australian study, 11% of cattle sampled at an abattoir were positive for protease labile strains of *D. nodosus*.<sup>47</sup> In another study, a *D. nodosus* isolate of bovine origin induced benign footrot in experimental cattle and sheep, and infection was transmitted to co-grazed calves and sheep, with resultant benign footrot.<sup>48</sup> In contrast, sheep with virulent footrot after experimental infection with a virulent strain transmitted infection to co-grazed sheep, causing virulent footrot, but did not transmit disease to co-grazed calves in the same paddock.<sup>48</sup> Molecular fingerprinting by pulsed field gel electrophoresis (PFGE) has also shown the same protease thermostable strain of *D. nodosus* can be isolated from co-grazing cattle and sheep.<sup>46</sup> In Norway, previously uninfected cattle co-grazed with sheep naturally infected with *D. nodosus* became infected with the same serogroup (serogroup A).<sup>49</sup> In Norway and Denmark, *D. nodosus* was isolated from 50% of bovine digital dermatitis lesions.<sup>50</sup>

*D. nodosus* is genetically diverse, with approximately one in every four isolates presenting a different molecular type based on PFGE results and a diversity of one in every seven isolates when tested by the infrequent restriction site (IRS) polymerase chain reaction (PCR) method.<sup>51</sup> A high genetic diversity has been reported from isolates in Malaysia<sup>52</sup> and New Zealand, the latter based on studies of genetic diversity in the fimbrial gene (*fimA*).<sup>53</sup> A single infected hoof may contain different strains of *D. nodosus* (protease thermostable and heat-labile), different molecular types (similar or different clonal types) and different serogroups.<sup>51,54</sup> In Western Australia, a third of all farms with sheep infected with footrot had sheep infected with more than one genetic type.<sup>51</sup>

The host immune response is related to lesion severity and duration of infection.<sup>23</sup> Sheep challenged with different antigenic fractions based on cell envelope antigens, polysaccharides, extracellular protease antigens or pilus antigen elicit a weak immune response but the latter generate the greatest immune response. There are ten serogroups (A-I, M) based on the pilus antigens and protection is serogroup specific.<sup>54</sup> Multivalent vaccines developed for treating footrot are largely unsuccessful due to antigenic competition, or inhibition of the immune response, or a reduced and less persistent immune response compared to that elicited by a monovalent vaccine.<sup>24</sup> A recent study indicates that sequential monovalent or bivalent vaccines prepared to serogroups specific to the flock of sheep generates a rapid clinical response in flocks where there are two or less serotypes. In flocks with greater than two serotypes, successive bivalent vaccines are required to eradicate the disease.<sup>55</sup>

# **Occurrence and Distribution**

Ovine footrot has been reported in many countries outside Australia and New Zealand, including Britain,<sup>56,57</sup> Brazil,<sup>58</sup> Canada,<sup>59</sup> Denmark,<sup>60</sup> France,<sup>61</sup> Germany,<sup>62</sup> Holland,<sup>63</sup> India,<sup>64</sup> Malaysia,<sup>52</sup> Nepal,<sup>65</sup> Portugal,<sup>66</sup> Spain,<sup>67</sup> Switzerland,<sup>41</sup> USA,<sup>17</sup> and in Sweden and Norway.<sup>49,68</sup>

Footrot occurs in the temperate rainfall areas of Australia predominantly in New South Wales (NSW), South Australia (SA), Western Australia (WA), Victoria and Tasmania. Its occurrence in Queensland is sporadic.<sup>1,69</sup>

The disease is treated differently by Australian states as revealed at a National Footrot Workshop held in Sydney in 2012 (sponsored by the Sheepmeat Council of Australia and Wool Producers Australia). Ovine footrot is notifiable in NSW, SA, Victoria and WA. Depending on state policy, programs aim to either eradicate (NSW) or control (SA, WA) virulent footrot. The number of properties in quarantine in NSW, SA and WA varied between 25 and 40 in 2010-2011. The prevalence of virulent footrot in NSW was estimated at 0.2% of

farms (J Seaman, personal communication, 2012). In SA, a 2008 survey found 6.25% of flocks with virulent footrot and 25% of flocks with benign footrot (I Sanderson, personal communication, 2012). In WA the prevalence of virulent footrot was 0.4% of sheep farms and for benign footrot was 6% of sheep farms (P Morcombe, personal communication, 2012). In Tasmania the disease is not notifiable and there is no control program; footrot is widespread and anecdotal evidence suggests the prevalence is increasing (A Bailey, personal communication, 2012). In Victoria, the disease is notifiable, but flocks with virulent footrot (diagnosed clinically as  $\geq$ 1% of sheep with score 4 or 5 lesions) are not quarantined. Current prevalence is not known (I McLaren, personal communication, 2012).

In New Zealand, footrot is endemic with low prevalence, but still of economic importance (W McDonald, personal communication, 2012). No official control or eradication programs exist. The disease is controlled by good farming practices with some vaccination or use of zinc sulphate footbathing (J Hickford, personal communication, 2012). Estimates in 2003/04 of the prevalence of footrot in merino wool producing farms and "mid-micron" farms, were 65.2% and 55.2%, respectively.<sup>70</sup>

#### **Gross Pathology**

Footrot may show some or all of the following pathology. Inflammation of the interdigital skin and skin horn junction may lead to a progressive degeneration of underlying tissues, distortion of the horn, underrunning of the soft horn of the heel and sole, which may extend under the hard horn of the walls and the toe resulting in separation of the horn from its germinal layers. Tissues may show erythema, a layer of white-grey, exudative necrosis and hyperkeratosis.<sup>1,8</sup>

Footrot begins as a mild inflammation of the interdigital skin. In benign footrot there is no or little progression of this inflammation. A small proportion of affected sheep may show separation of the soft horn at the heel and posterior sole but in contrast to virulent footrot there is reduced necrosis of the underlying soft tissues and less accumulation of necrotic exudate. Benign footrot characteristically shows mild lameness and rapid healing following treatment or when the environment is warm and dry. The condition ovine interdigital dermatitis (OID) may be confused with benign footrot. *D. nodosus* is not present in OID.<sup>39,71</sup>

A severe lesion diagnosed as virulent footrot is characterised by rapid progression of interdigital inflammation to full underrunning of the soft and hard horn usually within 7-14 days following initial infection under favourable conditions. Both digits and usually more than one foot are affected; lameness may be severe and may become chronic. A large proportion of the flock is affected and the disease spreads rapidly in a favourable environment. Some regression of lesions may occur when the conditions become dry but pockets of infection develop at the sole/wall junction.<sup>1,3,8,71</sup>

The amount and extent of damage to the interdigital skin, the skin horn junction and the horn forms the basis for a lesion scoring system. Mild lesions are scored 1 or 2, and the more severe lesions are scored at 3, 4 or 5.<sup>35,72-74</sup>

An intermediate form of footrot was once recognised by authorities in some Australian states in which a small proportion of affected sheep have score 3 or 4, but not (or very rarely) score 5 lesions.<sup>43,74</sup> However, for the purpose of national uniformity in diagnosis, a 1992 Working Party on the Co-ordination of Footrot Control in Australia recommended that footrot should only be described as either virulent or benign, and that strains of intermediate virulence be regarded as virulent.<sup>35</sup> Virulent footrot may occur throughout the year in most affected sheep if not treated. However, because the disease transmits and is seen more frequently in late spring and early summer in southern Australia when the temperature and moisture are conducive to footrot expression, feet should be examined for lesions and samples collected especially during this time. Any signs of lameness should be investigated for possible footrot.<sup>1,6,18,35</sup>

# **Diagnostic Tests (General)**

A diagnosis of footrot is made by clinical appraisal with hoof examination and interpretation of lesion scores and consideration of laboratory test results. Laboratory tests, including the examination of a Gram stained smear of hoof exudate and culture of the organism, are used to confirm the field diagnosis.

Tests for virulence of *D. nodosus* isolates include the gelatin gel thermostability test, the elastase test and the zymogram test; however, the requirement to undertake and report these tests is different in each Australian state according to varying legal and regulatory requirements.

There is still no single laboratory test for detecting virulent strains; however, in an extensive Australian study, the gelatin gel thermostability test and the elastase test gave the best correlation with virulence (a lesion score of 4 or 5) when validated using an *in-vivo* pen test under controlled environmental conditions.<sup>32</sup> The gelatin gel thermostability test had a sensitivity of 92% compared to a sensitivity of 74% for the elastase test, and the specificities were 68 and 71%, respectively.<sup>32</sup> The study indicated that clinical diagnosis of virulent footrot (by visual inspection of the hoof) has a sensitivity of 53% and a specificity of 77%. There was good agreement between field and laboratory diagnosis for virulent footrot, but poor agreement for diagnosis of benign footrot indicating that less than favourable conditions may mask virulent footrot.<sup>32</sup>

The Animal Health Committee (AHC) Working Party on Coordination of Footrot Control in Australia, in 1992, recommended the gelatin gel thermostability test as the best laboratory method for determining whether an isolate is virulent or benign.<sup>35</sup> To date, no other test has been shown to have greater sensitivity in detecting virulence, although the elastase test, which is used in some states, offers marginally better specificity. Quality assurance testing for the protease thermostability and zymogram tests is available through the Australian National Quality Assurance Program (ANQAP).

A serogroup-specific multiplex PCR has been reported for identifying strains of *D. nodosus* in a sheep flock particularly for use when developing autologous bivalent vaccines.<sup>75</sup> The primers consist of a common forward primer designed to detect the conserved amino-terminal region of the fimbrial gene (*fimA*) and nine reverse primers to detect the carboxyl-terminal regions of the gene, which relate to the serogroups A-I. However, this test has not yet been submitted to SCAHLS.

PCRs based on detection of the 16S rRNA gene of *D. nodosus* and the fimbrial subunit gene, *fimA*, and a real time PCR have also been reported<sup>68,76,77</sup> but have not undergone validation according to SCAHLS requirements.

# **Guidance on Safety and Containment Requirements**

Normal procedures apply to *D. nodosus* when working in a PC2 laboratory. Human infection with *D. nodosus* has not been reported.

# **Part 2 – Test Methods**

In Australia, diagnosis, treatment and regulation of ovine footrot is still addressed according to individual state policies and, despite numerous workshops and a national research project to validate diagnostic tests, no national consensus has been reached. This ANZSDP presents accepted methodologies used by some or all of the states where footrot exists.

#### Assessment of footrot based on flock prevalence

Although assessment of footrot based on flock prevalence is not a laboratory-based test, laboratory diagnosis may be used in addition to flock assessment and therefore is discussed here briefly.

In some Australian states the policy for the diagnosis of virulent footrot is based on flock assessment of the extent and progression of lesion severity.<sup>34,35</sup> The lesion scores of a minimum of 100 randomly-selected sheep from a suspect mob are recorded and an assessment is based on the prevalence of sheep with lesions of score 2 or greater, and prevalence of sheep with lesion scores of 4 or greater.<sup>34</sup> The guidelines suggest a flock in which the cohort of 100 has more than 1% sheep with severe lesions (score 4 or 5)<sup>34,35</sup> is a useful guide, although not an exclusive criterion, for considering virulent footrot. Under favourable conditions more than 10% of a flock with virulent footrot will usually have score 4 or 5 lesions, and can be up to 90%. In benign footrot, sheep will have score 1 and 2 lesions (interdigital dermatitis without underrunning) and a small percentage (not stated in the guidelines) will have score 4 lesions; however, these will regress without treatment.<sup>34,35</sup> Culture of *D. nodosus* from the lesion and subsequent laboratory tests to assess protease production (thermostability and elastase) may be performed, or molecular detection of the *intA*<sup>27</sup> gene may be undertaken to assess further virulence potential of a gelatin gel positive strain. Note that the *intA* PCR was not ratified by SCAHLS.

#### Assessment of footrot based on protease thermostability test

Other Australian states use a laboratory-based system for diagnosis of virulent and benign footrot, based on culture of *D. nodosus* from the lesion, followed by the gelatin gel test to assess protease thermostability and the zymogram test to differentiate the protease isoenzymes.<sup>31,33</sup> A definition of virulent footrot based on isolation of a protease thermostable strain recognises the maximum virulence potential of a strain independent of environmental conditions.

#### **Scoring footrot lesions**

Lesions are graded according to the degree of invasion (underrunning) and subsequent damage to the soft tissue and hard horn. Three lesion scoring systems have been described and are summarised in Table 1. The first (system 1) is the simplest with a 0-4 grading scale<sup>72</sup>, the second (system 2), known as the modified Egerton system for scoring lesions of footrot, is based on a standard scoring system approved by the Animal Health Committee and defined according to the Report of a Working Party of Footrot in Sheep and Cattle (1987) and published in the Australian Standard Diagnostic Techniques for Animal Diseases (ASDT).<sup>34,35</sup> Lesions are scored from 0 to 5 with three sub-scores for grade 3. The subscoring of grade 3 lesions in this system is used in more detailed investigations, including research studies of pathogenesis and vaccine responses.

The third system (system 3) is the most complex and is based on the sum of the weighted lesion scores (system 1) of the four feet, and is called the total weighted foot-score. It aligns lesion score severity with host humoral immune response and has been used to assess the response to vaccination in research trials.<sup>73</sup>

	System 1		System 2		
Score	Description	Score	Description	Additional subdivision	
1	Limited mild interdigital dermatitis	1	Slight to moderate inflamma interdigital skin and involvin epithelium	tion confined to g erosion of the	
2	More extensive interdigital dermatitis	2	Necrotising inflammation of also involves part or all of th wall of the digit	interdigital skin which e soft horn of the axial	
3	Severe interdigital dermatitis and underrunning of the horn of heel and sole	3	Necrotising inflammation with underrunning of part or all of the soft horn of the heel or sole which does not extend to the abaxial edge of the sole of the hoof.	3a: Separation at skin- horn junction with underrunning extending no more than 5 mm across sole 3b: Underrunning no more than halfway across heel or sole 3c: Extensive underrunning of heel or sole but not extending to abaxial edge of the sole	
4	Severe interdigital dermatitis and underrunning of the horn of heel and sole but with underrunning extending to the walls of the hoof	4	Underrunning extending to a	baxial edge of sole	
		5	Necrosis of deeper epiderma abaxial wall with under-runn	l layers (laminae) of ing of hard horn	

#### Table 1. Footrot scoring systems in current use in Australia

#### Collection of specimens for laboratory diagnosis

Material from the moist interdigital skin of the hoof is collected by either scraping with a sterile scalpel blade, a sterile wooden stick, or a cotton-tipped swab, although the former is regarded as the most sensitive method for culture.<sup>35</sup> The best sample is the whitish-cream dead tissue from the interdigital skin from the skin/horn junction from early cases of footrot. In more advanced cases samples are taken from the advancing edge of the underrun lesion or the bottom of cracks in the skin/horn junction area. Fly-struck feet should not be sampled as these yield poor results for bacteriology, and may be devoid of *D. nodosus*. The collected scrapings are placed or embedded into the top 2-3 mm of modified Stuart transport medium<sup>31</sup>, or the swab head is immersed fully into the medium and the swab stick broken off. The sample yield is often very small and it is important to use the modified transport medium as this contains a high concentration of agar to prevent dilution and dissipation of the sample, and assists in maintaining anaerobic conditions. The colour of the transport medium should

be cloudy grey or ice-blue. A strong blue colour indicates the medium has been oxidised and is no longer suitable for use.

The sample is transported to the laboratory on ice, or at ambient temperature, to arrive within 24-48 hours. If transport is delayed beyond 24 hours then the samples should be refrigerated at 4°C to assist in maintaining viability of *D. nodosus* and prevention of overgrowth by contaminating bacteria. Length of transport time affects the chance of successful culture of *D. nodosus*. Samples in transport medium that reach the laboratory within 3 days achieve best results, while those with transit times of more than five days may not be suitable for culture.<sup>31</sup>

### Gram stain for microscopic detection of D. nodosus

A smear is prepared from lesion material collected into modified Stuart transport medium or from laboratory cultures. The smear is air dried and fixed by passing through a Bunsen burner flame three times prior to the Gram stain procedure. The Gram-stained smear is examined under oil using the 100× objective. Cells of *D. nodosus* are Gram-negative, 0.6-0.8  $\mu$ m wide by 3-10  $\mu$ m in length and have rounded ends. Terminal swellings may be seen (Figures 1, 2). There is a tendency to resist decolourisation.<sup>1</sup> On laboratory media, especially under less than ideal conditions, pleomorphic forms including long convoluted forms or coccoid forms may be seen.<sup>78</sup>



**Figure 1.** *D. nodosus* in Gram-stained smears from hoof scrapings (Copyright © Western Australian Agriculture Authority, 2012)



Figure 2. Gram-stained smear from a culture of *D. nodosus* (Copyright © Western Australian Agriculture Authority, 2012)

#### Culture and identification of the organism

#### Principle of the test

*D. nodosus* requires an anaerobic environment for growth and is cultured on medium containing proteose-peptone, yeast extract and peptone, and essential compounds for growth: trypticase (trypsin-digested casein), arginine and serine (TAS).<sup>78</sup> The addition of ground ovine hoof, which can be substituted by trypticase, enhances growth.<sup>79</sup> However, ground hoof is recommended routinely as some strains may not grow or may not grow on subculture without the addition of ground hoof.<sup>31,79</sup>

In broth cultures, the addition of a reducing agent, thioglycollic acid, and the presence of magnesium ions enhances growth;<sup>78</sup> the latter can also be added to TAS agar.

*D. nodosus* can be grown on agars including Hoof agar,<sup>35,79</sup> TAS agar,<sup>78</sup> modified TAS agar,<sup>35</sup> and TASH agar (modified TAS agar with powdered hoof)<sup>31</sup> using isolation (TASH-IM) and maintenance medium (TASH-MM), and blood Eugon agar<sup>35,80</sup>. The use of a 4% final concentration of agar in isolation media reduces the growth and swarming of contaminants, due to inhibition of flagellar motility, but allows the twitching motility of *D. nodosus* to establish colonies away from the streak line.<sup>31,78</sup> The use of TASH agar containing 4% agar for initial culture (TASH-IM) and TASH agar containing 1.5% agar for subculture (TASH-MM) may provide the best range of nutrients for growth on solid medium.<sup>31</sup> For supplementary testing, *D. nodosus* can be subcultured into broth media such as TAS broth<sup>78</sup> and modified Eugon broth.<sup>35</sup>

*D. nodosus* grows optimally at 37-39°C,<sup>78</sup> with slower growth at ambient temperature. *D. nodosus* is an anaerobe but can tolerate short exposure (15 minutes) to air. Plates are examined under a binocular stereo microscope with  $10 \times$  magnification and suspect *D. nodosus* colonies are subcultured for pure growth.<sup>31</sup>

#### Reagents and materials

Agar plates (TASH-IM, TAS, Hoof agar or Eugon agar) are dried for 10 minutes but no longer than 15 minutes at 37°C. The plates need to be moisture-free, to prevent excessive spreading of contaminants. If possible, uninoculated plates should be stored in an anaerobic atmosphere to reduce the absorption of oxygen into the plates.

# Method

Prior to inoculation of plate media, plates are dried for 10-15 minutes at 37°C by removing lids and inverting on shelf of incubator. Minimal drying time is used to prevent the absorption of oxygen into the media.

For scrapings collected and placed into the top of a modified Stuart transport medium bottle, the tip of a sterile inoculating wire (stainless steel, 1.5 mm diameter and 75 mm in length; coat hanger wire is suitable) is used to collect a sample from the transport medium and inoculate two isolation medium plates (TASH-IM and/or hoof agar) as shown in Figure 3a. The initial inoculation streak (large arrow) is made onto the surface of the agar and then the inoculum is forced into the agar to the bottom of the plate. Secondary streaks are made (lines 1, 2, 3) into the surface to lightly etch the agar.<sup>31</sup>

When a swab has been used for the collection, flame-sterilized artery forceps are used to remove the swab, the swab tip is pressed to the edge of the transport medium bottle to remove excess moisture and then used to streak to isolation media as indicated in Figure 3b. Inoculation of two or more plates increases the sensitivity of culture. The residual material

(scraping in transport medium or the swab in transport medium) is held at  $4^{\circ}C$  in case reculture is required.



Figure 3a. Streak pattern employed using inoculating wire and scrapings. The large arrow shows the primary streak of inoculum. Small arrows (2, 3) show the order and direction of secondary streaks



Figure 3b. Streak pattern when using swabs. Numbers indicate order of streaks

When dealing with a large number of samples, if possible, the inoculated plates are placed into an anaerobe jar that is continually receiving a flow of carbon dioxide (CO<sub>2</sub>). Once all plates are inoculated, the CO<sub>2</sub> tube is removed, an anaerobic atmosphere-generating pack including indicator strip is added, the jar is sealed and incubated at 37°C for a minimum of 48 hours. If a continuous flow of CO<sub>2</sub> cannot be provided, the culture plates are placed into an anaerobic box with an appropriate gas-generating pack within 15 minutes of inoculation.<sup>31</sup> Commercial gas-generating packs produce an atmosphere of <1% oxygen and 9-13% CO<sub>2</sub>. Commercially available anaerobic atmosphere-producing packs/pouches and anaerobic boxes include AnaeroPack<sup>TM</sup> MCG (Mitsubishi Gas Chemical) and AnaeroGen Oxoid (both available from Thermo Scientific) and the GasPak EZ system (Becton Dickinson). All three packs or pouches are suitable for 2.5 or 3.5 L glass vacuum-sealing bottling jars, or commercially-available anaerobe boxes.

After 48 hours, a dissecting stereomicroscope with integrated vertical illumination and low power  $(10\times)$ ,<sup>31</sup> a magnifying glass or a jeweller's monocle are used to examine the surface of the agar for colonies typical of *D. nodosus*. Colonies have a diffuse ground glass appearance, particularly around the colony edge, and will grow out and away from the streak line (Figure 4).



**Figure 4. Colony (arrow) of** *D. nodosus* **on TASH-IM agar at 5 days of incubation** (Copyright © Western Australian Agriculture Authority, 2012)

For subculture to solid medium such as TASH-MM plate, hoof agar, TAS agar, modified TAS agar, TASH or blood Eugon agar for purification, two methods can be used. The suspect colony can be removed with a nichrome wire loop, or a colony with agar can be excised using a micro-spatula shaped from 0.5 mm nichrome wire and flattened at one end to resemble a hockey stick (Figure 5). The excised colony and agar plug are placed colony-side down onto the surface of the subculture plate (Figure 6). Up to three to four subcultures from one specimen can be applied to a plate.



Figure 5. Nichrome wire spatula or hockey stick fashioned from 0.5-0.9 mm diameter wire



Figure 6. Subculture of *D. nodosus* on TASH-MM agar at four days of incubation. Arrow indicates film of growth away from agar plug (Copyright © Western Australian Agriculture Authority, 2006)

The sub-cultures and the original isolation plates are incubated anaerobically for a minimum of 48 hours at 37 °C. Subcultures are examined for pure growth using a stereomicroscope or magnifying glass. Purity is checked using a Gram stain. Plates showing no *D. nodosus* colonies are re-incubated for a further 2-6 days before discarding and reporting as negative.

Pure growths are inoculated into TAS or HEPES TAS broth for testing for thermostable protease using the gelatin gel test and the zymogram test (see under 'Tests to characterise *D*. *nodosus* isolates'). For inoculation of broth from subculture plates, a colony with agar is excised, using either a nichrome wire hockey stick, or the nichrome wire fashioned into a large loop of diameter 8 mm to remove a circular plug approximately 10 mm in diameter.

Contaminated samples are re-subcultured from the colony edge on the subculture plate, or from the original plate. If no suitable colonies are available, a fresh TASH-IM plate can be subcultured from the underside of the agar of the original TASH-IM plate when the stab wire method is used (i.e. the agar is flipped into the lid of the plate and subcultured from the growth at the base of the agar), or if swabs are used, the repeat culture is performed from the original swab. Subcultures are incubated in an anaerobic atmosphere for a further 48 hours.

#### **Biochemical Tests**

Normally, biochemical tests are not performed on *D. nodosus*. The organism is non-motile but exhibits twitching motility, which is seen as a spreading growth on agar media. Results are negative for oxidase, catalase, urease, arginine decarboxylase (ADH), DNase, indole production, nitrate reduction, starch hydrolysis, aesculin hydrolysis and fermentation of glucose and sorbitol. Results are positive for ornithine decarboxylase and  $H_2S$  production. Proteolytic activity is seen on media containing gelatin, casein and albumin.<sup>2</sup> ADH and nitrate may be positive in some commercial strip tests.

#### Preservation of isolates

Cultures of *D. nodosus* can be maintained for short periods (2-3 weeks) on TASH-MM agar, hoof agar, TAS agar or Eugon agar under anaerobic conditions and stored in the dark at ambient temperature.

Long-term preservation requires freeze-drying or storage at -80°C. For storage at -80°C, treated beads, such as Protect Bacterial Preservers (Technical Service Consultants LTD, The Rope Walk, Schofield Street, Heywood, Lancs, OL10 England) have been used successfully (M. Palmer, personal communication, 2008). Other brands of beads should be pre-tested for suitability. The Protect Bead method involves emulsifying a loopful of growth from a 48-hour culture and mixing into the bead fluid, followed by removal of the fluid and freezing the beads at -80°C. Alternatively, growth may be suspended in Glycerol Lab Lemco (refer Media) and stored in 1 mL amounts in cryoprotectant vials at -80°C. For storage using freeze-drying, suspensions are prepared by harvesting 48 hour agar colonies. One method employs 1 mL inositol horse serum as the cryoprotectant in 2 mL Wheaton serum bottles. A sterile cotton-tipped swab moistened with cryo-preservation fluid is used to harvest cells of *D. nodosus*, which are suspended in the cryoprotectant fluid before being snap frozen in liquid nitrogen prior to lyophilisation (M. Palmer, personal communication, 2008). A second method uses skim milk as the cryoprotectant (K. O'Grady, personal communication, 2013).

# **Gelatin gel protease thermostability test**<sup>29,31,33</sup>

# Principle of the test

The gelatin gel test differentiates *D. nodosus* strains based on whether the extracellular proteases they produce are heat stable (thermostable, S) or heat labile (unstable, U). The test is used to differentiate virulent (S) and benign (U) strains of *D. nodosus* as determined by a number of research studies.<sup>29,30,33,81</sup> The use of the gelatin-gel test in a diagnostic laboratory to measure protease thermostability is described by Palmer *et al.*, (1993).<sup>33</sup> *D. nodosus* is grown in broth culture until a required cell concentration (ideally  $10^8$  CFU/ml) is reached,

usually at 48 hours, and then extracellular enzyme activity remaining after heat treatment of 0, 8 and 16 minutes is detected through the hydrolysis of gelatin. After incubation for 24 hours at 37°C, the clear zones of gelatin hydrolysis indicating protease activity are detected and measured. Measurement of protease activity is based on a modification of the radial diffusion method of Schumacher and Schill (1972)<sup>82</sup>. The amount of protease activity is related to the size of the zones and is calibrated from a standard curve (see under calibration). Each new batch of buffers and reagents must undergo calibration for quality control. The thermostable and equivocal strains retain protease activity after heating whereas strains that produce thermolabile protease retain no activity after heating for 16 minutes. The interpretation is given in Table 2.

#### **Reagents and Materials**

#### Inoculation of controls

A positive control strain (reference strain 198) and a negative control strain (reference strain 305) are used. Control strains are stored in a culture collection either freeze-dried or at -80°C. When new control broths are required, preserved cultures are reconstituted, grown in 100 mL volumes of TAS broth, and 5 mL aliquots are taken into plastic serum tubes.

The gelatin gel thermostability test can be performed in small batches (nine samples per plate) using Petri dishes, which has been validated using TAS broth (without optional components of thioglycollic acid and  $Na_2CO_3$ ), HEPES dilution buffer and agarose gelatin gel Tris buffer. Large batches can be performed using a glass plate (42 samples per plate), which has been validated using HEPES TAS broth, HEPES dilution buffer and agarose gelatin Tris buffer.

#### Inoculation of broth

The required numbers of HEPES-TAS or TAS broths are steamed in the autoclave with lids loose or boiled in a beaker containing 2 cm of water for 10 minutes to drive off any dissolved oxygen. Lids are re-sealed and the broths cooled to room temperature or kept at 4°C until used. A section of agar (7×20 mm or 10 mm diameter) containing growth of a pure *D. nodosus* colony is removed using an appropriate sterile device (microspatula, scalpel or large loop) as described previously under subculturing, and placed aseptically into a broth. The head-space is filled with CO<sub>2</sub> and sealed with the rubber stopper. Alternatively, if CO<sub>2</sub> is not available, the broths are incubated with lids loose in an anaerobe jar containing an anaerobic atmosphere generating pack. The broth culture is incubated for 2-4 days at 37°C. Usually 48 hours growth will achieve end of log phase of growth when the most protease is produced. Growth is indicated by slightly opalescent turbidity.

Ideally the gelatin gel test is done at the end of two days of growth; however, cultures may be stored up to one week at 4°C before testing, or frozen at -20°C if delaying testing for longer periods. Storage of samples may assist bulk testing.<sup>33</sup>

# Assessment of sample purity and cell concentration

Broths cultures used for the gelatin gel test and the zymogram test can be checked for purity using a Gram stain or wet preparation. The cell concentration can be estimated from these preparations or counted using a counting chamber. An ideal cell concentration is 10<sup>8</sup> cells/mL (usually obtained after 48 hours of incubation).

# Preparation of agarose-gelatin gel

Small batch testing can be performed in 150 mm diameter Petri dishes (Sarstedt No. 821184) for up to nine samples per plate, or bulk testing can be conducted using a  $200 \times 200 \times 3$  mm glass plate (e.g. window pane glass) for batches of 42 samples per plate.<sup>33</sup>

The agarose and buffer preparation and preparation of the Petri dishes and glass plates are detailed in Part 3. Various recipes for agarose-gelatin gel and buffer have been used for both methods. Both methods require calibration of zone sizes as part of the quality control of reagents and the test. See under calibration of gelatin gel test.

# Sample preparation and dispensing

In a labelled test tube 1.0 mL of broth culture is added to 1 mL of HEPES dilution buffer. The tubes are placed in a rack in rows corresponding to the number of samples tested on each plate. The positive (strain 198) and negative (strain 305) controls are prepared in the same manner and placed at the end of each row.

Sample numbers are marked on the test plate. Each sample requires 3 wells. The first well is the unheated sample (time 0), the second well is for the sample heated at 68°C for 8 minutes and the third well is for the sample heated at 68°C for 16 minutes.<sup>33</sup>

# Test Procedure

A 20  $\mu$ L volume of the first diluted broth is pipetted into the top left-hand well in the agarose-gelatin gel and the remaining tube of diluted broth is placed into a water bath set to 68°C±0.1°C. Start the timer (count up). The same tip can be used for all samples if it is rinsed in sterile water and wiped between samples.

Maintain a consistent interval rate (e.g. 20 seconds) and pipette 20  $\mu$ L of broth dilutions into well one (time 0) for each sample and place the tube into the water bath.

At exactly 8 minutes, pipette 20  $\mu$ L of heated sample into the second well (time 8 min) for each sample. Continue with this pattern for each sample.

At exactly 16 minutes, pipette 20  $\mu$ L of heated sample into the third well (time 16 min) for each sample. Continue with this pattern for each sample.

Leave the plate on the bench to absorb well contents for approximately 10 minutes.

Incubate the gel in a moist chamber (e.g. box containing dampened blotting paper) at  $37^{\circ}$ C for 18 hours.<sup>33</sup>

# Development of the gel

Develop the gel by flooding with or immersing in saturated aqueous ammonium sulphate, preferably heated. Heated solution (56-60°C) can be prepared either in a microwave oven or by leaving overnight in a 58°C incubator. Place flooded or immersed gel in a fume hood to avoid irritation from the vapours. Wear safety glasses or face shield, and gloves.

When the gel appears milky white, usually after 20 minutes, the gel is rinsed in tap water and drained. The milky-white colour is due to unhydrolysed gelatin whereas hydrolysed gelatin appears as clear zones around wells (Figure 7).<sup>33</sup>

If the gel cannot be developed immediately it can be stored in the fridge in a moist chamber for up to four days.

#### Reading results and validation of test run

The gel is placed over a black background or a light box so that the clear zones of gelatin hydrolysis can be seen easily (Figure 7). The diameter at the widest point of the zones of hydrolysis for 0, 8 and 16 minutes is measured using a ruler or callipers.





The controls are read first to validate the test. Sufficient cell growth and concentration of extracellular protease is indicated by the unheated zone size of all samples being greater than 15 mm. If it is less than 14 mm the test should be repeated using a new broth. Generally, the zone size of the 16 minute heated sample is the same difference in diameter from the 8 minute zone, as the 8 minute zone is from the unheated zone. If there is little difference between the 8 and 16 minute zones the test must be repeated with a new broth culture.

The most common cause of an unusually large 16 minute zone or no difference between the 8 minute and 16 minute times is the presence of highly thermostable protease produced by a contaminant organism in the broth. Minute colonies of proteolytic bacteria growing on the agarose gel at the point of inoculation are usually caused by bacterial contamination of the pipette tip.

The zone sizes used in the interpretation of results are obtained from a calibration (see Calibration of gelatin gel test). The relationship between protease concentration and zone diameter is not linear and are based on a mathematical equation derived from the results of measuring zone sizes of dilutions of unheated broth cultures of the control virulent strain 198. The initial validation of protease thermostability as a measure of virulence of *D. nodosus* has been determined from various research studies.<sup>29,30,33,81</sup> Validation of the gelatin gel test as a measure of protease thermostability in the diagnostic laboratory is described by Palmer (1993).<sup>33</sup>

# Interpretation of results

On the basis of zone sizes for *D. nodosus* broth cultures heated at  $68^{\circ}$ C for 8 and 16 minutes, isolates are classified in the gelatin gel test as either protease thermostable (S), protease thermolabile or unstable (U) or equivocal, based on the following characteristics:

Protease thermostable (S) positive result:  $\geq 20\%$  protease activity remains after 8 minutes and  $\geq 7\%$  after 16 minutes. Protease thermostable S strains (virulent strains) cause clearing around the 16 minute well. They have 20-47% protease activity remaining after heating for 8 mins at 68°C and must show a 3-4 mm difference in zone size between unheated and 8 mins heated wells. They have 7% or greater activity remaining after heating for 16 mins, and show a 7 mm or less difference in zone sizes between the 8 and 16 minute heating wells.

Protease thermolabile (unstable U) negative result:  $\leq 8\%$  protease activity remains after 8 minutes and no measurable activity after 16 minutes. These strains (benign strains) have no clearing or a very small zone around the 16 minute well. These strains have 0-8% remaining protease activity after heating for 8 mins at 68°C and show 7 mm or more difference in the zone size between unheated and 8 minute wells. Some incomplete clearing may occur in samples with an unheated zone of >20 mm.

Equivocal result: <20% but >8% protease activity remaining after 8 minutes. There is incomplete clearing at 16 minutes. These strains have an 8 minute zone size larger than the protease thermolabile strains and smaller than the protease thermostable strains. Isolates with an S3 zymogram profile may give an equivocal result in the gelatin gel test. All other zymogram types should be definitely positive or negative; if not prepare a new broth and retest.

Controls: Control strains must be used in the gelatin gel test and included on all plates assessed. The positive control (strain 198) must show a difference in zone sizes of  $\leq$ 7 mm for unheated and 16 minutes heated. The test must be repeated if the difference between the unheated and 8 minutes zone sizes for the positive control is >4mm. The negative control (strain 305) must show no measurable zone at 16 minutes heated; the test must be repeated if the unheated and 8 minute zone size for the negative control is <7mm.

Table 2 shows an example of typical zone sizes for unheated and heated inocula (at 8 and 16 minutes) that could be expected when interpreting unknown strains as protease thermostable and unstable.

Unheated 0 min	Heated 8 min		Heated	Heated 16 min	
	Unstable	Stable	Unstable	Stable	
≤14		Repeat broken	oth culture		
14	≤7	≥10	0	≥7	
15	8	11	0	8	
16	9	12	0	9	
17	10	13	0	10	
18	11	14	0	11	
19	12	15	0	12	
20	13	16	0	13	
21	14	17	0	14	
22	15	18	0	15	
23	16	19	0	16	

Table 2. Example of comparative zone sizes in the gelatin gel test relevant to inocula from typical stable and unstable strains that are unheated or heated for 8 and 16 mins

Where presence or absence of clearing around the 16 minute well conflicts with the result indicated by the difference between the unheated (0 minutes) and heated 8 minute zones, the

isolate should be re-tested. Some isolates may cause a zone around the 16 minute well without definite clearing; this is recorded as a negative / positive (-/+) on the record sheet.

In some cases, there may be a low level of unheated protease produced, as shown by an unheated zone diameter < 15 mm. In this case, the test is repeated using a new broth culture.

Results are interpreted with the assistance of the calibration chart specific for the current batches of HEPES and Tris buffer and agarose-gelatin gel (see Calibration of gelatin gel test).

# Calibration of the gelatin gel test<sup>33</sup>

# Principle of the Test

The gelatin gel thermostability test is a radial enzyme diffusion test where the zone of lysis is dependent upon volume of the wells, incubation temperature and time, concentrations and ratio of enzyme (protease) to substrate and the rate of diffusion through the gel determined by gel pore size and molecular size of the enzyme under test.<sup>83</sup> The relationship between the diameter of the zone of clearing and the concentration of protease is not linear at all stages of the test, and the estimation of protease activity is derived from calibration of  $log_{10}$  protease concentration and zone diameter using a standard curve. The gelatin gel test, as a measure of protease thermostability, was calibrated by Palmer *et al.*, (1993).<sup>33</sup>

While the gelatin gel test will give reproducible results when performed using a consistent technique, local variations in zone measurement conditions have caused laboratories to produce results that are consistently high or consistently low, especially when new buffers are prepared. For this reason each laboratory should calibrate their test using the following method. For each new batch of gelatin gel or dilution buffer (HEPES and/or TRIS buffer) it is necessary to calibrate them against the *D. nodosus* stable control reference strain.

# Test Procedure

A broth culture of protease thermostable reference strain 198 is diluted in dilution buffer in doubling dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64) in triplicate (Table 3). Volumes of 20  $\mu$ L from all dilutions are placed into wells on an agarose gel (glass plate or Petri dish method) and incubated as per the gelatin gel method. Samples are not heat-treated. After incubation the diameter of the unheated zone for each triplicate is measured and averaged. An arbitrary protease concentration of 1000 units is given to the undiluted sample. Therefore the dilutions will correspond thus: 1:2 = 500 protease units, 1:4 = 250 protease units, etc (Table 3).

The diameter of the zone size is graphed against  $log_{10}$  of protease concentration (Figure 8). This should be a straight line. The results are used to estimate the Y intercept and slope (from a spreadsheet program such as MS Excel) of the relationship between zone diameter and protease, using the following equation:

zone diameter = slope  $\times \log_{10}$  protease concentration plus intercept.

Dilution	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64
Protease conc.	1000	500	250	125	62.5	31.25	15.625
log <sub>10</sub> protease conc.	3.0	2.7	2.4	2.1	1.8	1.5	1.2
Average unheated zone diameter (mm)	23	20	18	16	15	13	9
			25				

#### Table 3. Example of calibration derived from graph

Y Intercept = 1.6Slope = (22.6-1.6)/3 = 7.0Thus zone diameter =  $7.0 \times \log_{10} \text{ protease} + 1.6$ 



≥20% protease

≥15

Figure 8. Example of calibration graph

To avoid calculating protease concentrations for each test, a table of zone sizes equivalent to 20% and 8% remaining protease (the lower limit for positive and upper limit for negative tests) is prepared for each of the unheated zone sizes found in the test i.e. 15-25 mm. For example, an unheated zone diameter of 20 mm with the calibration graph described in Figure 8 would generate cutpoints as shown in Table 4.

Unheated zone		8 minute heated zone (mr	n)
( <b>mm</b> )			
	Negative (unstable)	Equivocal	Positive (stable)

Table 4. Example from above calibration graph of a 20 mm unheated zone calculation				
-1 and $-1$ . Example invit above cann alive 21 and $0$ a $20$ into unicated whe calculation	Tahla / Evampla	from above caliby	otion granh of a 20	) mm unheated zone calculation
	I able 4. Example	II UIII ADUVE CAIDI	auon graph or a $20$	<i>initi uniteateu zone calculation</i>

These values were calculated as follows:

20

To convert zone diameter to protease concentration, the following formula is used:  $log_{10}$  protease concentration = (zone diameter-intercept)/slope.

<20% and >8%

14 or 15 mm

For example, for a zone diameter of 20 mm the calculation is:

≤8% protease

 $\leq 12$ 

(20-1.6) / 7 = 2.63 so protease concentration is antilog 2.63 = 427 units

Then, for a sample with an unheated zone size of 20 mm, the 8 minute zone sizes which correspond to 8% and 20% remaining protease will be:

8% (of 427) = 34.2 units = log 1.5340, so zone diameter =  $(7 \times 1.5340) + 1.6 = 12$  mm

20% (of 427) = 85.4 units = log 1.9315, so zone diameter =  $(7 \times 1.9315)+1.6 = 15$  mm

Similar calculations are done for all unheated zone sizes from 15 to 25 mm to produce a table that is used to classify isolates as positive, equivocal or negative in the gelatin gel test. An

example reference table, based on the data that gave a Y intercept of 1.6 and slope of 7.0 (as shown in Figure 8) is shown in Table 5.

Calibration for dil	Calibration for dilution buffer batch number Date			
Intercept	1.6			
Slope	7.0			
Gelatin gel no.	Batch Gabc			
<b>Dilution buffer</b>	Batch Bxyz			
Unheated Zone	8%	20%	Equiv	vocal
	Negative	Positive	Lower	Upper
15	7	10	8	9
16	8	11	9	10
17	9	12	10	11
18	10	13	11	12
19	11	14	12	13
20	12	15	13	14
21	13	16	14	15
22	14	17	15	16
23	15	18	16	17
24	16	19	17	18
25	17	20	18	19

Table 5. Example of a calibration run for a gelatin gel buffer or a dilution buffer batch

#### **Elastase protease test**<sup>25</sup>

#### Principle of the test

Elastase is a protease produced by some strains of *D. nodosus* and is detected using the elastase test which detects hydrolysis of elastin particles in TAS agar.<sup>25</sup> A virulent strain hydrolyses elastin in 4-7 days but this hydrolysis may take up to 14-21 days for intermediate strains. Intermediate strains cannot hydrolyse elastin without the presence of calcium chloride (CaCl<sub>2</sub>) in the medium. CaCl<sub>2</sub> is added to the elastase agar plate to enhance the hydrolysis of elastin and also to facilitate the detection of intermediate isolates.<sup>81</sup> The plates are read every four days up to 28-31 days.

# Test Procedure

An elastase agar plate (see media section) is marked into quadrants. A positive (strain 198) and negative (strain 305) control and two samples are tested per plate. A loopful of culture is streaked in a line about 2-3 cm in the middle of each quadrant. Streak should be made forwards and backwards along the line to ensure adequate growth. Plates are incubated in an anaerobic atmosphere at 37°C. Plates are examined every fourth day for up to 28 days. A positive result is a zone of clearing (degradation of elastin particles) around the growth streak (Figure 9).

#### Interpretation of results

Virulent isolates degrade elastin in 4-7 days. Intermediate isolates degrade elastin in 14-21 days. Benign isolates may show delayed elastase degradation at greater than 21 days, or a negative result.<sup>81</sup> More recently, some researchers have classified virulent isolates as those that produce a positive elastase test in 4-12 days (Dhungyel and Whittington, personal communication, 2012).

**Figure 9. Negative elastase test at 21 days (left). Positive elastase test at 7 days (right)** (Copyright © Western Australian Agriculture Authority, 2012)



#### Zymogram test

#### Principle of the test

*D. nodosus* produces extracellular proteases. The zymogram test detects the isoenzyme forms of these proteases, which are characteristic and can differentiate benign and virulent isolates.<sup>13,14,26,31,84</sup> This test is used in conjunction with the gelatin gel test. Four enzyme patterns from gelatin gel thermostable strains (S1-S4), and nine isoenzyme patterns from gelatin gel heat-labile strains (U1-8) are recognised.<sup>31</sup> The isoenzymes are separated using polyacrylamide gel electrophoresis and detected using a gelatin gel overlay. The test has been validated in different studies.<sup>13,31</sup>

#### *Reagents and materials*

A HEPES-TAS broth is inoculated with a pure culture of *D. nodosus* and incubated at 37°C for 48 hours.

Numbers of *D. nodosus* in the broth are estimated by microscopy using a wet preparation. Ideally this should be  $10^8$  cells/mL, but if fewer, grade as 'light 2, 3, or 4' where 2=75-50% of above, 3=25-50%, and 4=<25%.

#### Quality control

Control broth cultures of known zymogram types S1 and U1 (strain 198 and strain 305 respectively) must be run with each batch of unknowns. When a zymogram type other than S1 or U1 is found, the sample must be re-tested alongside the appropriate controls as follows. For a band pair higher than the U1 band pair, re-test alongside U5 and U8 controls. For a band pair just above the bottom band, test alongside U3, and S4 controls. For a band pair between those of S1 and U1, test alongside the S3 control. For a band pair just below S1 or U1 pairs, test alongside U2, U4 and S2 controls. When no band pair is present but the bottom band matches U1 and S1 controls, the isolate is designated U6, but should be re-tested using four times the inoculum to check for the presence of a weak band pair. If a weak band pair is obtained in the same position as an S1, the isolate is classified as a T strain, to signify less protease is produced compared to an S1 strain (Figure 10).

#### *Test procedure*

A number of protein gel electrophoresis systems are available. This method has been validated using the Hoefer SE vertical slab cell. Caution: Acrylamide monomer is a neurotoxin. Wear gloves when handling, and perform all procedures in a fume hood. Ensure

the exhaust from the vacuum pump is discharged into the fume hood. Prepare the separating gel by mixing the separating buffer, monomer and water in a 200 mL conical flask according to the proportions in Table 6. De-aerate the mixture under vacuum. Add TEMED (tetramethylethylenediamine) and ammonium persulphate. Pour the mixture into the gel plate assembly (i.e. between the two glass plates) without introducing any bubbles.

	Separating gel	Stacking gel
Separating monomer	15 mL	
Stacking monomer		6 mL
Separating buffer	15 mL	
Stacking buffer		3 mL
Distilled water	30 mL	3 mL
TEMED	30 µL	14 μL
10% ammonium persulphate	300 µL	70 µL

 Table 6. Formulation of ingredients for preparation of separating and stacking gels

Adding the molten gel to the glass plate sandwich may be facilitated by using a 50 mL syringe and an 18G needle. Add a layer of de-aerated water onto the surface of the separating gel using a syringe and a 21G needle. Allow the gel to polymerise for 30 minutes before pouring off the water layer. Rinse the top of the gel and leave at an angle to allow to drain completely.

#### Preparation of agarose-gelatin gel

Weigh 0.2 g gelatin (Sigma G-2500), 0.2 g agarose (Biorad 162-0100) into a 250 mL conical flask or Schott bottle. Add 23 mL distilled water and 2 mL gelatin agarose buffer (note 5 mL of water is lost during microwaving to give a final total volume of 20 mL). Boil in a microwave oven and allow cooling to approximately 60°C.

Cut a piece of  $100 \times 180$  mm acetate film (as used for overheads) and mark one end with the date of testing; and if 2 gels are being run, either A or B. Pour the cooled gel as a thin layer,  $90 \times 170$  mm, on the film which is placed on a level piece of polystyrene foam to prevent the gel cooling and setting before it is spread. To prevent run-off, do not spread the gel right up to the edge.

# Loading samples and electrophoresis

Place the gel into the electrophoresis tank and add buffer to the tank and to the upper gel. Arrange the samples in a rack. Usually this is done in numerical order. If a particular comparison is being made of an unusual unknown, then this should be loaded beside relevant controls. Fill wells with stacking buffer diluted 1:4. Load samples into the bottom of the wells using a pipette and a gel loading tip. The volume of sample loaded into each well is based on the estimation of the cell concentration obtained from the broth culture, which is related to the amount of protease produced. These numbers are a guide only. For samples estimated as optimum cell concentration add 15 µL, and for low numbers of cells load 20-40 µL. The U1 controls are loaded into wells 1 and 2, and the S1 controls are loaded into wells 19 and 20. Also load 5 µL of tracking dye into well 1. Electrophoresis is carried out for 5 hours. Electrophoresis is carried out for the first hour at 20 milliamps until the tracking dye enters the separating gel. The current is increased to 30 milliamps and electrophoresis carried out for about 4 hours or until the tracking dye reaches the bottom of the gel. If two gels are run simultaneously then the current is doubled. Warning: High voltages may be generated by the power supply, so take care to switch the power off before touching or disconnecting the leads, or connections, to avoid electric shock. Remove the gel from the tank.

# Development of protease bands

Gently remove the upper glass plate exposing the polyacrylamide gel. Lay the gelatin-agarose gel on top of the polyacrylamide gel and ensure good contact between the gels by removing all bubbles. Place the gels in a moist chamber and incubate at 37°C for 75 minutes. Remove the agarose-gelatin gel and develop the protease bands by placing into heated saturated ammonium sulphate solution. The saturated ammonium sulphate solution is heated to about 60°C in a microwave oven. Bands of hydrolysed gelatin should be seen in about two minutes. Rinse the gel with tap water.

Warning: Hot ammonium sulphate solution emits an irritating vapour.

# Interpretation of Results

As shown in Figure 10, the zymogram patterns usually consist of three main isoenzyme bands; a fast moving band which is common to all types, and a slower moving pair of bands. Compare the position of the band pair of unknowns with those of the controls to determine the result. Generally, this is possible by making a visual comparison, but if there is any doubt, measure the distance between the centre of the lower band of the pair and the centre of the other lower band, and compare that with similar measurements of controls.





Figure 10. Schematic of isoenzyme patterns (not to scale)

Record patterns of unknowns onto the worksheet. Be aware that because the gel has been manipulated, the apparent order of samples may be reversed. Begin reading at the end that starts with the U1 controls.

# In vivo pen test for assessment of virulence potential of strains of D. nodosus<sup>32</sup>

#### Principle of the Test

Expression of the disease is influenced by the characteristics of the strain (production of virulence factors), environment (in particular temperature and moisture) and host immunity. To assess an organism's true virulence potential under conditions where the environmental factors of temperature and moisture can be controlled, the *in-vivo* pen test can be used. This was used in a national trial to assess diagnostic tests.<sup>32</sup>

#### Reagents and Materials

#### Bacteria

Strains of *D. nodosus* for inoculation are tested for protease stability, zymogram pattern and any additional bacterial marker, such as serotype, that will ensure laboratory differentiation of all strains in the trial. The strains must be ultimately identified by culture collection number and have a history of fewer than four subcultures between isolation and inoculation. The strain number is coded for double blind design.

#### Sheep

A single line of Merino sheep (approximately 23 micron wool, preferably about 18 months old with about 6 months of wool growth) should be obtained from a district where they are unlikely to have been exposed to *D. nodosus* infection. Preferably, sheep are obtained from a flock that has had no introductions or history of benign or virulent footrot for five years. Sheep showing lameness, foot abscess or other injury or inflammation are rejected when allocating sheep to pens.

#### Sheep pens

Each pen is isolated by a barrier to prevent direct contact between sheep in adjoining pens and to prevent water or effluent contact between pens. Pens of approximately  $3 \times 4$  m in size and are situated in an animal house with a concrete floor and weather-proof roof are ideal. Each pen has individual drainage into a secure effluent disposal unit, and a water trough and feed bin. A foot bath containing disinfectant, scrubbing brush or pick is placed at the entry to each pen. Boots and hands are disinfected before entry into and exit from each pen. Use of the animal house is controlled by one person and is locked to prevent unauthorised entry.

Two thirds of the floor in each pen is covered with a deep foam mat. To ensure that the feet of the sheep are maintained in a macerated condition for the duration of the trial this mat is re-charged with water to run-off daily. Each pen is hosed out daily to ensure that there is no excess faecal material in the pen. This enables sheep to rest in the pen without becoming grossly contaminated with dung.

The average temperature of the pen floor should be kept at approximately 19°C, with a mean minimum temperature of 17.5°C and mean maximum temperature of 20.5°C.

# Preparation of sheep and allocation to pens

The sheep are individually identified by numbered ear-tags and randomly allocated to each treatment. Ten sheep are allocated to each pen. There are four pen types according to the treatment:

Pen type	Treatment
Benign control	Sheep infected with strain 305
Virulent control	Sheep infected with strain 198
Negative control	Uninfected
Test	Sheep infected with test strain

Each pen is clearly identified with a code number for each treatment. The negative control ensures that there is no residual infection in the trial sheep. The sheep are typically fed a mixture of hay and milled grain, at a rate calculated to maintain body weight. The feet of the sheep are water macerated by maintaining the sheep on wet mats for seven days before inoculation.

# Test procedure

#### Sheep inoculation

Ten plates of each isolate to be inoculated are grown in pure culture for four days on hoof agar (TASH-IM). The left front and left rear feet of each sheep are inoculated. This involves scarifying the interdigital skin with a blunt scalpel blade, without drawing blood, after the sheep have been on the wet mats for seven days. The agar containing colonies of *D. nodosus* is cut into a plug, representing half a plate of growth. The plug is placed onto cotton wool and applied, plug down, to the scarified interdigital area of the foot. The cotton wool-plug is held in place with 50 mm-wide non-stretch gauze bandage. The bandages and cotton wool plugs are removed seven days after inoculation.

The negative control sheep are treated in the same way as infected sheep, including water maceration, interdigital scarification and bandaging, but do not have *D. nodosus* culture applied to the foot.

The trial is conducted blind, such that inspections and recordings are carried out by observers who do not know the identity of the *D. nodosus* strain in each pen.

# Data recording

The feet of all sheep are inspected every seven days. At each inspection, lesion severity of every foot is recorded according to the standard 0-5 rating system with score 3 lesions being divided into 3a, 3b and 3c categories (System 2, Table 1).<sup>32</sup> Paring of under-run lesions is done only on the day of termination of the trial for each pen to confirm prior lesion estimations. Changes to lesion score after paring are individually recorded.

In each pen, samples of interdigital skin scrapings are taken from two sheep with the most severe lesions when bandages are removed, and then each fortnight after inoculation. Cultures of *D. nodosus* from these samples are tested for appropriate markers to check for cross contamination between pens.

Minimum and maximum air and floor (beneath the mat) temperatures are recorded daily. Subjective evaluations of abnormal lesions, or events that may affect the result, are recorded at each inspection.

#### **Termination**

Each pen of sheep is terminated from the trial 42 days after inoculation, or when at least five of the sheep in a pen have score 4-5 lesions. The sheep are disposed of directly to an abattoir.

### Interpretation of results

Tabulate the individual foot-scores of each sheep at each inspection. Calculate total weighted foot scores (score 1 and 2 lesions are recorded as 1 and 2; score 3a, 3b and 3c lesions are recorded as 9; score 4 and 5 lesions are recorded as 16. The sum of the highest score for each foot is calculated and the result is referred to as the total weighted footscore (TWFS). The scores of each sheep at each inspection range from 0 to 64.<sup>73</sup> TWFS correlates the severity of the lesions with the number of lesions (infected feet) per sheep and provides a more accurate indication of the severity of footrot in a flock than the simple, or unweighted, foot-scoring method, or calculating the number or proportion of affected sheep.<sup>73</sup>

# Part 3 Media and Reagents

#### Stuart transport medium

Scrapings or swabs from the interdigital space of the ovine hoof may yield only a small amount of sample that is better preserved in Stuart transport medium, containing an increased concentration of agar to assist in preventing dilution or dissipation of the specimen.<sup>31</sup>

BBL Transport Medium	7.0 g
Agar, Calbiochem	1.5 g
Distilled water	500 mL

Dissolve ingredients by heating in the microwave (for 1 bottle 10 minutes on 'high'). Place in a 54°C water bath for 5 minutes to cool before dispensing into Bijou bottles or 5 mL polypropylene tubes and completely filling the bottles. Cap tightly. Autoclave at 121°C for 15 minutes. Label the bottles with batch number, expiry date and store at room temperature for 12 months for glass bijou bottles and 6 months for plastic tubes. Discard any bottles that turn blue.

An alternative method is as follows.

Stuart transport medium (modified)	
(Oxoid CM0111, Thermo Scientific)	16.0 g
L-cysteine (Sigma 8755)	1.0 g
6M NaOH	3.0 mL
Purified (reverse osmosis (RO) or de-ionized (DI)) water	to 1 L

Dissolve Stuart transport medium (modified) in 500 mL water. Weigh L-cysteine and dissolve in 6M NaOH. Add L-cysteine to Stuart transport medium, then add water to 1000 mL. Adjust pH to 7.4 with HCl. Boil until ingredients are dissolved and medium is clear. Dispense into glass Bijou bottles with metal screw-top lids or 5 mL polypropylene 5 mL tubes completely filling the bottles. Autoclave at 121°C for 15 minutes with lids slightly loose. Tighten lids soon after removal from the autoclave. This makes approximately 122 bottles. Store at 4-8°C. Discard after 6 months. Do not use if any blue colour is evident as this indicates oxidation of the medium.

Stuarts transport medium modified (Oxoid CM0111) contains 10.0 g/L sodium glycerophosphate, 0.5 g/L sodium thioglycollate, 0.5g/L cysteine hydrochloride, 0.1 g/L calcium chloride, 5 g/L agar and 0.001 g/L methylene blue.

#### Agar media for cultivation of D. nodosus

A number of media (TAS agar and modifications, hoof agar and Eugon agar) for *D. nodosus* culture have been formulated for use in different laboratories in Australia and are detailed here. *D. nodosus* has specific growth requirements for proteose-peptone, yeast extract and the essential compounds of trypticase, arginine and serine (TAS) to promote growth.<sup>78</sup> Powdered ovine hoof aids growth<sup>79</sup> but can be substituted with trypsin-digested casein (trypticase, 1.5%) but not with acid-hydrolysed casein (Casamino acids, 0.5% w/v) which is less effective.<sup>78</sup> The addition of magnesium (as either sulphate or chloride 0.2 mg/mL) improves growth in liquid cultures. The use of 4% agar aids in the isolation of *D. nodosus* as the bacterium can outgrow other swarming bacteria whose swarming ability is inhibited by the

use of 4% agar. Therefore, addition of 4% agar is recommended for the isolation medium and 2% agar for the maintenance medium.  $^{31,78}$ 

Different brands of bacteriological agar vary in their suitability for growth of *D. nodosus*. The following brands have been found suitable: Difco Bacto agar (currently available as Bacto agar, Becton Dickinson (BD) Cat# 214010), Marcor bacteriological grade agar (Product no. 1031, Bleakley Fine Chemicals), Serva Kobe 1 research grade agar (Cat # 11395, Gallay Scientific), Gibco bacteriological agar (Cat # 152-00001 M).<sup>35</sup> Laboratories are advised to validate each batch of agar. Some agars have calcium and magnesium ions in different proportions ranging from nil to <3,000 units and although differences in growth according to agars were reported<sup>35</sup> it is not known whether the range of magnesium and calcium ions in different brands of agar is responsible for batch to batch variation. Technical agar no. 3 (Oxoid, Thermo Scientific code LP0013) has a high mineral content.

A selective medium containing lincomycin has been described<sup>80</sup> but has not been tested on Australian isolates.

#### Preparation of ground sheep hoof horn

Some footrot media (TAS Hoof agar and modifications and Hoof agar) use ground sheep hoof horn, which is prepared according to Thomas (1958).<sup>79</sup>

Collect ovine feet from an abattoir (they can be stored frozen) and wash to remove excessive dirt. Place the feet in a steel tub and cover with water. Autoclave at 121°C for 15 minutes, to soften the hoof horn for easy removal from the foot. If necessary, rewash the horn and, while still soft, cut into 5-10 mm strips using hoof paring shears. Dry the strips of hoof in a 60°C oven, a 37°C drying cabinet or in the sun, for 3-5 days until hard and brittle. Grind to powder using a hammer mill with a 2-3 mm screen (medium then fine screen), with appropriate personal protective equipment (face mask, protective glasses). Store the powder indefinitely in large, dry, screw-capped, plastic jars. An amount of 88 g of ground hoof makes approximately 50-55 L hoof agar. The method of grinding the hoof is important, as other methods such as acid or alkaline hydrolysis for preparing the hoof horn do not provide optimal growth.<sup>79</sup>

# **TAS Hoof agar (TASH) plates**<sup>30,31</sup>

TAS agars, originally described by Skerman<sup>78</sup> as both an isolation medium (IM) and a maintenance medium (MM), have been modified in subsequent studies<sup>11,30,31</sup> by:

- the addition of ground sheep hoof (3% and 1.5% in IM and MM respectively)
- a final agar concentration of 4% in the isolation medium (IM), which aids in reducing overgrowth of swarming bacteria, compared with 2% in the MM.
- the replacement of washed packed bovine red blood cells (5.0 mL/L) in IM (to increase opaqueness and improve visible detection of *D. nodosus* colonies) with powdered haemoglobin.

# **TAS agar (Skerman, 1975)**<sup>78</sup>

	( <b>IM</b> )	( <b>MM</b> )
Trypticase (trypsin-digested casein)	15.0 g/L	15.0 g/L
Lab Lemco powder	5.0 g/L	5.0 g/L
Proteose-peptone	5.0 g/L	5.0 g/L
Yeast extract	2.0 g/L	2.0 g/L
L-arginine-HCl	5.0 g/L	5.0 g/L
DL-serine	1.5 g/L	1.5 g/L
MgSO <sub>4</sub> .7H <sub>2</sub> O	2.0 g	2.0 g
Washed, packed bovine red blood cells (RBCs) <sup>a</sup>	0	5.0 ml/L
Agar (Difco-Bacto, Oxoid) <sup>b</sup>	40.0 g/L	20.0 g/L
Powdered hoof horn (optional)	2.0 g/L	2.0 g/L
Purified (RO or DI) water to	1 L	1 L
pH before autoclaving	7.8	8.0

<sup>a</sup> The original medium included RBCs as an aid to visualising the colonies, but the blood can be omitted or replaced with haemoglobin powder.

<sup>b</sup> The original medium used agar at 15.0 g/L and 22.0 g/L for IM and MM, respectively. However, the use of 4% agar in the IM aids in reducing overgrowth of swarming bacteria.

Mix ingredients in a Schott bottle and add water. Autoclave for 15 minutes at 121°C. Cool to 50°C and pour into 55 mm Petri dishes. Dry plates at 37°C for 15 minutes and store under anaerobic conditions. Excessive drying of plates may lead to oxidative changes that result in restricted growth of colonies. Similarly, incubation in jars containing used catalysts whose effectiveness has declined, may lead to restricted or aberrant growth.<sup>78</sup>

# TAS base for TAS Hoof (TASH) agar plates<sup>30,31</sup>

TAS base (500 g) is prepared:

Trypticase peptone (trypsin-digested casein, B D 211921)	225.0 g
Lab Lemco powder (Oxoid, L29)	75.0 g
Proteose-peptone (BD 211684)	75.0 g
Yeast extract (BD 212750)	30.0 g
L-arginine (Sigma A-5131)	75.0 g
DL-serine (Sigma S-4375)	22.0 g

Grind L-arginine and DL-serine together in a mortar and pestle before adding to the other ingredients. The final materials are mixed with a mechanical mixer or shaker for 3-4 hours. BD=Becton Dickinson.

# TASH-IM and TASH-MM agar<sup>29,30,31</sup>

	( <b>IM</b> )	( <b>MM</b> )
Agar (BD 214010)	24 g	9 g
Ground sheep hoof horn	9.0 g	4.5
TAS base	20.25 g	20.25 g
Haemoglobin powder (Oxoid L53)	1.5 g	1.5 g
HEPES acid <sup>a</sup>	1.8 g	1.8 g
Purified (RO or DI) water to	600 mL	600 mL
pH before autoclaving 7.6		

<sup>a</sup> HEPES is 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; store in a desiccator.

Add dry ingredients to a 1 L Schott bottle containing a magnetic stir bar. Shake to mix ingredients. Add the water and adjust to pH 7.6 using approximately 1.0 mL of 10 M NaOH. Microwave the mixture until the agar is dissolved (for 4 bottles 15 minutes on 'high'). Autoclave for 15 minutes at 121°C. Cool to 50°C in a water bath. Mix the medium with a magnetic stirrer and, working in a laminar flow cabinet, pour the agar into 55 mm Petri dishes to a depth of about 3 mm (½ the depth of the dish). Leave the lids off and cool the plates for approximately 15 minutes to avoid excess condensation when replacing the lid.

Label the base of the plates with the batch number and expiry date. Place the plates lid-down in an anaerobic jar. Suitable preserve jars such as Le Parfait jars may be used. Gas the jar with nitrogen for 20 minutes using chromatography tubing for gas transport. Add an anaerobic gas generating sachet and seal the lid of the anaerobic box or jar. Label with the type of media and the preparation date and store at room temperature for a maximum of 4 months. Storage under an anaerobic atmosphere reduces the absorption of oxygen into the media.

Excessive drying of plates may lead to oxidative changes that result in restricted growth of colonies. Similarly, incubation in jars containing used catalysts whose effectiveness has declined, may lead to restricted or aberrant growth.<sup>78</sup>

# **Hoof agar plates**<sup>35,79</sup>

Protease peptone (BD 211684)	5.0g
NaCl	2.5g
Lab Lemco (Oxoid L29)	2.5g
Yeast extract (BD 212750)	0.5g
Ground sheep hoof horn	7.5g
Bacto Agar (Becton Dickinson 214010)	20.0g
Purified (RO or DI) water	to 500 mL

Dissolve peptone, NaCl, Lab Lemco and yeast extract in 500 mL water on a stirrer. Adjust pH to 7.8-8.0 using 10M NaOH. Weigh agar and hoof powder and add to dissolved ingredients. Steam medium at 100°C for 15 mins. Autoclave for 15 minutes at 121°C.

As this medium contains 4% agar it must be poured hot (approximately 80°C). Work over a flame while pouring the medium into sterile Petri dishes. Keep the hoof particles in a uniform suspension by gently stirring the flask. Allow medium to set on the bench 2-3 hours before stacking. Shake plates to remove any condensation, label and leave to dry overnight. Before packaging, check plates for condensation and if present dry at 56°C for 8 minutes. Place into plastic bag and seal. Store refrigerated. If possible plates should be stored under anaerobic conditions. Re-dry plates briefly before use.

# **Blood Eugonagar**<sup>35,80</sup>

Eugon agar <sup>a</sup> (BBL or Becton-Dickinson)	45.4 g/L
Yeast extract (Difco, Oxoid or BBL)	2.0 g/L
Bovine blood (10% v/v)	100 mL
Purified (RO) water	to 1 L

Dissolve yeast extract in distilled water and adjust pH to 8.0. Add yeast extract solution to Eugon agar (use a 3 L flask or Schott bottle). Autoclave for 15 minutes at 121°C. Cool to 50°C and add bovine blood. Dispense aseptically into Petri dishes (16-20 mL per plate). Dry

plates overnight at room temperature. Store at 4°C for up to 4 weeks. Re-dry before use to remove surface moisture but do not over-dry plates.

<sup>a</sup>Eugonagar (BBL) contains trypticase peptone (pancreatic digest of casein) 15 g, phytone (papaic digest of soybean meal) 5 g, NaCl 4 g, sodium sulphite 0.2 g, L-cysteine 0.3 g, dextrose 5.5 g, agar 15.0 g.

# **TAS Broth (Skerman, 1975)**<sup>78</sup>

Trypticase (trypsin-digested casein, BBL 4311921)	15.0 g/L
Lab Lemco powder (Oxoid L29)	5.0 g/L
Proteose-peptone (Difco, Oxoid L46 or BD 211684)	5.0 g/L
Yeast extract (Difco, Oxoid L21 or BBL BD 212750)	2.0 g/L
L-arginine-HCl (Sigma A6969)	5.0 g/L
DL-serine (Sigma A 4375)	1.5 g/L
Magnesium sulphate (MgSO <sub>4</sub> .7H <sub>2</sub> O)	2.0 g/L
Thioglycollic acid 95% v/v (optional)	0.63 mL
Na <sub>2</sub> CO <sub>3</sub> 20% w/v (optional)	10 mL
Purified (RO or DI) water	to 1 L
pH before autoclaving using 10M NaOH	7.8

TAS broth can be used in the gelatin gel test. The media is dispensed into 5 mL volumes in glass of clear plastic tubes or bottles (100 mm, 14 mm optical density, 1 mm thick wall) and autoclaved at  $121^{\circ}$ C for 15 minutes.

There are a number of ways to maintain anaerobic conditions in the broth and prevent the absorption of oxygen into the medium. The original medium of Skerman  $(1975)^{78}$  included the reducing agent thioglycollic acid (which reduces the oxygen to improve the growth of anaerobes). After autoclaving, the solution is dispensed into bottles, sealed and then injected with sterile Na<sub>2</sub>CO<sub>3</sub>. If thioglycollate and injection with Na<sub>2</sub>CO<sub>3</sub> are not used then the lids are tightened immediately on removal from the autoclave to create a partial vacuum and stored under anaerobic conditions. Alternatively, the medium is dispensed under carbon dioxide or nitrogen before capping and storing. For media not stored under anaerobic conditions, this must be treated to remove dissolved oxygen by boiling or steaming the medium for 10 minutes with loose lids, then the lids are tightened immediately and allowed to cool before use.

# **Modified Eugon broth**<sup>35</sup>

Eugon broth <sup>a</sup> (BBL)	30.0 g
L-Lysine (BDH)	2.5 g
L-Arginine (BDH)	2.5 g
Trypsin 1:230 (Difco)	10.0 g
Yeast Extract (Difco, Oxoid L21 or BBL)	10.0 g
Purified (RO or DI) water	to 1 L

Add reagents to purified water and make up to 1 L. Autoclave the media for 15 minutes at 121°C. Filter while hot through cotton wool and then through a D1 pad using a Buchner funnel. Make up the volume to 1 L with distilled water and adjust the pH to 7.8-8.0 using 10 ml/L sodium hydroxide. Dispense the clear solution into 200 mL volumes in Schott bottles and autoclave for 15 minutes at 121°C. Tighten the caps when cool and store at 4°C until required. Before use, loosen the caps and boil the medium to drive off dissolved oxygen.

<sup>a</sup>Eugonbroth (BBL) contains trypticase peptone (15.0 g), phytone (5.0 g), NaCl (4.0 g), Na<sub>2</sub>SO<sub>3</sub> (0.2 g), L-cysteine (0.3 g), dextrose (5.5 g).

# Elastin agar (Stewart, 1979)<sup>25</sup>

Trypticase (BBL)	15.0 g
Polypeptone peptone (BBL)	5.0 g
Beef extract (BBL or DIFCO)	5.0 g
Yeast extract (BBL or Difco)	2.0 g
L-arginine HCl	5.0 g
DL serine	1.5 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	2.0 g
Calcium chloride (anhydrous)	1.5 g
Bacto agar (Difco)	15.0 g
Elastin powder (bovine neck ligament, insoluble, Sigma)	3.0 g
Purified (RO or DI) water	to 1 L

Dissolve all reagents except the Bacto agar and the elastin powder in a Schott bottle containing a magnetic stirrer. Adjust pH to 7.8-8.0 by adding 10 M NaOH. Add agar and elastin powder and dissolve while stirring on a magnetic stirrer. Continue mixing for up to 30 minutes until the elastin is well dispersed. Autoclave for 20 minutes at 121°C. Cool to 50°C. Mix the solution thoroughly to evenly disperse the elastin particles. Aseptically pour into sterile Petri dishes using a volume of 21 mL per plate or a depth of 3 mm. Dry plates and store in an anaerobic atmosphere at 4°C.

# Gelatin gel thermostability test media<sup>14,30,31,33</sup>

#### **HEPES TAS Broth**

TAS base (see under Plate Media)	20 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	1.2 g
HEPES sodium salt	7.8 g
CaCl <sub>2</sub> .2H <sub>2</sub> O 10% w/v solution <sup>a</sup>	9 mL
Cysteine HCl	0.3 g
Purified (RO or DI) water	to 600 mL

<sup>a</sup> Add 10 g CaCl<sub>2</sub>.2H<sub>2</sub>O (the contents of 1 vial) to 95 mL of purified water. To prevent weighing errors due to absorption of water from the air, dry the chemical in an oven or freeze dryer and distribute in aliquots (e.g.10 g) in sealed bottles (e.g. Wheaton serum vials with stoppers that can be crimp sealed) or store in a desiccator.

This medium can be used for growth of cultures for the gelatin gel and zymogram tests. Add all ingredients except the cysteine to a 1L Schott bottle, and heat in the microwave oven until boiling (for one bottle, 10 minutes on high). Cool the solution for 5 minutes while bubbling nitrogen through the solution to maintain an anaerobic atmosphere in the bottle. Add the cysteine while maintaining the flow of nitrogen through the broth.

Dispense 8 mL of the solution into a 15 mL serum vial ensuring the nitrogen is also flowing into the vial. Cap immediately with a rubber stopper to prevent air entering the top of the vial. Stack the capped vials into a suitable autoclaving rack fitted with a lid to keep the stoppers in place while autoclaving. Autoclave for 15 minutes at 121°C, and allow to cool before removing from the autoclaving rack. Store broths at room temperature for a maximum of 4 months in dark boxes labelled with batch number and expiry date.

# **HEPES dilution buffer**

HEPES free acid (Calbiochem 391338)	1.325 g
HEPES sodium salt (Calbiochem 391333)	24.5825 g
$4M CaCl_2.2H_2O^a$	10 mL
Purified (RO or DI) water	to 1L

Dissolve each salt individually in part of the warmed purified water (approximately  $45^{\circ}$ C) and make to the required volume in a volumetric flask. Normally this buffer requires no adjustment for pH and will give a pH of 8.5 (according to the amounts of acid and salt as calculated by the Henderson-Hasselbalch equation). If necessary, adjust pH with conc. HCl or 10M NaOH to pH 8.5 at 40°C. Store for 4-6 months at room temperature or for 12 months at 4°C. Caution: Acrylamide monomer is a neurotoxin. Wear gloves when handling, and perform all procedures in a fume hood. Ensure the exhaust from the vacuum pump is discharged into the fume hood.

<sup>a</sup>4M CaCl<sub>2</sub> = 5.88 g to 100 ml distilled or RO water.

HEPES is 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, a zwitterion organic chemical buffering agent. HEPES acid and HEPES sodium salt are stored in a desiccator.

#### Agarose-gelatin gel for glass plate or Petri dish

Agarose (Sigma A4679 or A2929)	0.4 g	2 g
Gelatin (Sigma G-2500)	0.4 g	1.5 g
Tris buffer (agarose-gelatin buffer)	4 mL	20 mL
Purified (RO or DI) water	to 45 mL	to 180 mL

The volumes are 45 mL for one 200×200 glass plate or 180 mL for eight Petri dishes.

In a conical flask or Schott bottle mix the agarose, gelatin and Tris buffer in the water to make to the appropriate final volume (45 or 180 mL). Boil in a microwave oven until the gelatin is dissolved completely (boil until 'froth' disappears; after boiling the final volume will be about 40 mL). Alternatively, free steam in an autoclave for 15 minutes, mix and resteam for a further 10 minutes.

Sigma A4679 agarose is recommended for immunoelectrophoresis and immunodiffusion applications as it has a large pore size to facilitate rapid diffusion. Sigma A2929 agarose is recommended for electrophoresis of large molecules of DNA such as in pulsed field gel electrophoresis.

# Preparation of glass plates

On a 200×200 mm glass plate rule a 20 mm margin down each side and along the top and bottom using a chinagraph wax pencil or a piece of candle wax.

Warm the glass plate over a Bunsen burner flame and place on a level surface. Pour the gelatin-agarose gel mixture onto the glass plate and spread the gel to the ruled margins. When set, punch 4 mm diameter holes into the gel in a grid pattern of 42 wells 20 mm apart (centre to centre of each well) and 7 rows of 6 columns. A Perkin-Elmer vacuum pump fitted with a 4 mm punch is suitable for making the wells. Dry the gel for 1-2 hours at room temperature

Growth of protease-producing contaminant bacteria may occur on the gel during incubation if ingredients become contaminated. If this occurs prepare new ingredients or add 0.5 mL of a 1.6% sodium azide solution to the heated gel before pouring to prevent contamination. Beware, as sodium azide is toxic and should only be used as a last resort.

# Preparation of Petri dishes

Gently mix and pour 25 mL gel into  $150 \times 20$  mm plastic Petri dishes (Sarstedt No. 821184). Set for 30 minutes, then dry at 37°C for 20 minutes with lids off. When gels are cooled, punch  $9 \times 4$  mm wells (evenly spaced in vertically-aligned groups of three across the plate) using a template and hole puncher under vacuum.

# Tris buffer (for agarose gelatin gel using glass plate)

Tris base	4.43 g
Tris HCl	0.54 g
CaCl <sub>2</sub> .2H <sub>2</sub> O 10% solution in water	3 mL
Purified (RO or DI) water	to 200 mL

Dissolve reagents in water and make up to final volume. This formula is set for pH 8.8 at 37°C and no adjustment of pH is necessary. Alternatively, Tris HCl may be omitted and replaced by 4.84 g Tris base adjusted to pH 8.8 with HCl. This buffer consists of 0.2 M Tris, 0.01 M CaCl<sub>2</sub>, pH 8.8 at 37°C.

To prepare a 10%  $CaCl_2.2H_2O$  solution, add 10 g  $CaCl_2.2H_2O$  to 95 ml purified water. Note  $CaCl_2.2H_2O$  is hydroscopic.

Store at room temperature for a maximum of four months.

# Tris buffer (for agarose gelatin gel using Petri dishes)

Trizma base (Sigma T1503)	24.22 g
4M CaCl <sub>2</sub> .2H <sub>2</sub> O solution <sup>a</sup>	10 mL
Purified (RO or DI) water	to 1 L

(<sup>a</sup> 5.88 g CaCl<sub>2</sub>.2H<sub>2</sub>O in 10 mL RO water)

Dissolve each salt individually in warmed (45°C) volume of purified water and combine to make up to 1L. At 37°C adjust pH with HCl to 8.8. Autoclave 15 minutes at 121°C.

Note: The pH values of all buffers are temperature- and concentration-dependent. Trizma base can replace Tris-HCl.

# Zymogram Test Media and Reagents<sup>13,31,33,84</sup>

Note that chemicals (especially acrylamide and bis-acrylamide) used in the zymogram test procedures are toxic; wear gloves to avoid skin contact and work in a fume hood to avoid breathing their vapour.

# Sodium azide (1.6%w/v)

Add 1.6 g sodium azide to 100 mL of pure water. Store at room temperature for 12 months.

# Saturated ammonium sulphate

A saturated solution is prepared in 500 mL Schott bottles. The solution is 'topped up' with distilled water as required. Ensure a layer of undissolved ammonium sulphate is present on the bottom of the bottle by adding more chemical as required. Store at room temperature indefinitely.

# **Electrophoresis lower tank buffer**

(63mM Tris, 0.05 HCl, pH 7.47)

Tris base	30.2 g
De-ionised water	to 1 L

The formulation results in a buffer of the correct pH. If necessary, adjust pH to approximately 7.7 with HCl. Make up to 4 L in the lower tank with de-ionised water, and make final pH adjustment to pH 7.47). Replace buffer after 3 months, or when run time exceeds 4 hours.

#### Electrophoresis upper tank buffer stock solution (10× stock)

(37.6 mM Tris, 40mM glycine, pH 8.89)

Tris	45.6 g
Glycine	30 g
De-ionised water	to 1 L

The formulation results in a buffer of the correct pH. Dilute the stock solution 1:10 (70 mL stock to 630 mL purified water) for use in the test. Store at room temperature for 3 months.

#### Electrophoresis upper tank buffer (1× for immediate use)

Tris	3.2 g
Glycine	2.1 g
De-ionised water	to 700 mL

The formulation results in a buffer of the correct pH.

#### **Electrophoresis separating buffer (4× concentration)**

(947 mM Tris, 0.289 N HCl, pH 8.48)

Tris base	22.94 g
De-ionised water	to 200 mL

Dissolve Tris base in 150 mL purified water and adjust to pH 8.48 with dilute HCl. Make up to 200 mL with de-ionised water. Store refrigerated for 3 months.

#### **Electrophoresis stacking gel buffer (4× concentration)**

(158 mM, Tris, 0.256 N H<sub>3</sub>PO<sub>4</sub>, pH 6.9)

Tris base	3.84 g
De-ionised water	to 200 mL

Dissolve Tris in 150 mL purified water, and adjust to pH 6.9 with phosphoric acid. Make up to 200 mL with purified water. Store for 3 months at 4°C.

# Electrophoresis monomer solution for separation gel

(40% T, 5% C bis)

40% acrylamide/bis-acrylamide solution 19:1 is available as a ready-prepared solution (BioRad 161-0144 or Sigma A9926). Alternatively it can be prepared as follows:

Acrylamide	38.0 g
Bis-acrylamide	2.0 g
Purified water	to 100 mL

Dissolve the acrylamide in purified water. Add the bis-acrylamide and stir until dissolved. Store for 12 months at 4°C.

Warning:- Acrylamide and bis are toxic – wear gloves to avoid skin contact and work in a fume cupboard to avoid breathing vapour.

#### **Electrophoresis monomer solution for stacking gel**

Separating gel monomer	20 mL
2% bis-acrylamide (BioRad 161-0142) (or 0.4 g Bis)	20 mL
De-ionised water	to 120 mL

Add gel monomer and bis-acrylamide and make up to 120 mL with distilled water. Store for 12 months at 4°C.

# **Tracking dye**

(5% sucrose, 0.1% Bromophenol blue)

Sucrose	5 g
Bromophenol blue (0.1% solution)	1 mL
Purified water	to 10 mL
Divide into 0.25 mL aliquots and store frozen indefinitely.	

vide into 0.25 mL aliquots and store frozen indefinitely

#### TEMED

TEMED is Tetramethylethylenediamine (BioRad 161-0801). No preparation is required. Expiry is two years after opening.

#### **Ammonium persulphate**

Aliquot into 20 g amounts in containers. Storage life is two years.

For use weigh 0.1 g into a 5 mL plastic tube. Add 1 mL of de-aerated purified water. Use within one week.

# Media for preservation of D. nodosus

#### **Inositol horse serum for freeze-drying**

Inositol	5.0 g
Horse serum	100 mL

Dissolve inositol in horse serum. Filter through a 0.45 µm filter followed by further filtration through a 0.22 µm filter for sterilisation. Check sterility by culture onto blood agar. Dispense into 5 mL sterile bottles and store at 4°C.

#### Glycerol Lab Lemco broth for storage of cultures at -80°C

Lab Lemco broth (Oxoid)	0.64 g
Glycerol	20 mL
Purified (RO or DI) water	80 mL

Add all reagents together. Pipetting of glycerol may be facilitated by pre-warming. Dispense into 2 mL volumes into 5 mL sterile bottles. Autoclave at 121°C for 15 minutes. Store at 4°C.

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