AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

ANIMAL HEALTH COMMITTEE

SUB-COMMITTEE ON ANIMAL HEALTH LABORATORY STANDARDS

Ovine Footrot

Clinical Diagnosis and Bacteriology

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1. Introduction

Footrot is an important disease affecting sheep in temperate climates. It is transmitted by *Dichelobacter nodosus* (Dewhirst *et al.*, 1990) formerly *Bacteroides nodosus*, a Gram-negative obligate, anaerobic bacillus with knob-like ends. Footrot results in a debilitating lameness with marked loss of productivity and reduced market value. The disease is a chronic bacterial infection causing inflammation of the epidermal tissues of the hoof with underrunning of the horn progressing from an initial interdigital dermatitis. The lesions of footrot result from a combined invasion by *D. nodosus* and a gastrointestinal inhabitant, *Fusobacterium necrophorum*. Although other environmental bacteria, including *Spirochaeta penortha* and a motile fusiform subsequently invade the primary lesions, *D. nodosus* is the essential transmitting agent of footrot. In its absence lesions of footrot do not develop. *D. nodosus* lives only in diseased hooves and survives for no longer than 7–14 days in faeces, soil or pasture. Healthy sheep can acquire *D. nodosus* either from animals showing clinical signs of footrot or from animals prior to the onset of clinical signs (L.J. Depiazzi pers. comm. 1991). All strains of *D. nodosus* can cause disease in sheep and goats. Lesions differ in severity according to the virulence of the strain of *D. nodosus* involved. Benign (thermolabile protease) strains commonly cause lesions in cattle and deer. The role of cattle as an important reservoir for transmitting the more virulent forms of footrot to sheep is currently being further investigated since thermostable protease strains of *D. nodosus* have been isolated from cattle (Stewart *et al.*, 1982, 1984, 1986; R.K. Mitchell, pers. comm. 1991; R.I. Walker and I.J. Links, pers. comm. 1991) (see also 3.).

2. Predisposing Causes

Normal healthy skin is not susceptible to infection with *D. nodosus*. Invasion by this organism requires some prior damage to the interdigital skin. Water maceration resulting from sheep running continuously under wet conditions enables *F. necrophorum* to invade the interdigital skin. This invasion causes sufficient tissue damage to permit infection by *D. nodosus*. If *D. nodosus* is not present either in carrier sheep or other animals grazing with the flock, the superficial dermatitis caused by *F. necrophorum* resolves spontaneously.

Outbreaks of footrot are usually confined to regions that have a sufficient annual rainfall (usually above 500 mm) to produce a lush sward of pasture during the warmer, more humid months. Under Australian conditions, spread of footrot in the temperate and mediterranean climatic zones is seasonal with outbreaks occurring in the late autumn (April–May) particularly when good rains have fallen but more commonly in late spring (Oct.–Nov.) when 70% or more of an unvaccinated flock will develop footrot in an outbreak if no prophylatic precautions are taken. In general at least three months of rainfall averaging 50 mm per month in the cooler part of the year is required for the spread of the disease. Prolonged rainfall promotes a lush growth of clover rich pasture and keeps the soil wet for long periods. Transmission of footrot ceases as the environment dries out with the consequent dehydration of the interdigital skin even though there may be sufficient moisture for continued plant growth.

Transmission of footrot occurs only when the mean ambient temperature rises above 10°C. In winter if the temperature is consistently below this level, disease outbreaks are not observed to any great extent because the low temperature inhibits transmission. However, established infection can persist through both summer and winter. In endemic districts in Australia the period for transmission can be as short as three to four weeks but is usually about 6–12 weeks if sheep are exposed continuously to wet pasture during warm weather.

The transmission of footrot to susceptible sheep and the development of new cases requires the presence of:

(a) *D. nodosus* from infected sheep;
(b) sufficiently wet conditions to allow invasion of the interdigital skin by *F. necrophorum*; and
(c) mean daily temperatures above 10°C.

3. Clinical Description

There are at least two clinical forms of footrot — virulent and benign (Egerton and Parsonson, 1969).

3.1. Virulent Footrot

The initial lesion is usually a mild inflammation of the interdigital skin which is followed by a break in the skin–horn junction on the axial aspect of one or both digits. Separation of the adjacent soft horn from the underlying epithelium then occurs and within a few days this separation extends to the axial margin of the sole or around the back of the heel. At this stage, the sheep may be lame and the affected foot may be warmer than normal. During the ensuing 5–10 days, separation of the horn may occur across the entire sole to the toe and abaxial wall and the lameness will become more obvious. Eventually the distal lamellar portion of the abaxial wall may be undermined, leaving the stratum corneum attached along the proximal portion of the abaxial wall. Both digits on the one foot are usually involved. In the advanced stages the skin between the digits shows a diffuse superficial erosion. On feet which have been affected for several weeks or months

the hoof becomes long and misshapen. The non-horny portion of the infected sole in advanced cases is covered by soft, necrotic epithelial tissue and usually there are several eroded areas with more or less sharply defined edges. Lesions of virulent footrot have a distinctive fetid or foul-smelling odour and a small amount of white or greyish, soft, moist necrotic exudate can be invariably found between the separated tissue layers.

3.2. Benign Footrot

Benign footrot is also sometimes called non-progressive footrot or scald. The infection does not usually extended beyond the interdigital skin. The interdigital lesions resemble those of virulent footrot although the latter are usually more severe. In a small proportion of affected sheep, separation of the soft horn at the heel and posterior sole occurs. This separation is accompanied by much less necrosis of the underlying soft tissues and there is little accumulation of necrotic exudate under the separated horn and lameness is less severe than with virulent footrot. A further characteristic of benign footrot is that it causes only mild lameness and rapidly regresses after topical treatment or after the advent of dry environmental conditions, whereas virulent footrot causes severe chronic lameness and tends to persist after the onset of dry conditions unless intensive treatment is undertaken.

More recently it has been shown that there are strains of *D. nodosus* intermediate in virulence between those causing benign and virulent footrot (Stewart *et al.*, 1982, 1984) (see 3.4.). Thus footrot is a continuum of clinical conditions from severe disease to inapparent infection and always involves the presence of *D. nodosus*. The disease can be classified as virulent, intermediate or benign, based on the extent, nature and progress of lesions through a flock. However, expression of the disease in the field is a result of a three-way interaction between host, environment and virulence of the pathogen. There is variation in natural resistance to footrot within and between breeds with Merinos being more susceptible to footrot than British breed sheep. Consequently the classification of the disease can be more easily applied to the former. Environmental factors such as temperature, moisture, topography, pasture and soil type also influence the full expression of virulence. Thus clinical evidence of benign footrot can be associated with either benign (thermolabile protease), intermediate or virulent (thermostable protease) strains of *D. nodosus*, whereas clinically virulent footrot can only be related to either intermediate or virulent (thermostable protease) strains of *D. nodosus* (see 6.2.4.1.). An Animal Health Committee (AHC) Working Party on the *Co-ordination of Footrot Control* in Australia has recommended at the Standing Committee on Agriculture (SCA) Meeting No. 149 in 1992 that the cut-off point needs to be standardised between the States. The chosen cut-off point, below which eradication is not economically advisable is between virulent/intermediate footrot and benign footrot. For the purpose of national uniformity in diagnosis, the above Working Party also considered that footrot should be described as either virulent or benign: that strains of intermediate virulence should be regarded as virulent. This, then clearly establishes the necessity to obtain data on virulence of *D. nodosus* from laboratory diagnostic tests prior to providing advice for control of footrot.

3.3. Definition of Scoring System

The Report of the Working Party on Footrot in Sheep and Cattle (as minuted in AHC Meeting No. 38, Item 5.1, 1987) recommended the following scoring system be used for classification of lesions. A library of colour prints is available to assist in standardisation.

3.3.1. Score 1

Slight to moderate inflammation which is confined to the interdigital skin and involves erosion of the epithelium.

3.3.2. Score 2

A necrotising inflammation of the interdigital skin which also involves part or all of the soft horn of the axial wall of the digit.

3.3.3. Score 3

A necrotising inflammation with underrunning of part or all of the soft horn of the heel and sole which does not extend to the abaxial edge of the sole of the hoof. If necessary a more precise subdivision into 3a, 3b, and 3c can be used for research into vaccines and pathogenicity studies:

3a. Separation at the skin–horn junction, with underrunning extending no more than 5 mm;
3b. underrunning no more than halfway across the heel or sole; and
3c. more extensive underrunning of the heel or sole but not extending to the abaxial edge of the sole of the hoof.

3.3.4. Score 4

Underrunning extending to the abaxial edge of the sole of the hoof.

3.3.5. Score 5

Necrotising inflammation of the deeper epidermal layers (laminae) of the abaxial wall with consequent underrunning of the hard horn of the hoof.

3.4. Definition of Virulence Categories of Clinical Footrot

The definitions are taken from the Report of the Working Party on Footrot in Sheep and Cattle (as minuted in AHC Meeting No. 38, Item 5.1, 1987).

3.4.1. Virulent Footrot

(a) A significant percentage of sheep affected.
(b) Rapid spread of the disease during favourable conditions.
(c) Rapid development of lesions during favourable conditions. Within 7–14 days interdigital dermatitis can develop into lesions with severe underrunning with scores of three to five.
(d) Usually both digits and more than one foot affected.
(e) Significant production losses usually occur in the affected animals. Sheep spend long periods lying down and they often separate from the flock.
(f) Severe lameness is a feature. They may kneel when grazing and when driven they drop to the rear of the flock. Although there is some self cure of the lesions as the season dries off, others relentlessly progress or do not regress. A feature of the disease is that pockets of infection develop at the junction of the sole and wall.
(g) The foot lesions are highly susceptible to fly strike which in turn can predispose to body strike.

3.4.2. Intermediate Footrot

(a) Variable percentage of flock affected depending upon climatic conditions.
(b) Only a small percentage of affected sheep may have severe lesions with scores 3 and 4 and very rarely score 5.
(c) Lameness is commensurate with severity of lesion.
(d) 'Self cure' tends to occur as the season dries off although a few severely affected sheep will remain affected.
(e) Effect on production can vary from minimal to moderate according to the severity of the lesions.
(f) Strains of intermediate virulence are commonly found in the footrot control regions where compulsory quarantine and treatment programs are in progress. However, intermediate virulence strains do exist at a lower frequency outside these areas and their main significance is that the clinical disease they produce is more difficult to distinguish from virulent footrot. The clinical differentiation of virulent footrot is less of a problem.

3.4.3. Benign Footrot

(a) Benign footrot is often erroneously called foot scald, the latter being a term which embraces the diseases ovine interdigital dermatitis (OID) and benign footrot.
(b) The main lesion of benign footrot is interdigital dermatitis (as for OID).
(c) There is little or no underrunning. There is often ridging of the horn of the posterior axial wall.
(d) A large proportion of the flock can be affected but only under conditions favourable to spread.
(e) Usually more than one foot affected. Lesions very rarely progress to severe underrunning (score 4) even during favourable conditions.
(g) Lameness is usually not readily apparent except when sheep first rise after resting. The disease disappears as the season dries off or with footbathing.
(h) Production effects are minimal.

4. Clinical Diagnosis

For the field diagnosis of footrot the following procedure is recommended (The Report of the Working Party on Footrot in Sheep and Cattle as minuted in AHC Meeting No. 38, Item 5.1, 1987).

4.1. History

Conditions under which sheep have been run, the origin of the sheep, and contact with other ruminants.

4.2. Examination

Sheep are examined in the paddock, preferably while grazing and undisturbed, to ascertain prevalence of lameness.

At least 10 individual sheep, lame and in poor condition, are selected, placed in a sitting position and all four feet closely examined for interdigital, skin horn, soft horn or hard horn lesions. If suspect lesions are found, paring of the horn may assist. However, to determine the prevalence of the disease in the flock or to confirm a diagnosis of intermediate footrot, a larger number of sheep will need to be examined. The number examined will depend on the expected prevalence [refer to Cannon and Roe (1982)]. If 10% of the flock are expected to have underrunning lesions then for a flock size of 100 sheep, 25 sheep will need to be examined to be 95% certain of including at least one positive. If the expected prevalence is 2% then 78 out of 100 sheep will need to be inspected.

J.R. Egerton (pers. comm. 1993) considers that provided footrot has had an opportunity to express itself fully (i.e. at least 25% of the flock are affected with score 2 lesions or greater), the disease can be classified, on the basis of percentage feet with score 4 lesions, as either virulent footrot (>10%), intermediate footrot (1–10%) or benign footrot (<1%). Tables have now been devised for assisting in diagnosing the different forms of footrot following estimation of the prevalence of score 4 lesions for 20, 50 and 100 randomly selected sheep with lesions of score 2 or greater. The 90% confidence limits of the estimate of score 4 lesions is determined by the number of sheep examined (J.R. Egerton, pers. comm. 1993).

Examination of feet affected with virulent footrot reveals some or all of the following changes. The interdigital skin and skin horn junction may be inflamed showing erythema, erosion, exudative necrosis and hyperkeratosis. There may be underrunning of the soft horn of the heel and sole extending under the hard horn of the toe and abaxial wall. The underlying tissues may be erythematous and covered by a layer of white–grey necrotic exudate. In more chronic cases there may be pockets of this necrotic material and several layers of separated horn.

In benign footrot the lesions are characterised by inflammation virtually confined to the interdigital skin. The varying degrees of inflammation range from hyperkeratosis of the posterior interdigital skin and skin horn junction to more severe lesions of both these sites with erythema and exudative, erosive hyperkeratosis. These changes are usually much milder than in virulent footrot. The horn of the axial wall of the heels can possess a corrugated appearance. Slight separation of the soft horn of the axial wall at the heel is common but only rarely does this separation extend completely across the sole.

With intermediate footrot the lesions more closely resemble those of benign footrot except that there are a small percentage of sheep (up to 10%) with underrunning of the sole of the hoof to a varying extent.

Numbers of affected feet, and sheep are recorded.

4.3. Follow-up Examination

The presence of *D. nodosus* infection in a flock can usually be determined by one visit but differentiation of these infections is impossible when only interdigital lesions are present.

Interdigital lesions may occur alone in the following circumstances:

(a) when benign footrot is present;
(b) when intermediate footrot is present;
(c) when virulent footrot has been recently introduced and underrunning has not developed; and
(d) when virulent footrot lesions have not developed fully because of unfavourable environmental conditions.

A number of marked sheep will need to be re-examined in 10–14 days provided environmental conditions are favourable for the development of lesions.

Alternatively a number of affected sheep can be placed on wet straw or wet foam rubber mats in small pens and re-examined after 10–14 days. All affected sheep in (c) and (d) above should develop extensive underrunning and a proportion of affected sheep in (b) may develop underrunning lesions. Those in category (a) will not. *However, this procedure is unlikely to be successful unless the ambient temperature in the pen or shed is >10°C and the bedding kept moist.*

4.4. General

A flock diagnosis of virulent footrot can usually be made from the history and examination of foot lesions except when either interdigital lesions alone are present or most lesions have healed. Thus diagnosis of virulent footrot is straightforward when there are a number of sheep with extensive underrunning lesions because the nature of the latter is so characteristic.

Flock diagnosis is based on the most severe lesions present. If one sheep has score 5 lesions the provisional diagnosis is virulent footrot. However, the situation can be complicated by the fact that strains of intermediate virulence cause severe underrunning lesions in some sheep. Therefore, diagnosis for assessment of virulence should be based on the clinical examination of the flock and not individual sheep.

5. Differential Diagnosis

A number of other diseases can cause lameness in sheep. Those occurring in Australia include:

(a) Ovine interdigital dermatitis (OID);
(b) Foot abscess;
(c) Toe abscess (lamellar suppuration);
(d) contagious pustular dermatitis (CPD), (contagious ecthyma or scabby mouth);
(e) Shelly toe;
(f) Strawberry footrot;
(g) Post dipping lameness;
(h) Toxic laminitis; and
(i) Parasitic infestations (*Strongyloides* and *Trombicula*).

A review of the differential diagnosis of mixed bacterial infections occurring in sheep's feet (Egerton, 1969; Stewart, 1989) is given in Table 1.

6. Laboratory Diagnosis

Footrot is a notifiable and quarantinable disease in South Australia, Western Australia and parts of New South Wales and Victoria. In the latter two States, Strategic Plans for the regulatory control of footrot have also recently been implemented so that there is now a major national emphasis for substantially reducing the prevalence of virulent footrot. Laboratory confirmation of footrot for regulatory and legal purposes has depended on microscopic examination of Gram-stained smears for detecting the presence of *D. nodosus*. Laboratory tests for determining the virulence of *D. nodosus* following isolation and culture are now being increasingly used in Regional Veterinary Laboratories in footrot endemic States. However, the need for national uniformity in diagnosis requires that these tests be standardised. An AHC Working Party on *Co-ordination of Footrot Control* in Australia has recommended at the SCA meeting No. 149 in 1992 that protease thermostability, as determined by the gelatin gel test, be used as the

Table 1. Differential diagnosis of mixed bacterial infections occurring in sheep's feet under wet conditions[1] (adapted from Egerton 1969 and Stewart 1989)
A. pyogenes = Actinomyces pyogenes; D. nodosus = Dichelobacter nodosus; F. necrophorum = Fusobacterium necrophorum

Disease	*Animals affected*	*Tissue affected*	*Bacterial flora*	*Clinical signs*
Ovine interdigital dermatitis (OID, 'Scald')	All classes	Interdigital skin	*A. pyogenes* *F. necrophorum* and diphtheroids	Transient lameness in a few affected sheep. Disappears when pastures dry out. May be present in all feet.
Benign footrot ('Scald', non progressive footrot)	All classes	Interdigital skin	OID flora, *D. nodosus* of low virulence, spirochaetes and motile fusiform bacteria	Low to high morbidity rate. May be present in all feet. Mild lameness in proportion of affected sheep. Cases resolve without treat ment when pastures dehydrate, but recur in next wet, warm season. Minimal production loss.
Intermediate footrot This clinical entity should be regarded as virulent (SCA, 1992)	All classes	Interdigital skin, matrix of horn	OID flora, *D. nodosus* of intermediate virulence, spirochaetes and motile fusiform bacteria.	Small proportion of flock affected with severe (underrunning, grade 4) lesions. (<10%). May be present in all feet. Lameness commensurate with severity of lesion. Persists in a few sheep in the absence of treatment. Minimal to moderate production loss.
Virulent footrot	All classes	Interdigital skin, matrix of horn	OID flora, virulent *D. nodosus* spirochaetes and motile fusiform bacteria	High proportion of flock affected. May be present in all feet. Causes marked lameness. Persists in the absence of treat ment. Marked production loss in chronically affected sheep
Foot abscess	Rams, ewes in late pregnancy	Subdermal tissues and distal interphal-angeal joint. Sinuses opening at the coronet	*A. pyogenes* and *F. necrophorum*	Low proportion of flock affected (less than 10% usually). Occurs most commonly in hind feet. Causes acute lame ness.Chronic cases characterised by marked swelling and discharging sinuses
Toe 'abscess' (Lamellar suppuration)	All classes	Sensitive laminae of toe region. Sinuses may discharge above coronet	Various bacteria including *F. necrophorum*	Low proportion of flock affected. Front feet most commonly involved. Causes acute lameness.

[1] Growth of aerobic bacteria on the surface of the wet interdigital skin provides suitable conditions for invasion of the epidermis by *F. necrophorum*. This invasion results in OID. In flocks where *D. nodosus* is present invasion of OID by this organism results in benign, intermediate or virulent footrot depending on the virulence of the strain present. Bacterial invasion beyond the epidermis results in foot abscess. Toe abscess does not arise from a primary OID but through a break in the continuity of the horn. Table reproduced with kind permission of J.R. Egerton.

basis for defining the virulence of *D. nodosus* as virulent or benign (Standing Committee on Agriculture, 1992) (see also 3.2. and 6.2.4.1.).

6.1. Smear Examination

Smears should be prepared from the inflamed interdigital skin where footrot is suspected, or from the active sites beneath the underrun sole or necrotic lamellae. Material can be collected with the blunt side of a scalpel blade, a cotton tipped swab, swab stick or a wooden applicator stick (2.5 x 150 mm) and spread thinly on a slide which should be air dried. It is advisable to prepare one slide from each of at least three cases which are then stained by the Gram stain (see 9.1.). Slides should be correctly identified in relation to the property from which they were obtained prior to staining. For confirmation of diagnosis the fluorescent antibody test may be used. Lyophilised FITC sheep IgG anti-*D. nodosus* reagent (from CSL Ltd, see 9.11.) is commercially available as a special product on request.

D. nodosus is identifiable in smears from active cases of footrot as a large Gram-negative rod (0.6–0.8 x 3–10 μm) with swollen ends. There is some variability in length if smears are not taken from an active area. The organism may be sparse or absent in smears from old chronic cases. A characteristic mixed flora is usually present in Gram-stained smears of lesion material. Besides organisms with the cellular morphology of *D. nodosus*, spirochaetes and Gram-negative, fusiform filaments and rods resembling *F. necrophorum* and the motile fusiform, respectively, are usually present. *Examination of smears does not enable the virulent and benign forms of the disease to be distinguished.*

6.2. Bacteriological Examination

Where it is required to determine whether the benign, intermediate or virulent forms of the disease exist in a flock it may be necessary to isolate *D. nodosus* from a number of affected animals and characterise the isolates for colony morphology and proteolytic activity.

6.2.1. Isolation of *Dichelobacter nodosus*

Collect specimens from active sites of the lesions usually under the separated sole or wall of the

hoof by scraping the area with either wooden applicator sticks, disposable scalpel blades or cotton tipped swabs. For drier lesions applicator sticks are best. The use of a disposable scalpel blade may be better for lesions of benign footrot involving only the interdigital skin. For transport to the laboratory, specimens should be deposited into the top 5 mm of Thorley's (1976), modified Stuart or SBL transport media (see 9.2.) protected from light and sent at ambient temperature to reach the laboratory within 48 hours of collection where they are inoculated onto 4% hoof agar (HA) (see 9.3.), 4% trypticase arginine serine (TAS) agar (see 9.3.) or modified TAS agar (see 9.3.) plates as soon as possible.

The transport medium (TM) should not be used for sample collections when the methylene blue indicator turns blue, showing that oxygen is present. Lesion material from a number of feet on the same sheep can be pooled into the same bottle of TM. However, it is preferable that only one sample from one foot per sheep be placed in the one TM with up to 10 samples being obtained for each suspect flock (I.J. Links, pers. comm. 1991).

Good isolation rates are obtained by inoculating well dried 4% HA plates (one plate per specimen) directly in the field and putting them into an anaerobic jar containing catalyst, gas generator pack and indicator. One anaerobic gas generator pack should be used per jar. The anaerobic jars are returned to the laboratory that day or as soon as possible and incubated at 37°C for four to six days. At the laboratory either the gas generator pack system or vented anaerobic jars gassed three times with 90% hydrogen (H_2) and 10% carbon dioxide (CO_2) are used.

Primary plates should be inoculated by one of the methods shown in 9.4. Colonies of *D. nodosus* grow out from the streak lines as translucent, flat granular semi-circular colonies and often exhibit concentric zones with a fimbriate edge (Thorley, 1976).

In Method 1, three or four (or more if colony morphology varies) colonies should be picked off each plate with a sterile straight wire, subcultured onto 1/2 or 1/4 of either a well dried 4% HA (see 9.3.), TAS agar (see 9.3.) or blood Eugonagar (see 9.3.) plate to obtain pure colonies of *D. nodosus* and incubated anaerobically as soon as possible. The subculture plates are examined after a further three or four days of anaerobic incubation. For serogrouping purposes up to 10 colonies per foot should be examined.

HA plates are preferred over TAS agar as they have a long shelf life, they can be stored aerobically and they allow more leeway between inoculation and gassing of the anaerobic jar. HA plates must be well dried before storage at 4°C (in sealed plastic bags) or before use. They should be preferably used within three to four weeks. The advantage of subculturing onto blood Eugonagar from the primary isolation plates is that purification of colonies of *D. nodosus* is facilitated. The blood Eugonagar plates can be stored aerobically at 4°C for up to four weeks.

In Method 2 (RVL, Albany, WA) a stereo microscope (x10) equipped with a light source is used to examine for pure microcolonies or outgrowths of *D. nodosus* on the surface of the modified TAS isolation agar (see 9.3.) plate previously inoculated using a stabbing technique (see 9.4). Once located, a small slab of agar is cut from the isolation agar plate so as to include the colony and transferred colony side down onto the surface of a modified TAS maintenance agar plate (see 9.3.). After anaerobic incubation for two days at 37°C, a slab of agar containing pure growth of *D. nodosus* is added to HEPES TAS broth (see 9.3.) and incubated for a further two days. A useful tool for the above procedures can be made from 0.5 mm diameter nichrome wire, the end of which has been flattened and bent so as to form a small blade or spatula (D.R. Pitman, pers. comm. 1991). The addition of hoof horn particles to modified TAS isolation agar medium (see 9.3.) has improved the success rate for isolation of *D. nodosus*. The use of small (55 mm) instead of conventional Petri dishes for isolation and maintenance agar enables less culture medium to be used and three times as many agar plates can be incubated in the one anaerobic jar. The small plates and inoculation procedure also make it easier to scan for *D. nodosus* colonies under the stereo microscope (D.R. Pitman, pers. comm. 1991).

6.2.2. Colony Morphology

Although not absolute, there is a correlation between colony morphology on low strength (2%) agar and virulence. In general, isolates from virulent footrot produce large fimbriate colonies on this medium whereas those from cattle and benign footrot in sheep tend to produce smaller non-fimbriate (entire edge) colonies. Colony morphology should also be assessed on 4% agar and the colonies typed according to the presence or absence of a fimbriate edge and whether their surface is rough (beaded) or smooth (mucoid). Good descriptions of colonies are provided by Thorley (1976), Skerman *et al.* (1981) and Stewart *et al.* (1986). These are summarised in 9.5.

6.2.3. Surface Translocation

Twitching motility is a virulence factor, mediating progressive invasion of epidermal tissues of the hoof by surface translocation and is correlated with colony size (Depiazzi and Richards, 1984/1985). Using an agar stab inoculation technique virulent strains have a high level of twitching motility and produce large colonies. Benign and intermediate strains have low motility and form small colonies. The combination of protease thermostability tests (see 6.2.4.1.2.) and colony size can assist in differentiating intermediate from

virulent and benign strains (Depiazzi and Richards, 1984/1985). The measurement of colony diameter is not recommended for routine diagnosis because of the necessity for repeated measurements on a number of subcultures but the method is included to assist in gathering further information on intermediate footrot (see 9.6.).

6.2.4. Proteolytic Activity

Three methods are available.

6.2.4.1. Proteolytic enzyme stability

6.2.4.1.1. Degrading proteinase test. The original method for the degrading proteinase (DP) test (Depiazzi and Richards, 1979; Richards *et al.*, 1980) measured the proteolytic activity of broth cultures of *D. nodosus* for some time after organism death to assess the stability of the enzyme(s). In general, isolates of *D. nodosus* capable of producing virulent footrot produce stable enzymes in TAS broth without calcium supplementation whereas those from ovine benign footrot and most cattle isolates are rapidly degraded. The test was rather prolonged since it was performed on broth cultures at intervals over a 12-day incubation. It has now been superseded by the protease thermostability test since there is a good correlation between the activity of heated cultures at day 2–4 of incubation and those for unheated cultures at day 12–13.

6.2.4.1.2. Protease thermostability tests (see 9.7.). These are modified DP tests and compare heated broth cultures with unheated broth cultures after two to four days of culture growth provided a sufficiently high cell density has been achieved. Protease thermostability was originally determined using the hide powder azure method and heating cultures at 55°C for 30 min (see 9.7.1.). More recently, the gelatin gel method with cultures heated at 67–68°C for eight and 16 min (see 9.7.2.) has replaced the former. Isolates capable of producing virulent and intermediate footrot produce thermostable proteases, whereas those from benign strains are thermolabile. A result can be obtained within 8–14 days from time of sampling when the modified tests are used, utilising heating instead of prolonged incubation.

6.2.4.2. Elastin agar test (see 9.8.).

This test examines isolates for elastase production determined by clearing of elastin particles in TAS agar. In general, freshly isolated ovine virulent isolates cause complete digestion of the elastin particles beneath the streak line whereas ovine benign and most cattle isolates do not cause detectable clearing. Elastin digesting activity of freshly isolated virulent strains is usually detectable within four to seven days but may take up to 14 days or longer for cultures of intermediate strains.

Strains of intermediate virulence can vary in their elastolytic activity. Some strains cause slight clearing of elastin particles in the presence of calcium chloride ($CaCl_2$) (Stewart *et al.*, 1982; 1986) whereas with others, the degree of clearing may be reduced and delayed in comparison to the more rapid clearing by virulent strains (Claxton, 1986; Stewart *et al.*, 1986). Further intermediate strains possess no activity in the presence of calcium chloride (Stewart *et al.*, 1986). However, the incorporation of calcium chloride in the elastin agar medium is advantageous enabling some intermediate strains to clear elastin. At least one bovine isolate has been shown to have a thermostable protease causing clearing of elastin particles (Stewart *et al.*, 1982; 1986).

6.2.4.3. Zymogram

The zymogram method (see 9.9.) is a rapid electrophoretic technique which utilises polyacrylamide gel as the supporting medium and a contact print activity assay for resolving extracellular proteases of *D. nodosus* into component isoenzymes according to their electrophoretic mobility. The number and positions of the isoenzymes, the so-called electrophoretic pattern, is characteristic and different for benign and virulent strains.

The method reported by Every (1981, 1982) utilised horizontal slab gel electrophoresis but a simpler more practical technique was developed by Kortt *et al.* (1983). They adopted a vertical polyacrylamide slab gel in a simple non-dissociating (non-denaturing) and non-reducing electrophoresis system. This simple system separates proteins in their native (non-denatured) form according to differences in charge and size. Thus different but related proteins such as isoenzymes of similar molecular weight can be separated due to differences in ionic charge. These proteins still retain their functional activity and consequently they can be detected among a myriad of other proteins by a specific activity assay. The mini-gel vertical slab electrophoresis system allows the analysis to be completed two to three times faster than with conventional vertical slab gels. The system can run two slab gels for analysis of 20 samples at one time, i.e. 10 samples per gel. However, the advantage of the larger gel system is that 40 samples (20 per gel) can be electrophoresed. For a detailed description of the electrophoretic procedures, the Bio-Rad, Hoefer or Pharmacia LKB mini-gel or standard gel vertical electrophoresis instruction manuals should be consulted. Bio-Rad (1984) also provides useful information.

The electrophoretic method consists of a resolving or separating (lower) gel and a stacking gel along with the discontinuous buffer system of Davis (1964) or that of Laemmli (1970) omitting the sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$). The discontinuous buffer system employs different buffer ions in the gel compared to those in the electrode buffer reservoirs. There are discontinuities of both buffer composition and pH. The sample is loaded onto a large pore size stacking

gel polymerised on top of the small-pore resolving gel. The stacking gel acts to concentrate relatively large volumes of dilute protein sample resulting in better band resolution than is possible on gels without a stack.

The protease isoenzyme bands are detected by placing the polyacrylamide gel slab on a layer of gelatin-agar and incubating at 37°C for one hour to allow hydrolysis of the gelatin. The unhydrolysed gelatin is precipitated with acid mercuric chloride ($HgCl_2$/HCl) or hot saturated ammonium sulfate solution [$(NH_4)_2SO_4$]. For safety reasons the latter is preferred (M.A. Palmer, pers. comm. 1991). Thus the typical zymogram shows clear areas corresponding to enzyme activity (which appear black in photographs) against an opaque or milky white background of unhydrolysed gelatin.

Benign and virulent isolates of *D. nodosus* can be distinguished from each other by differences in their protease isoenzyme patterns in the zymogram (Fig. 1). The cultures do not require concentration before electrophoresis. Virulent isolates of *D. nodosus* possess four isoenzyme bands, whereas benign strains have a zymogram pattern with five bands of activity of which the first four bands have lower mobility than protease bands 1, 2 and 3 of virulent strains. Band 5 from benign and virulent strains have similar electrophoretic mobilities. Virulent strains can be divided into types 1 and 2 on the basis of variation in the virulent zymogram pattern. All virulent strains contain bands 1, 2 or 3. Compared with type 1, the type 2 strains lack band 5 but have an additional band (4) migrating between bands 3 and 5 (Fig. 1*a*).

A characteristic feature of the pattern of benign strains is the presence of the two lower mobility bands 1 and 2 which are not present in any of the virulent strains. However, in some benign strains bands 1 and 2 are difficult to detect. A second feature is that bands 3 and 4 of benign strains consistently differ in mobility compared with bands 1, 2 and 3 of virulent strains and this difference can be used for differentiation in the absence of benign bands 1 and 2 (Fig. 1*a*).

Depiazzi *et al.* (1991) has demonstrated one zymogram pattern associated with thermostable proteases and four distinctive patterns with thermolabile proteases (Fig. 1*b*). Each of the patterns was composed of three major isoenzymes, one of which was common to all types. The other two isoenzymes were formed into pairs, consisting of distinct electrophoretic bands separated by a relative mobility of 3–4%. The zymogram band patterns, differentiated on the basis of mobility of paired major isoenzymes, were characteristic of individual isolates. More recently two patterns have been found in isolates with thermostable proteases and seven patterns in isolates with thermolabile proteases. One of the patterns in the latter was also present in one thermostable protease isolate. More than 95% of strains with stable enzymes (subtype S1) and more than 90% of strains with unstable enzymes (subtype U1) have the typical virulent zymogram (Z1) and benign (Z3) pattern, respectively (L.J. Depiazzi and M.A. Palmer, Proceedings of the Footrot Technical Workshop, Regional Veterinary Laboratory, Wagga Wagga, May 1991, to be published).

6.2.4.3.1. Advantages of the zymogram.

(a) Robust and reproducible method.
(b) It is now quick and relatively simple with the availability of the mini-gel system.

6.2.4.3.2. Disadvantages.

(a) The apparatus is relatively expensive.
(b) The zymogram does not discriminate between virulent and intermediate strains.
(c) Fewer samples can be processed.

The zymogram is a useful research tool but it is not recommended as a technique for routine diagnosis.

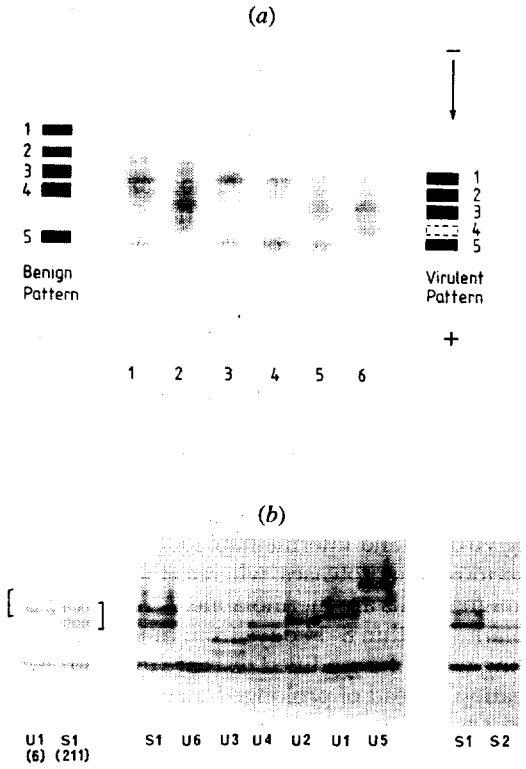

Figure 1. Comparison of the protease isoenzyme patterns in the zymogram of broth cultures of *D. nodosus* benign and virulent isolates. **(a)** The benign strains are (1) 291, (3) 134 and (4) 269; the virulent strains are (2) 240, (5) 238 and (6) 243. Arrow denotes direction of migration. Reproduced with publishers permission from A.A. Kortt, J.E. Burns and D.J. Stewart (1983). *Research in Veterinary Science*, 35, 173. **(b)** Zymogram patterns in isolates with stable proteases (subtypes S1, S2) and isolates with unstable proteases (subtypes U1, U2, U3, U4, U5, U6). Isolates 6 (U1) and 211 (S1) (shown at left) possess the most common zymogram patterns. Brackets indicate pairs of bands used in identification of the type of zymogram pattern. Reproduced with kind permission of M.A. Palmer, RVL, Albany.

6.3. Serology and Serogrouping

The quantative serological investigation of antibody responses of vaccinated sheep, by the K agglutination tube (Egerton, 1973) or microtitre test is of value when the efficacy of *D. nodosus* vaccines is being monitored. Serogrouping by the slide, tube or microtitre agglutination test (see 9.10.) is useful as an epidemiological tool and for investigating the likelihood of new serogroups being responsible for vaccine breakdowns.

The present Australian serological classification scheme for typing *D. nodosus* consists of nine major serogroups A–I. All serogroups except B, D and I can be subdivided into two subgroups on the basis of cross absorption tests. Serogroup B is the most complex containing four subgroups whereas serogroups D and I contain only one subgroup. Strains in different subgroups in the same serogroup possess distinct and shared K antigens (Claxton *et al.*, 1983; Claxton, 1986). Currently, commercial footrot vaccines contain strains representative of the major serogroups of *D. nodosus*. The basis for multistrain footrot vaccines is that the pili are the principal serological antigens mediating the K agglutination reaction and they are also the major immunoprotective antigens eliciting serogroup restricted protection against footrot (Stewart, 1973; Walker *et al.*, 1973; Stewart, 1978).

British workers have extended Claxton's classification scheme to include a further nine distinct serotypes J–R giving a total of 17 serotypes (Day *et al.*, 1986; Thorley and Day, 1986). In Claxton's typing scheme some cross reactions among subgroups within a serogroup were of fairly low titre compared to the homologous titre and it is probable that Day *et al.* (1986) and Thorley and Day (1986) have assigned strains in these subgroups to new serotypes J–R. However, the rationale for restricting the number to nine serogroups, A–I, is that related subgroups (serotypes) with shared pilus antigens will induce cross protection against the other subgroup from the same serogroup, i.e. the pattern of cross protection is reflected in these groupings. More recently one of Claxton's subgroups (serogroup H, subgroup 2) has been assigned to a new serogroup on the basis of lack of either pilus antibody cross reactivity or cross protection with subgroup H1 (Stewart *et al.*, 1991). Furthermore, the pilin gene sequence homology between subgroups H1 and H2 is lower than that for strains within other serogroups (Hoyne *et al.*, 1989). Subgroup H2 corresponds to serotype O in the extended classification scheme of Day *et al.* (1986) and Thorley and Day (1986).

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9. Appendixes

9.1. Appendix 1 — Staining Procedures

9.1.1. Gram Stain — Kopeloff's Modification (after Holdeman *et al.*, 1977)

9.1.1.1. Reagents

9.1.1.1.1. Alkaline crystal violet.

(a) Solution A: Dissolve 10 g crystal violet in 1 L distilled water.

(b) Solution B: Dissolve 50 g sodium bicarbonate ($NaHCO_3$) in 1 L distilled water.

9.1.1.1.2. Iodine. Dissolve 4 g sodium hydroxide (NaOH) in 25 mL distilled water. Add 20 g iodine and 1 g potassium iodide (KI), dissolve well and then gradually add 975 mL distilled water, mixing well with each addition.

9.1.1.1.3. Acetone–alcohol. *N.B.* Other workers have found acetone alone to be satisfactory. Mix 300 mL acetone (C_3H_6O) with 700 mL 95% ethanol (C_2H_5OH).

9.1.1.1.4. Counter stains.

(a) Safranin: To 20 g safranin add sufficient 95% ethanol to dissolve the safranin. Then add 1 L distilled water; or

(b) Carbol fuchsin: (not as satisfactory for *D. nodosus*).

(i) Solution A. Mix and dissolve 10 g basic fuchsin in 100 mL 95% ethanol. Keep in a stoppered bottle overnight at 37°C.

(ii) Solution B. Mix and dissolve 5 g phenol (C_6H_5OH) in 100 mL distilled water.

For use. Pour 10 mL (i) into 100 mL (ii). Dilute one volume of this strong carbol fuchsin with 10–20 volumes distilled water.

9.1.1.2. Method

(a) Air dry smears and *very lightly* heat fix.
(b) Flood with alkaline crystal violet (i) and add about five drops of (ii).
(c) After one minute tip off crystal violet and thoroughly rinse off residual stain with iodine solution.
(d) Cover slide with fresh iodine.
(e) After one minute tip off iodine and gently rinse slide with tap water.
(f) After removal of excess water, decolourise smear by holding slide at a steep angle and pouring acetone–alcohol over it from the upper end so as to cover the whole surface. Continue either until no colour is released or for no more than 15 s.
(g) Rinse thoroughly under running tap water.
(h) Flood slide with safranin or carbol fuchsin and leave for 10 s.
(i) Rinse in tap water, blot and air dry.
(j) Examine under oil immersion.

9.1.2. Fluorescent Antibody

9.1.2.1. Method

Non-fluorescing glass slides, cleaned in concentrated nitric acid (HNO_3) and washed several times in distilled water should be used.

(a) Air dry smears and fix in anhydrous acetone for 10 min.
(b) Allow acetone to evaporate.
(c) Overlay area to be stained with the appropriately diluted conjugate (Manufacturer's recommendation).
(d) Incubate slides for 30 min at room temperature (25°C) in a humid chamber.
(e) Rinse in phosphate buffered saline (PBS, pH 7.4) and wash in a stirred PBS bath for 30 min.
(f) Mount in buffered glycerin (nine parts glycerin and one part PBS pH 7.4).
(g) Examine under UV illumination with appropriate exciter and barrier filters and dark field condenser. Non-fluorescing immersion oil is required between the top lens of the condenser and the lower surface of the slide.
(h) Scan using x10 eyepiece and x10 objective and confirm the morphology of fluorescing organisms using a x40 objective.

All serotypes of *D. nodosus* should fluoresce with the conjugate. Typically, the cell is outlined with more brightly fluorescing 'polar caps' (Roberts and Walker, 1973) giving a 'safety pin' appearance. The conjugated antiserum is specific for *D. nodosus*.

9.2. Appendix 2 — Transport Media

9.2.1. Thorley's TM (Thorley, 1976)

PBS pH 6.8	0.05 mol/L
Cysteine HCl	0.05% w/v
Agar	0.35% w/v

(a) Add the cysteine HCl and agar to the PBS and dissolve by boiling.
(b) Dispense into 5.0 mL screw-capped Bijou bottles which are filled to the brim.
(c) Sterilise by autoclaving for 15 min at 121°C (15 psi) and immediately tighten caps.
(d) If bottles are stored for any length of time it may be necessary to boil before use to remove dissolved oxygen. Most consistent results are obtained by using TM within a few days of preparation.

9.2.2. Modified Stuart Transport Medium [Regional Veterinary Laboratory (RVL) Hamilton, Vic.]

Sodium glycerophosphate, $C_3H_7O_6PNa_2.6H_20$)	10 g
Sodium thioglycollate, $C_2H_3O_2SNa$ (Sodium mercaptoacetate)	1 g
$CaCl_2$	0.1 g
Methylene blue (stock solution)	0.002 g
L-Cysteine HCl (BDH)	0.5 g
L-Cystine (dissolve in 3 mL 6 mol/L NaOH)	1.0 g
Distilled water	1 L
'Ionagar' No. 2	2 g

Dissolve all ingredients except Ionagar No. 2 in 1 L distilled water. Adjust pH to 7.4. Add 'Ionagar' No. 2 and boil until agar is dissolved. Fill Bijou bottles to overflowing. *Use metal caps on all bottles.* Autoclave at 121°C (15 psi) for 15 min. Label and date.

9.2.3. SBL TM [CSIRO Animal Health Research Laboratory (AHRL), Parkville, Vic.]

Sodium glycerophosphate	10 g
Thioglycollic acid, $C_2H_4O_2S$	0.5 mL
$CaCl_2$ (1% aqueous stock solution, 0.09 mol/L)	10 mL
Methylene blue (0.1% aqueous stock solution)	2 mL
Cysteine HCl monohydrate	0.25 g
Distilled water	950 mL
Bacto agar	10 g

Adjust pH to 7.2. Steam and replace air with nitrogen, autoclave for 15 min at 121°C, tighten lids and store at 4°C.

N.B.:

(a) Stuart TM (modified) can be purchased as powder or tablets (Oxoid). The RVL, Albany, WA, use the Becton Dickinson BBL brand of Stuart TM but the agar concentration is increased to 6.0 g/L.
(b) A culture sampling and transport system containing a swab and separate plastic capped tube with Stuart TM (Transwab, TSS001X, Johns Division of Hardie Health Care Products) appear satisfactory (J.A. Vaughan, pers. comm. 1990).
(c) Anaerobic specimen collectors (Vacutainer, Becton Dickinson) work very well but they are relatively expensive for routine diagnostic purposes.

9.3. *Appendix 3 — Culture Media*

9.3.1. Hoof Agar (HA)

9.3.1.1. Preparation of ground ovine hoof horn (after Thomas, 1958)

(a) Sheep feet collected from the abattoir in garbage bins are either washed and soaked in water for two to three days to soften the hoof horn or autoclaved for 15 min at 121°C (15 psi). The hoof horn is then manually removed; or hoof clippings collected following hoof trimming can be used after washing to remove dirt.
(b) Cut into narrow strips 0.5–1 cm wide.
(c) Thoroughly dry at 37°C or in sunlight. This may take three to four days.
(d) Grind in a hammer mill using 2 mm screen.
(e) Store the resulting powder in a dry screw capped jar until required.

9.3.1.2. Preparation of medium

Proteose peptone (Difco or Oxoid L46) or Polypeptone peptone (BBL)	10.0 g
NaCl	5.0 g
Lab Lemco powder (Oxoid L29)	5.0 g
Yeast extract (Difco, BBL or Oxoid L21)	1.0 g
Washed, ground ovine hoof horn	15.0 g
Agar (Difco-Bacto, Marcor, Serva-Kobe 1, or Gibco)	40.0 or 20.0 g
Distilled water to	1 L

Different brands of bacteriological agar incorporated into hoof medium have been found to vary in their suitability for both isolation (4%) and maintenance of growth (2%) of *D. nodosus*. Bacto agar (Difco) has traditionally been used in hoof medium but following its recent unavailability assays were conducted by Veterinary Laboratories in New South Wales to find a substitute. The best results, comparable to Bacto agar were obtained with Marcor bacteriological grade agar (Product No. 1031, Bleakley Fine Chemicals, Silverwater, NSW) (R.J. Whittington, K.C. O'Grady, J.R. Egerton and C.L. Kristo, pers. comm. 1990) and Serva Kobe 1 research grade agar (Cat. No.11395, Gallay Scientific, North Melbourne, Vic.) (F.A. Cockram and L. Whittingham - Pugh, pers. comm. 1990). Gibco bacteriological agar (Cat. No. 152-00001 M) was also satisfactory. (F.A. Cockram and L. Whittingham-Pugh, pers. comm. 1990). Marcor agar in hoof medium has been used for some time by Arthur Webster Pty Ltd for growth of *D. nodosus*. (A. Ray, pers. comm. 1990). These results will require further verification by other laboratories. They do, however, highlight the importance of validating each brand and batch of agar for its ability to support the growth of *D. nodosus*.

(a) Dissolve the peptone, NaCl, meat extract and yeast extract in 1 L distilled water at 56°C in a water bath.
(b) Adjust pH to 7.8–8.0, using 10 mol/L sodium hydroxide.
(c) Weigh agar and hoof powder into conical flasks and add dissolved ingredients. For convenience and ease of dispensing, the medium can be prepared in 500 mL volumes in 2 L flasks or Schott bottles or in 2.8 L Erlenmeyer flasks (Crown Corning) which hold 1 L of medium.
(d) Plug with cotton wool and autoclave for 15 min at 121°C (15 psi). Ensure that *slow* exhaust is used or medium will 'bump' badly and blow plugs.
(e) After cooling to about 80°C (4% agar) or 50°C (2% agar) dispense medium aseptically into sterile petri dishes, taking care to ensure that hoof particles are suspended as uniformly as possible throughout the medium. The temperature at which the 2% HA medium is poured can be controlled by a water bath or 56°C incubator. The 4% HA must be poured hot.
(f) Thoroughly dry the inverted open plates in a 56°C incubator to remove all surface moisture before use or before storage at 4°C in sealed plastic bags. Stored plates should be redried before use.

9.3.2. Trypticase Arginine Serine (TAS) Agar (Skerman, 1975)

Trypticase (BBL)	15.0 g
Lab Lemco powder (Oxoid L29)	5.0 g
Proteose peptone (Difco or Oxoid L26)	5.0 g
Yeast extract (Difco, Oxoid L21, BBL)	2.0 g
L-Arginine HCl	5.0 g
DL-Serine	1.5 g
Magnesium sulfate, $MgSO_4.7H_2O$	2.0 g
Agar (Difco-Bacto, Oxoid)	40.0 or 20.0 g
Distilled water to	1 L

(a) Dissolve all ingredients except agar in glass distilled water and adjust pH to 7.8 with 10 mol/L sodium hydroxide.
(b) Weigh agar into conical flasks and add dissolved ingredients. Autoclave for 15 min at 121°C (15 psi) cool quickly to about 50°C (or 80°C for 4% agar).
(c) Mix and dispense into petri dishes.
(d) Carefully dry plates and store under anaerobic conditions until required. Plates should be dried at 37°C only until surface moisture has been removed. Excessive drying of TAS agar plates at elevated temperatures (56°C) immediately before inoculation leads to oxidative changes that restrict growth in a similar manner to agar cultures incubated in anaerobic jars with inefficient catalyst.

9.3.3. Modified TAS Agar

Trypticase peptone (BBL)	6.0 g
Lab Lemco powder (Oxoid)	2.0 g
Proteose peptone (Difco)	2.0 g
Yeast extract (Difco)	0.8 g
L-Arginine HCl	2.0 g
DL-serine	0.6 g
Distilled water	350.0 mL
10 mol/L NaOH	1.6 mL
Agar (Calbiochem)	16.0 or 6.0 g
Ground ovine hoof horn	3.0 g

9.3.3.1. Haemoglobin solution

HEPES acid	1.2 g
Haemoglobin powder (Oxoid L53)	1.0 g
Water	50.0 mL

Make to a paste in mortar and pestle, centrifuge and filter through Whatman No. 1 paper.

(a) Add the haemoglobin solution to modified TAS agar, autoclave, cool to 60°C, mix thoroughly and the medium is then dispensed into petri dishes.

9.3.4. TAS Broth (Skerman, 1975)

Trypticase	15.0 g
Lab Lemco powder (Oxoid L29)	5.0 g
Proteose peptone (Difco, Oxoid L46)	5.0 g
Yeast extract (Difco, Oxoid L21, BBL)	2.0 g
L-Arginine HCl	5.0 g
DL-serine	1.5 g
$MgSO_4.7H_2O$	2.0 g
Distilled water to	1 L

This medium is the same as for TAS agar except that agar is omitted. After adjusting the pH to 7.8 with 10 mol/L sodium hydroxide, dispense in 5.0 mL amounts into screw capped bottles or either cotton plugged or plastic capped test tubes and autoclave at 121°C for 15 min. After autoclaving, the lids of screw capped bottles are immediately tightened which creates a partial vacuum and facilitates storage. Test tube cultures are stored in an anaerobic jar with a gas generator pack or appropriate gas mixture. If not stored anaerobically the medium may have to be boiled before use to drive off dissolved oxygen. Alternatively the medium can be dissolved and dispensed under carbon dioxide or nitrogen into serum bottles which are then capped and crimped.

9.3.5. HEPES — TAS Broth

This medium is the same as for TAS broth except for the addition of the following per litre.

HEPES sodium salt	13.0 g
$CaCl_2$ (anhydrous)	1.0 g

(a) Dissolve on magnetic stirrer/heater.
(b) Boil for two minutes under oxygen-free carbon dioxide or nitrogen.
(c) Cool to about 80°C under carbon dioxide or nitrogen.
(d) Add 0.5 g L-Cysteine HCl monohydrate.
(e) Dissolve under carbon dioxide or nitrogen.
(f) Dispense 9 mL into 10 mL carbon dioxide or nitrogen-filled serum bottles while the medium is still hot.
(g) Fill headspace with nitrogen.
(h) Cap and crimp.
(i) Sterilise by autoclaving.

Whereas 10 mmol/L calcium chloride is incorporated in HEPES-TAS broth by the RVL, Albany, WA (Depiazzi and Richards, 1984/1985) this ingredient has been excluded from the recipe used by AHRL, CSIRO Division of Animal Health.

9.3.6. Blood Eugonagar (BEA)

Eugonagar (BBL)	45.4 g
Yeast extract (Difco, Oxoid or BBL)	2.0 g
Distilled water to	1 L

The yeast extract is dissolved in distilled water and the pH adjusted to 8.0. This solution is added to the Eugonagar, weighed in a 3 L flask which is then plugged and autoclaved at 121°C for 15 min.

The medium is cooled to 50°C, 10% v/v bovine blood is added and the mixture is dispensed aseptically into plastic petri dishes, each dish receiving 16–20 mL of medium. Once the agar has set, the plates are dried overnight at ambient temperature and stored at 4°C until required. Plates can be stored for up to four weeks and redried before use to remove all surface moisture. Be careful not to overdry the plates.

BBL Eugonagar contains the following ingredients in 45.4 g:

Trypticase peptone (Pancreatic digest of casein)	15.0 g
Phytone (Papaic digest of soybean meal)	5.0 g
NaCl	4.0 g
Sodium sulfite (Na_2SO_3)	0.2 g
L-Cystine	0.3 g
Dextrose	5.5 g
Agar	15.0 g

9.3.7. Modified Eugonbroth (MEB)

Eugonbroth (BBL)	30.0 g
L-Lysine (BDH)	2.5 g
L-Arginine (BDH)	2.5 g
Trypsin 1:250 (Difco)	10.0 g
Yeast extract (Difco, Oxoid L21 or BBL)	10.0 g

The MEB ingredients are added to distilled water, mixed thoroughly and the volume made up to 1 L. The suspension is autoclaved for 15 min at 121°C and filtered while hot through cotton wool and then through a D1 pad using a Buchner funnel.

The volume is returned to 1 L with distilled water, the pH is adjusted to 7.8–8.0 using 10 mol/L sodium hydroxide and the clear solution is dispensed in 200 mL volumes into screw-capped or cotton wool plugged 300 mL bottles. The medium is autoclaved for 15 min at 121°C, the caps are tightened when cool and the bottles are stored at 4°C until required. Before use, the caps are loosened and the medium boiled for 20 min to drive off dissolved oxygen.

BBL Eugonbroth contains the following in 30 g:

Trypticase peptone	15.0 g
Phytone	5.0 g
NaCl	4.0 g
Na_2SO_3	0.2 g
L-cystine	0.3 g
Dextrose	5.5 g

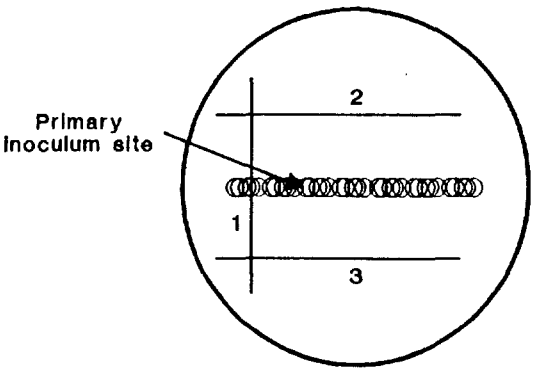

Figure 2. Surface inoculation technique for primary plates (Method 1).

9.4. Appendix 4 — Methods of Inoculating Primary Plates

9.4.1. Method 1

(a) Make sure surface of hoof agar plate is dry by placing open plate upside down in incubator for at least 10 min before use. It is important that the plates are quite dry to prevent excessive spreading of contaminants.

(b) Inoculate hoof agar plate as per Fig. 2. If lesion exudate is sent in TM either on a cotton tipped swab or as necrotic material in the top few millimetres of TM use the swab directly for the former or a sterile bacteriological loop for the latter.
(i) Inoculate heavily with cotton tipped swab or loop.
(ii) Turn plate at right angles and using same swab, inoculate as four or five straight lines from (ii) across plate. (These lines should be about 1 cm apart). The streak lines need to be widely spaced (about 1 cm apart) to permit the *D. nodosus* colonies to grow out from the streak lines and be distinguished by their typical morphology.
(iii) Turn plate at right angles and, again using same swab, inoculate as two or three straight lines (about 1 cm apart).

(c) Label plate with sheep identification and foot sampled.

(d) Place inoculated plates with agar uppermost in the anaerobic jar (Oxoid, BBL or Le Parfait preserve jar), put catalyst container down side of jar, insert indicator strip (read directions) and GasPak envelope (anaerobic system, BBL) or Gas Generating Kit (anaerobic system, Oxoid) (read instructions). Preserving jars [Le Parfait Supreme (Bocal) 3 L capacity] make excellent anaerobic jars. *Oxoid and BBL both supply catalyst — indicator — gas generator systems. N.B.* The catalyst for BBL is now contained on the back of the GasPak envelope.

(e) Dispense 10 mL water into GasPak envelope and quickly close jar tightly — be sure lid is on squarely.

(f) Forward to laboratory immediately. If jar will not reach laboratory the same day it may be preferable to make arrangements locally to incubate the jar for 48 hours prior to despatch.

(g) The catalyst-free anaerobic pouch system (Difco) is suitable for transporting small numbers of plates (J.A. Vaughan, pers. comm. 1990).

(h) Incubate anaerobic jar at laboratory for four to six days at 37°C. If indicator paper has not turned white overnight, check that there is no dirt around top of jar, replace gas generator pack and indicator and reincubate. Alternatively at the laboratory the evacuation/replacement system for anaerobiasis can be used and involves gassing vented anaerobic jars three times with 90% hydrogen, 10% carbon dioxide. Anaerobiasis is achieved more rapidly with the latter method and also it is more effective with broth cultures.

(i) Subculture *D. nodosus* colonies (see 6.2.1.).

9.4.2. Method 2 (RVL, Albany, WA)

(a) Stab-inoculate, as per diagram, by streaking a single line of inoculum onto the surface of a 55 mm plate of modified TAS isolation agar (9.3.) and then mash the inoculum through the agar to the petri dish base along this streak. A number of other streaks (see Fig. 3.: 1, 2 and 3) are cut into the surface, away from the primary site of inoculation. Two isolation agar plates are inoculated from each TM bottle. A suitable tool for the above procedure can be made from 1.5 mm diameter stainless steel welding wire cut into 75 mm lengths.

(b) Incubate isolation agar plates anaerobically for two days at 37°C in an atmosphere of 90% hydrogen and 10% carbon dioxide.

(c) When *D. nodosus* colonies are evident proceed with subculturing (see 6.2.1., Method 2).

(d) If no *D. nodosus* is evident the primary plates are reincubated for another two days. If no *D. nodosus* is present after this, the plates are discarded. When *D. nodosus* is observed, proceed with subculturing (D.R. Pitman, pers. comm. 1991).

9.4.3. 'Flipping' Procedure (Subculture of Contaminated Growth)

This procedure is adopted if there is evidence of *D. nodosus* present, using Method 2, but a pure isolate is not obtained after four days of incubation of the primary isolation plate.

A 75 mm length of 1.5 mm diameter stainless steel rod (as used in the primary inoculation procedure), is placed between the wall of the petri dish and the 4% agar slab. This enables the latter to be levered out of the petri dish base into the lid, thus exposing the underside growth.

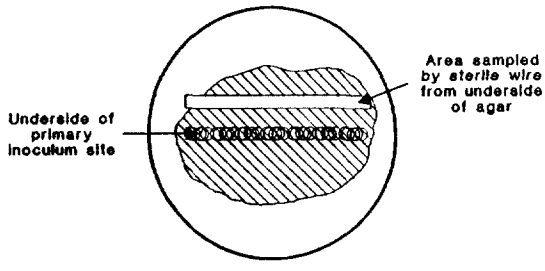

Figure 3. Stab inoculation technique for primary plates (Method 2).

This underside colony is then sampled using the above mentioned wire rod so as to include in the inoculum both the inner regions and the periphery of the colony (Fig. 4). A new plate of isolation agar is then inoculated using a procedure similar to that for primary inoculation. Again incubate for two days, examine for *D. nodosus* growth and subculture where possible (D.R. Pitman, pers. comm. 1991).

9.5. Appendix 5 — Colony morphology (Thorley, 1976; Skerman et al., *1981; Stewart* et al., *1986)*

Primary colonies growing in mixed cultures on 4% agar are distinguishable as flat crescentic outgrowths with granular outer zones, fimbriate edges, clear internal bands and papillate to fused-papillate central regions. Primary colonies from benign and virulent forms of footrot may be indistinguishable.

On subculture, two basic colony types are identifiable, described by Thorley (1976) as fimbriate and non-fimbriate according to the appearance of the edge of the colony. On Thorley's high-strength agar medium the outer zone of fimbriate colonies consists of a thin, spreading finely granular growth terminating peripherally in a fine diffuse fimbriate edge consisting of migrating microcolonies. In some cases there may be clear areas between the outer and inner parts of the fimbriate colony (Thorley, 1976). On low-strength agar isolates from virulent footrot retain the above characteristics seen in primary culture, although overall diameters (3–6 mm), the presence of a diffuse granular periphery and a tendency to form pits in the agar underlying the central papillae vary within and between strains. This colony type, designated by Skerman *et al.* (1981) as a B (beaded) type due to close studding with multiple papules in the centre of the colony is stable on selective subculture. Organisms from such colonies are highly piliated and are generally capable of inducing lesions of virulent footrot.

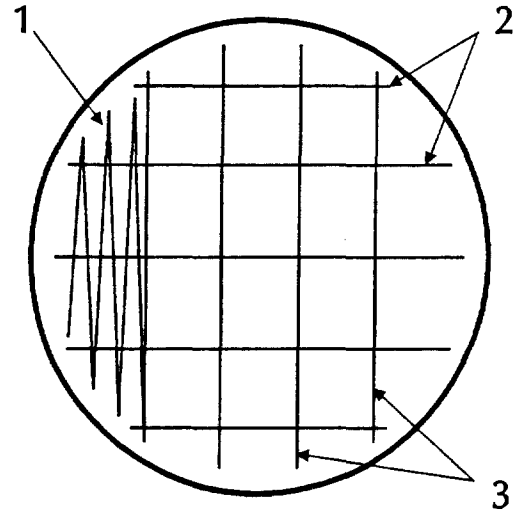

Figure 4. 'Flipping' procedure for subculturing *D. nodosus* from the underside of the stab-inoculated agar.

The second colony type has a conical profile with a shiny, elevated central region of a confluent mucoid texture. The outer region of the colony has an even, smoother surface and the non-fimbriate margin varies from even to dentate. Pitting is not found on low-strength agar. This colony represents the *non-fimbriate* type of Thorley (1976) and has been designated M (mucoid) by Skerman *et al.* (1981), to highlight the coherent nature of this central conical region as opposed to the multiple papillae of the B type. M type colonies (6–10 mm in diameter) are typical of those isolated from benign footrot and from cattle. Organisms in such colonies have far fewer pili than those from B-type colonies. M type are also stable on selective subculture and are capable of producing varying degrees of interdigital inflammation with lesions usually resolving spontaneously in two to three weeks.

A third colony type has been described as occurring after repeated non-selective subculture. This colony has a generally circular outline, a hemispherical profile and an entire even edge. Colonies are small (1.5–3 mm), translucent and non-pitting. They have been designated C (circular) type by Skerman *et al.* (1981) and are stable on subculture. Organisms from C-type colonies appear to be devoid of pili and are avirulent. Colonies of this type are frequently isolated in South Australia.

Colony morphology is useful for discriminating virulent, intermediate and benign strains (Stewart *et al.*, 1986). On hoof agar containing 4% agar, colonies are classified on the width of the fimbriate zone, the width and texture of the mid-zone, (coarse granular, fine granular, narrow, smooth, absent) the nature of the centre (beaded, slightly rough, smooth conical) and their relative size. The most virulent strains have a wide fimbriate zone and a coarse granular mid-zone, beaded centre and have the largest colonies. Intermediate and benign strains have a narrow fimbriate zone (or it may be absent) fine granular or narrow smooth mid zone, slightly rough or smooth conical central zone and the colony size varies.

When the agar content of the medium is reduced to 2%, the fimbriate zone is virtually absent in all but the most virulent strains. The mid-zone also tends to be reduced and is absent in a proportion of benign strains. The texture of this zone is similar to that of the 4% agar colonies except for a change from a finely granular to a smooth surface in some intermediate strains. The central zones of colonies of all avirulent, benign and intermediate strains are a smooth dome, but in the virulent strains, colonies are more variable: the beaded centre is retained in strains showing this feature on 4% agar, while other virulent strains (high virulence intermediate) have a smooth, domed centre similar to the strains of lesser virulence.

On hoof agar containing 2% agar and on blood Eugonagar virulent strains can be distinguished from intermediate and benign by the presence of a wide moat of agar corrosion in which the moat is lacking or less evident. Moat formation refers to a circular zone of liquifaction of agar beneath the mid zone and is visible without disturbance of the colony. After removal of the colonies larger and deeper crater-like erosions of agar are generally evident with the virulent strains than with those of the less virulent categories. Colonies from an avirulent strain do not cause any erosion. On blood agar, colonies of the virulent strains also possess a granular mid zone and fimbriate edge whereas intermediate and benign strains do not. Intermediate strains either have a more elevated smooth mid zone or a narrow smooth one. Benign strains usually have no mid zone although sometimes a narrow one is present. On the different media, while there is a general trend towards the virulent strains forming the largest colonies and the benign strains the smallest there is considerable overlap between the several virulence classes. The following abbreviations can be used to describe colony morhology (Table 2).

9.6. Appendix 6 — Surface Translocation

Colony size is used to measure the degree of surface translocation or motility of *D. nodosus* isolates.

(a) Prepare 90 mm plates of modified TAS maintenance agar containing haemoglobin (see 9.3.).

(b) Subculture *D. nodosus* from a plate culture in a 3 x 3 configuration, resulting in three replicates per isolate and three isolates per plate. Sterile microhaematocrit capillary tubes or 1 µL disposable plastic loops (Disposable Products or Difco) are satisfactory implements for inoculations. The three inoculations per isolate are performed from left to right without acquisition of additional inoculum.

(c) Include high and low motility isolates as a reference in each anaerobe jar.

Table 2. Abbreviations for colony morphology terminology

Abbreviation	*Description*
HF	Highly fimbriate
HFRC	Highly fimbriate raised centre
F	Fimbriate
FRC	Fimbriate raised centre
SF	Small fimbriate
SFRC	Small fimbriate raised centre
NF	Non-fimbriate
NFRC	Non-fimbriate raised centre
FE	Fimbriate etching
FERC	Fimbriate etching raised centre
SNFE	Small non-fimbriate etching

(d) Incubate anaerobically at 37°C for 72 hours.

(e) Record the diameter of the three colonies of each isolate. The diameter of the middle colony should equal the mean diameter of all three colonies.

(f) The foregoing procedure is repeated four times using different batches of media.

(g) Colony size of a *D. nodosus* isolate is calculated as the mean of all results for that isolate.

9.7. Appendix 7 — Protease Thermostability Test (Stewart *et al.*, 1982; Depiazzi and Richards, 1984/1985)

9.7.1. *Hide Powder Azure Method*

Three- or four-day broth cultures are heated for 30 min at 55 ± 0.2°C in a water bath and their proteolytic activity is compared with that of unheated cultures by the method previously used at CSIRO AHRL (see 9.7.1.2.). This method differs from the thermostability test of Depiazzi and Richards (1984/1985). For the latter the medium is buffered and contains calcium ions (HEPES TAS broth, see 9.3.) and the cultures are heated at 40°C and 70°C for 15 min.

9.7.1.1. Requirements

9.7.1.1.1. Three- to four-day-old TAS broth culture (10^8 organisms/mL) of *Dichelobacter nodosus*. The TAS broth pH 7.8 (see 9.3.) is slightly modified from that of Skerman (1975) in that the L-arginine content has been reduced to 2.5 g. The TAS broth (5.0 mL) is inoculated from either primary plates inspected at x15 magnification to ensure that *D. nodosus* which are picked off have not been partially overrun by spreading contaminants or, preferably, from plates subcultured at least once to avoid this problem. Three colonies from each of the isolation plates or several colonies from each of these subcultured colonies are picked into separate broth cultures and incubated anaerobically at 37°C.

9.7.1.1.2. Hide Power Azure (HPA). (Sigma Chemical Company).

9.7.1.1.3. 0.05 mol/L TRIS buffer pH 7.8.

9.7.1.1.4. Thermostatically controlled heated water bath. ('Thermomix', B. Braun Australia Pty Ltd, Castle Hill, NSW).

9.7.1.2. Procedure

(a) Heat 0.3 mL of a well grown three- to four-day-old TAS broth culture of *D. nodosus* at 55°C for 30 min in a water bath; cover tubes with Parafilm M (American Can Company).
(b) Weigh out 20 mg HPA in centrifuge tubes with screw cap.
(c) Add 5 mL 0.05 mol/L Tris buffer (pH 7.8) to tubes and wait five minutes.
(d) Add 100 µL unheated or heated TAS broth aliquot to the appropriate tube of buffer and HPA, cap and shake.
(e) Place tubes in 'Thermomix' water bath at 37°C for 45 min. Shake tubes every five minutes.
(f) Stop reaction by placing tubes in ice.
(g) Spin tubes in a refrigerated centrifuge at 3000 rpm for 10 min, to sediment undigested HPA.
(h) Pour off supernatant and read at OD 595 nm in a Spectronic 20 (Bausch and Lomb).
(i) Calculate percentage stability.

9.7.1.3. Notes

(a) *D. nodosus* cultures should have a high cell density (about 10^8 organisms/mL).
(b) *D. nodosus* virulent and benign control cultures should be included with each test run.
(c) Incubation temperatures are critical, (use water bath with thermometer accurate to 0.1°C).
(d) During culture growth and during heating, calcium chloride must not be present either in the medium or in the Tris buffer, otherwise calcium ions will stabilise the protease and interfere with the results of the test.
(e) It is necessary to have unheated broth cultures as controls to check that proteolytic activity is present before heat treatment.

9.7.2. Gelatin Gel Method

Although requiring overnight incubation the gelatin gel method is a simpler, more reproducible method and more samples can be processed in one day than that using HPA. The gelatin gel method was originally developed by M.A. Palmer at the RVL, Albany, WA, with further modifications being made by AHRL, CSIRO Division of Animal Health and RVL, Albany.

9.7.2.1. Requirements

(a) Two-day-old HEPES-TAS broth (see 9.3., RVL) or three- to four-day-old TAS broth (see 9.3., 9.7.1., AHRL) cultures (10^8 organisms/mL) of *D. nodosus*. Calcium chloride is present in the former but not the latter.
(b) Agarose gelatin gel.
(c) Agarose gelatin gel buffer.
(d) HEPES test buffer with detergent (AHRL) or without detergent (RVL).
(e) Gelatin precipitant, saturated ammonium sulfate solution.
(f) 16 x 16 cm prewarmed glass plate or transparent tray (RVL) or 150 mm petri dishes (AHRL).
(g) Screw cap test tubes (Cat. No. 45066A, Kimble glassware, 13 x 100 mm or glass tubes of similar dimensions).
(h) Water bath with thermometer accurate to ±0.1°C ('Thermomix').
(i) Quebec colony counter (American Optical) (RVL) or similar.
(j) Control virulent (thermostable protease) and benign (thermolabile protease) broth cultures.

9.7.2.2. Reagents

9.7.2.2.1. Agarose–gelatin gel.

Agarose (Calbiochem) (Bio-Rad Cat. No. 162-0100, RVL)	1.2 g
Gelatin (Difco)	0.75 g
Agarose gelatin buffer	10.0 mL
Distilled water	90.0 mL

9.7.2.2.2. Agarose–gelatin gel buffer (pH 8.8).

Tris base (Sigma)	2.84 g, (4.84 g, RVL)
$CaCl_2$ (anhydrous)	0.1 g, (0.01 g, RVL)
Distilled water	100.0 mL

9.7.2.2.3. HEPES test buffer with or without detergent.

HEPES acid (BDH, Calbiochem)	2.650 g
HEPES sodium salt (BDH, Calbiochem)	49.165 g
$CaCl_2$ (anhydrous)	2.2 g
Zwittergent 3–14 (Calbiochem) (omitted by RVL)	0.07 g
Distilled water	2 L

Adjust to pH 8.5 at 40°C (adjust at 68°C, RVL). Dissolve and filter (Whatman No. 1) before use.

9.7.2.2.4. Gelatin precipitant. Saturated ammonium sulfate solution.

9.7.2.3. Method 1 (AHRL)

(a) Prepare plates with a layer of gelatin gel on day of test by pouring 25 mL per 150 mm petri dish test and dry at 37°C for 1.5 hours before use.
(b) Dilute test samples (0.5 mL) in HEPES test buffer (2.0 mL) in glass test tubes.
(c) Mix dilutions by shaking and place 20 µL onto surface of gelatin gel. Three test samples can be done per plate. When dried the gel is slightly hydrophobic so the drop should not spread.
(d) Place dilutions in 67°C water bath which has been equilibrated for two hours prior to commencement. Tubed dilutions remain in water bath for full 16 min.
(e) After eight minutes in 67°C water bath, shake, remove 20 µL and drop onto gelatin gel 20 mm beneath initial sample.
(f) After a further eight minutes at 67°C, shake, remove 20 µL and drop onto gelatin gel 20 mm beneath second sample.
(g) Allow drops to dry and incubate gelatin gel plates on a flat surface in a moist chamber at 37°C, overnight.

(h) Flood gel with hot (50°C) saturated ammonium sulfate solution to precipitate remaining gelatin.

(i) Measure all zone diameters to the nearest mm and calculate percentage stability in relation to 'clearing' at zero time (diameter of clear zone, zero minutes = 16–20 mm).

9.7.2.3.1. Interpretation. (TAS broth without calcium chloride). The thermostable proteases of virulent/intermediate strains after heating at 67°C for 16 min cause clearing of the gelatin gel whereas the thermolabile proteases of benign strains either cause no clearing or a trace amount of incomplete clearing. The percentage change in diameter of clearing is used in this version of the test for comparing the thermostability of proteases from virulent and intermediate strains. However, L.J. Depiazzi and M.A. Palmer (pers. comm. 1991) consider this parameter inappropriate because the relationship is not linear between protease concentration and the diameter of the zone of clearing.

9.7.2.4. Method 2 (RVL, Albany)

(a) Rule an area of 160 x 160 mm on the glass plate using a piece of wax, leaving a border for labelling. Warm plate before pouring 40 mL molten gelatin agarose gel into the ruled area and spread evenly with orange stick. When the gel is set punch 3 mm diameter holes in a grid pattern 25 mm apart (i.e., six rows of six holes). This will accommodate 12 tests. Allow surface of gel to dry at room temperature for at least two hours before use.

(b) Prepare wet preparation of test broths. Check microscopically (x1000) for purity and approximate cell numbers. Dilute well grown (10^8 cells/mL) test broths (0.5 mL) in HEPES buffer (1.5 mL). Dilutions may be varied or broths used undiluted if cell numbers are low. However total volume of dilution must always be 2.0 mL.

(c) Mix first dilution by shaking, place 20 µL into top, left well in gelatin gel and place dilution in water bath at 68°C. Repeat for all samples keeping interval between samples constant.

(d) After first sample has been incubated for eight minutes, shake, remove 20 µL and place in well beside well containing the unheated sample. Replace tube in water bath. Repeat for all samples keeping interval between samples as before.

(e) Repeat (d) after a further eight minutes incubation at 68°C.

(f) Incubate gel in a moist chamber at 37°C overnight.

(g) Flood gel with hot (60–70°C), saturated ammonium sulfate solution to precipitate remaining gelatin.

(h) Measure zones of proteolysis around wells, while viewing the gel against a dark background and lit obliquely from below. (A 'QUEBEC' colony counter works well).

Table 3. Interpretation of gelatin gel (Method 2)
Figures refer to diameter (mm) of clear zone

Unheated sample	*Heated 8 min @ 68°C* Unstable	Stable	*Heated 16 min* Unstable	Stable
15	≤ 7	≥ 9	- ve	+ weak
16	≤ 8	≥ 10	- ve	+ weak
17	≤ 9	≥ 11	- ve	+ ve
18	≤ 10	≥ 12	- ve	+ ve
19	≤ 11	≥ 13	- ve	+ ve
20	≤ 12	≥ 14	- ve	+ ve
21	≤ 13	≥ 15	- ve	+ ve
22	≤ 14	≥ 16	- ve	+ ve

9.7.2.4.1. Interpretation. (HEPES–TAS broth with calcium chloride) (see Table 3). When a sample gives a result between the limits shown at eight minutes or the reaction at 16 min does not fit, the culture should be grown in a fresh broth and retested. The most likely source of error is contamination of the original broth with another protease producing bacterium.

For multiple samples:

(a) Prepare all dilutions of crude protease.

(b) From then on, stagger incubation times for each individual sample.

9.8. Appendix 8 — Elastin Agar Test (Stewart, 1979)

The elastin agar (EA) test uses TAS medium (see 9.3.) containing 1.5% Bacto agar (Difco) and 0.3% elastin powder from bovine neck ligament (Sigma Chemical Company). Clearing of elastin particles is enhanced and may occur earlier when 0.15% calcium chloride is incorporated in the elastin agar medium (Stewart *et al.*, 1986). The addition of calcium chloride also facilitates the detection of a proportion of intermediate isolates. It is, therefore, advisable that calcium chloride (anhydrous, 1.5 g/L) be included in the EA plates. Dissolve all ingredients, except agar in distilled water and adjust pH to 7.8 with 10 mol/L sodium hydroxide. The medium is allowed to soak for 30 min with vigorous agitation using a magnetic stirrer, to disperse the elastin particles (3 g/L). Weigh agar (15 g/L) into a conical flask, add dissolved ingredients, mix well and autoclave for 121°C for 15 min. After cooling to 50°C and before dispensing 15 mL of EA medium per plate, it is important that the elastin is again well dispersed.

The test medium is inoculated in a single streak line with two strokes of a 10 µL sterile loop (Disposable Products or Difco) forward and back to provide heavy confluent growth and then incubated anaerobically at 37°C. The inoculum can be either transferred directly from typical large uncontaminated colonies on primary plates or preferably following an additional subculture on solid medium. Four isolates can be inoculated on each EA plate with one positive control isolate included in each anaerobic jar of plates.

Elastase positive isolates completely digest elastin particles in the agar beneath the confluent

line of colonies with clearing extending away from the colonies for a variable distance up to 12 mm. The relative width of the zone of clearing of elastin particles in the EA plates is recorded as: +, present; or –, absent. Numbers indicate width on a scale: 1+ (least) to 4+ (greatest). A score of 1+ includes any degree of clearing.

Clearing of the elastin particles should be evident within four to seven days for virulent isolates but may take 14–21 days or longer for intermediate strains. Plates should be read at four, seven, 10–11, 14, 18, 21, 25, 28 and 31 days.

9.9. Appendix 9 — Zymogram

9.9.1. *Method 1* (AHRL, CSIRO Division of Animal Health)

(a) Grow the organism in TAS broth (see 9.3.) to a density of 10^8 organisms/mL, transfer aliquots to Eppendorf tubes and remove bacteria from the culture supernatant by centrifugation in a Microfuge at 10 000 *g* for 15 min. However this centrifugation step is not essential and can be omitted.

(b) It is essential that a standard culture of a benign and virulent strain be included as controls on each slab gel.

(c) The apparatus is the Bio Rad Mini-Protean II Dual Slab Cell (mini-gel) vertical electrophoresis system and a Model 200/2.0 constant voltage power supply.

9.9.1.1. Reagents

All stored at 4°C.

9.9.1.1.1. Filtered glass distilled water or reagent grade water.

9.9.1.1.2. 30% Acrylamide/bis-acrylamide (stock solution for separating and stacking gels). Acrylamide (Bio-Rad) 30 g dissolved in 70 mL of filtered distilled water, then add 0.8 g bis-acrylamide (Bio-Rad). Stir until dissolved. Store in dark bottle.

9.9.1.1.3. 1 mol/L Tris-HC1 pH 8.8 (separating buffer). To 24.2 g Tris base (Sigma) add 180 mL of filtered, distilled water or reagent grade water, bring to pH 8.8 at room temperature using concentrated hydrochloric acid (32%, 10 mol/L) then make up to final volume of 200 mL with either filtered distilled water or reagent grade water.

9.9.1.1.4. 0.5 mol/L Tris-HCl pH 6.8 (stacking buffer). To 6.05 g of Tris base (Sigma) add 80 mL of filtered distilled water or reagent grade water, bring to pH 6.8 at room temperature using concentrated hydrochloric acid, then make up to 100 mL.

9.9.1.1.5. 10% Ammonium persulfate. 0.1 g ammonium persulfate [$(NH_4)_2S_2O_8$, Sigma] dissolved in 0.9 mL of filtered distilled, or reagent grade, water (will last four days if stored at 4°C).

9.9.1.1.6. Electrode buffer.

Tris base (0.05 mol/L) (Sigma).	3 g
Glycine (0.2 mol/L) (Ajax Chemicals)	14.4 g
Glass distilled water	1 L

9.9.1.1.7. TEMED (N,N,N',N'-tetramethyl-ethylene-diamine).

9.9.1.1.8. Glycerol/bromophenol blue. 90% Glycerol + 10% of 0.5% solution of bromophenol blue, e.g. 9 mL glycerol + 1 mL of 0.05% solution of bromophenol blue. Store at -20°C. Before use, stand at room temperature for about three to five minutes.

9.9.1.1.9. Gelatin/agarose (for detection of protease isoenzyme activity).

Agarose	2.4 g
Gelatin	1.0 g
$CaCl_2.2H_2O$	0.146 g
or $CaCl_2$ (anhydrous)	0.110 g
Sodium azide, NaN_3	0.04 g
Tris buffer, 0.5 mol/L, pH 7.8	8 mL

Make up to 200 mL with filtered distilled water or reagent grade water and steam to dissolve. Dispense into about 50 mL volumes and store at 4°C. Before use, melt in microwave.

9.9.1.1.10. Mercuric chloride/HCl.

Mercuric chloride, $HgCl_2$	15 g
Concentrated HCl (32% or 10 mol/L)	20 mL
Glass distilled water	80 mL

9.9.1.2. Procedure

(a) Apparatus is Bio-Rad Mini-Gel System. The gel is non-dissociating (no sodium dodecyl sulfate) and non-reducing (no dithiothreitol).

(b) Allow reagents to reach room temperature before use.

(c) Prepare plates and spacers in gel holder apparatus.

(d) Pour 5% separating gel:

Filtered glass distilled water or reagent grade water	5.6 mL
Acryl/bis-acryl (30% stock)	1.7 mL
Tris HCl, 1 mol/L, pH 8.8	2.5 mL
TEMED (see 9.9.1.1.7.)	5 µL
Fresh 10% $(NH_4)_2S_2O_8$	50 µL

This volume is enough for two gels. Mark on the glass plate 1.0 cm below comb (to show where separating gel is to be added). Remove comb and add 5% separating gel with a Pasteur pipette, ensuring no bubbles. Add one Pastuer pipette of filtered distilled water or reagent grade water along top of separating gel. This gives a flat surface and prevents oxygen inhibiting polymerisation. Polymerisation of the gel has occurred when the water–gel interface is clearly defined by refraction. Allow to set at room temperature one hour.

(e) Tip off water and place comb in position. Pour 3% stacking gel:

Filtered distilled water or reagent grade water	2.6 mL
Acryl/bis-acryl (30% stock)	0.4 mL
Tris HCl, 0.5 mol/L, pH 6.8	1.0 mL
TEMED	5 µL
Fresh 10% $(NH_4)_2S_2O_8$	20 µL

Allow to set at room temperature for one hour.

(f) Prepare samples as follows.

Culture supernatant	50 µL
Glycerol/bromophenol blue	5 µL

Load 25 µL maximum into each well and run 150 V for about 40 min (until dye front is at the base of the gel).
Remove plates from apparatus, leave the non-dissociating gel on one glass plate and carefully overlay non-dissociating gel on top of a single large glass plate that is covered with gelatin/agarose substrate (about 13 mL) and with a thin film of water on the surface to ensure uniform contact. Remove excess water and incubate in a moist plastic box in a 37°C water bath for one hour.

(g) Carefully remove non-dissociating gel and develop gelatin/agarose with hot, saturated ammonium sulfate (M.A. Palmer pers. comm. 1991) or mercuric chloride/hydrochloric acid. The former is recommended for safety reasons. These precipitants cause the gelatin agar to turn milky white with clear zones where proteolysis has occurred. Exposed substrate plates can be stored in a moist atmosphere at 4°C for two days without deterioration.

(h) View or photograph against a dark background.

9.9.2. Method 2 (CSIRO Division of Biomolecular Engineering, Vic.)

9.9.2.1. 30% Acrylamide/0.8% bis-acrylamide (stock solution for separating gel)

Acrylamide	30.0 g
Bis-acrylamide	0.8 g
Glass distilled water to	100 mL

Store at 4°C.

9.9.2.2. Separating buffer (pH 8.9)

HCl, 1 mol/L	48.00 mL
Tris	36.60 g
TEMED	0.23 mL
Glass distilled water	100 mL

9.9.2.3. 10% Acrylamide/2.5% bis-acrylamide (stock solution for stacking gels)

Acrylamide	10 g
Bis-acrylamide	2.5 g
Add glass distilled water to	100 mL

Store at 4°C.

9.9.2.4. Stacking buffer (pH 6.7)

HCl, 1 mol/L	48.00 mL
Tris	5.98 g
TEMED	0.46 mL
Add glass distilled water to	100 mL

9.9.2.5. Ammonium persulfate

$(NH_4)_2S_2O_8$	0.28 g

Add glass distilled water to 100 mL in volumetric flask. Make up freshly each day.

9.9.2.6. Electrode buffer (50x)

Tris	30 g
Glycine	143.5 g
Add glass distilled water to	1 L

Take 20 mL of stock solution and make up to 1 L in a measuring cylinder.

9.9.2.7. Bromophenol blue/sucrose/sample

Bromophenol blue 0.1%	5 µL
Sucrose	a few granules
Sample	50 µL

9.9.2.8. Gelatin/agarose activity plates

Gelatin type A (Sigma G2625)	0.30 g
Tris-HCl, 0.02 mol/L, 5 mmol/L $CaCl_2$ buffer with 0.02% NaN_3	50 mL

Stir for one hour at room temperature and then heat to about 70°C.

Add Agarose A (Pharmacia)	0.72 g.

When cloudiness disappears, add 9.2 mL to each glass tray which has spacers 1.5 mm thick glued around the periphery. The unused activity plates covered with a glass plate can be stored at 4°C for three weeks. The glass trays can be made from the glass plates and spacers used for the slab gels. The trays can be constructed by securing the spacers to the perimeter of the plate with glue, adhesive tape or Vaseline. They should be washed with glass distilled water and rinsed with ethanol to prevent growth of bacteria during incubation of the activity plate.

9.9.2.9. 15% Mercuric chloride/2 mol/L hydrochloric acid

$HgCl_2$	15 g
Concentrated HCl	20 mL
Water	80 mL

9.9.2.10. 5% Separating gel

30% Acrylamide/0.8% bis acrylamide	3.5 mL
Resolving buffer	2.5 mL
Glass distilled water	10.0 mL

De-aerate by gently shaking under partial vacuum. Then add:

$(NH_4)_2S_2O_8$	5.0 mL

This volume is sufficient for two gels. Pour the gel into the glass plate sandwich and overlay with glass distilled water. Allow gel to set (30–40 min).

9.9.2.11. 2.5% Stacking gel

10% Acrylamide/2.5% bis-acrylamide	2 mL
Stacking buffer	1 mL
Glass distilled water	3 mL

De-aerate by gently shaking under partial vacuum. Then add:

$(NH_4)_2S_2O_8$	2 mL

After removal of the water layer with a syringe and 21G needle, place comb in position and pour the stacking gel. Allow the gel to set (25–30 min). Remove the comb and suck out excess liquid with syringe and a flat tipped needle.

9.9.2.12. Loading and running the gel

Load 25 µL into each well. Run the gel at 15 mA/gel (for a power pack with constant current) and until the dye front just runs off the bottom of the gel.

9.9.2.13. Developing the zymogram

Overlay separating gel slab on gelatin/agar activity plate and incubate for 50–55 min at 37°C. Separate from substrate and flood with acid mercuric chloride solution.

9.9.3. Method 3 (RVL, Albany)

9.9.3.1. Stock solutions

9.9.3.1.1. Acrylamide/bis-acrylamide.

Acrylamide	29.2 g
N'N'-bis methylene acrylamide	0.8 g
Water	100 mL
Amberlite MB-1 (BDH-55007)	3.0 g

Mix on magnetic stirrer for one hour. Filter and store at 4°C in the dark (30 days max).

9.9.3.1.2. Separating buffer (1.5 mol/L TRIS-Cl pH 8.8).

TRIS-base	18.15 g
Water	50.0 mL

Adjust to pH 8.8 with 1 mol/L hydrochloric acid and make up to 100 mL with water.

9.9.3.1.3. Stacking buffer (0.5 mol/L TRIS-Cl pH 6.8).

TRIS-base	3.0 g
Water	50 mL

Adjust to pH 6.8 with 1 mol/L hydrochloric acid.

9.9.3.2. Separating gel preparation

12% gel, 0.375 mol/L TRIS, pH 8.8 (enough for one 1.5 mm x 14 cm x 14 cm slab)

Water	13.4 mL
Separating buffer	10.0 mL
Zwittergent 3–14 (Calbiochem) 20 mmol/L	0.4 mL
Acrylamide:bis	16.0 mL
De-aerate with vacuum pump. Then add	
10% $(NH_4)_2S_2O_8$, fresh	0.2 mL
TEMED	0.02 mL

Polymerise about one hour, then prepare stacking gel.

9.9.3.4. Stacking gel preparation

4% Gel, 0.125 mol/L TRIS, pH 6.8 (enough for one 1.5 mm thick stacking gel).

Water	6.1 mL
Stacking buffer	2.5 mL
Zwittergent 3-14 (Calbiochem) 20 mmol/L	0.1 mL
Acrylamide:bis	1.3 mL
De-aerate with vacuum pump. Then add	
$(NH_4)_2S_2O_8$ (fresh), 10%	0.05 mL
TEMED	0.01 mL

Mix and pour onto separating gel. Polymerise one hour and remove comb. After washing with water, fill with stacking buffer and leave at 4°C overnight. (*N.B.* Dilute stacking buffer 1:2 for above).

9.9.3.5. Electrode buffer (TRIS-Glycine pH 8.3) (concentrated x10)

TRIS-base (0.025 mol/L final concentration)	30.0 g
Glycine (0.325 mol/L final concentration)	144.0 g
Zwittergent 3–14 (Calbiochem)	0.7 g
Water	1 L

9.9.3.6. Protease substrate preparation

9.9.3.6.1. Agar gelatin buffer.

TRIS-base	2.42 g
$CaCl_2$ anhydrous	0.05 g
Water	50 mL

Adjust pH to 8.8 with HCl. Make up to 100 mL.

9.9.3.6.2. Agar gelatin substrate.

Agarose	1.2 g
Gelatin (Sigma G-2500)	0.75 g
Agar gelatin buffer	10.0 mL
Water	100 mL

Boil to dissolve. Pour (20 mL) on 160 x 60 mm flexible plastic film.

9.9.3.6.3. Gelatin precipitant.

Saturated ammonium sulfate solution.

9.9.3.7. Equipment

(a) Hoefer Dual Vertical Slab Gel Electrophoresis Unit (Cat. No. SE 600) with capacity for two 160 x 140 mm gels. Each gel will run 20 samples (16 unknown and four control samples).
(b) Power supply Pharmacia ECPS 2000/300.
(c) Lower tank buffer maintained at 4°C using Grant FC20 flow cooler with an FH15 heater/temperature controller.

9.9.3.8. Procedure

(a) Fill wells in acrylamide gel with half strength (0.25 mol/L) stacking buffer.
(b) Inoculate 15 µL of well grown (two-day) cultures of *D. nodosus* in modified TAS broth into the bottom of wells. For weakly growing or contaminated broths, this may be increased up to 40 µL (maximum).
(c) Include stable protease subtype 1 (S1) and unstable protease subtype 1 (U1) as broth culture controls at both ends of the gel, and place tracking dye in one outside well.
(d) Run at 20 mA until dye reaches top of separating gel, then at 30 mA until dye reaches bottom of gel (about five hours).
(e) While running gel, prepare gelatin–agarose gel as follows:
(i) Dissolve 20 mL volume by boiling and continue boiling to remove bubbles (we find a domestic microwave ideal for this).
(ii) Allow to cool slightly before pouring as a layer about 1.5 mm thick on a 160 x 60 mm piece of flexible plastic film. The film used to make overhead transparencies works well, e.g. 3M Transparency Film PP2410.
(iii) Pouring the gel too hot will result in a rippled effect on the surface, which will make poor contact with the acrylamide gel.
(iv) To avoid the gel setting before pouring is complete, lay the plastic film on a warm surface.
(f) After the electrophoresis run is completed, remove one glass plate from acrylamide gel and lay gelatin gel on top, so the top edge is at or slightly higher than the top of the separating gel. Avoid trapping air bubbles between gels by slightly bending gelatin gel and applying from one side to the other or from the centre to the outside. To squeeze out any remaining bubbles run the back of one finger across the back of the gelatin gel. *Do not* flood gel with water before adding gelatin gel as there will be enough moisture present to give good diffusion of protease.

Excess water may allow lateral diffusion of protease and could also result in the gelatin gel moving around on the acrylamide gel during handling.

(g) Place gels in a moist chamber and incubate together in a 37°C incubator for one hour.

(h) Remove gelatin gel and develop by flooding with hot saturated ammonium sulfate (about five minutes).

(i) When interpreting results only the position of the prominent pair of bands is used to classify isolates though other weaker bands will often be present (Fig. 1*b*).

9.9.3.9. Problems and solutions

(a) Smile effect (band patterns curve upward at both ends of gel) due to centre of the gel running hotter than either end. Solution is to decrease power setting.

(b) Diffuse tracking dye or protein bands. The solution is to prepare fresh reagents.

(c) Vertical streaking of protein due to sample overload. The solution is to dilute the sample.

(d) Top reservoir leaking. Solution:
(i) Turn off power pack and add more buffer.
(ii) Before pouring gel, make sure that the inner glass plate is butting up against notch in the U-shaped gasket of inner cooling core.

(e) Separating gel leakage during pouring. Make sure spacers butt against sides of clamp assembly and that glass plate sandwich is placed correctly in casting slot.

(f) Low current. Make sure that jack connections are tightened up. Bubbles on cathode wire indicate that electrophoresis is working.

(g) If samples fail to move into gel, check that anode and cathode connections to power pack and gel apparatus are correct.

(h) When loading gel, do not have any bubbles in syringe, as this may cause sample to overflow into next well.

(i) For correct identification of sample lanes, remember which is left and right of gel, in relation to loading of samples.

(j) For good banding patterns, use three- to four-day-old well-grown cultures with a cell density of 10^8 organisms/mL.

9.10. Appendix 10 — Serology and Serogrouping

9.10.1. Preparation of Broth Antigens

A well grown (three- to four-day-old) culture of the appropriate strain of *D. nodosus* on 2.0% HA is used to heavily inoculate 20 mL McCartney bottles or test tubes containing 4.0 mL of freshly boiled and cooled MEB or TAS broth. Isolated fimbriate colonies are selected and broth inoculated with a sterile loop.

These cultures, in loose capped bottles, are incubated anaerobically either using the GasPak system (BBL) or vented anaerobic jars evacuated and gassed three times with a 90% hydrogen and 10% carbon dioxide gas mixture. Maximum growth is obtained after 18–48 hours incubation at 37°C after which 20 mL volumes of broth are inoculated with 2 mL of culture. When maximum growth is achieved, these 20 mL cultures are used to inoculate 200 mL volumes of broth which are in turn incubated anaerobically for 18–48 hours until maximum growth is achieved. The 220 mL cultures are then killed by the addition of formalin to give a final concentration of 1 in 80 (1.25%).

After standing at room temperature for at least four hours, these killed broth cultures are centrifuged at 10 000 *g* and 4°C for 20 min. The supernatant is carefully decanted and the deposited cells are gently resuspended in PBS, pH 7.4, to a concentration of about 5×10^9 bacteria/mL. Formalin is then added to a concentration of 1 in 80 and the antigen concentrates are then stored at 4°C. For the agglutination test, the suspension is standardised photometrically so that the final dilution has an optical density (OD) of 0.3 at a wavelength of 520 nm and which is equivalent to 10^8 organisms/mL.

9.10.2. Preparation of Plate Antigens

Each strain of *D. nodosus* is inoculated onto well-dried 2.0 % HA plates, giving up to four strains per plate. The most fimbriate colonies are selected for inoculation. The inoculated plates are incubated anaerobically at 37°C for three to four days, after which, the cells are harvested.

Harvesting is carried out by dispensing 0.5–1.0 mL sterile PBS pH 7.4 onto the surface of the plate and scraping the surface growth off into the PBS with a sterile scalpel blade. Care is taken to ensure that only plates without contaminants are harvested. The suspension from each plate is collected with a sterile Pasteur pipette and dispensed into a Bijou or a McCartney bottle. The cell suspensions are standardised in the same manner as the broth antigens and formalin is added to a concentration of 1 in 80.

9.10.3. Preparation of Antisera

9.10.3.1. Antigen

An unwashed, formalin-killed suspension of *D. nodosus* grown on HA and containing 5×10^9 organisms/mL of PBS is emulsified with Freund's incomplete adjuvant (FIA) in the ratio of 1:2.

9.10.3.2. Animals

Well-grown white rabbits or sheep are used. Preferably more than one animal should be used for each strain of *D. nodosus*.

9.10.3.3. Inoculation

Each rabbit is inoculated with 0.5 mL of the appropriate antigen by deep intramuscular injection into each hind leg (1 mL per rabbit). The rabbits are bled on the day of inoculation to demonstrate that they have no existing antibody to *D. nodosus*. Each rabbit is bled again 21 days after inoculation to determine the K-agglutinin titre to the injected *D. nodosus* strain. If the homologous titre is at least 5120 the rabbit is

bled out by cardiac puncture under pentobarbitone sodium anaesthesia and 4.0 mL aliquots of the serum stored at -20°C in the screw cap plastic vials. If the homologous titre is less than 5120 the rabbit is rebled at 28 days. If the titre is again below 5120 the rabbit is usually discarded. If the titre is 5120 or greater, the rabbit is bled out and the serum stored as described.

For sheep, two injections of 2 mL are given subcutaneously with an interval of 28 days between injections. The sheep are then bled to check their agglutinating antibody response and if satisfactory 100 mL of blood is taken by needle from the jugular vein and the serum stored in aliquots of 20 mL at -20°C. Serums for day-to-day use are preserved with 1/10000 merthiolate and stored at 4°C.

9.10.4. Absorption of Antisera

In order to absorb an antiserum, either of two procedures can be used.

(a) Formalised suspension (0.9 mL) containing about 10^9 organisms/mL of the appropriate strain of *D. nodosus* is added to 0.1 mL serum in a 10 x 87 mm glass tube. After incubation, with intermittent shaking for four hours at 37°C in a waterbath, the tube is centrifuged at 3000 *g* for 20 min and the supernatant carefully transferred to a second tube. A further 1 mL of antigen suspension is added and after mixing, incubated for a further four hours at 37°C. Centrifugation, collection of supernatant and addition of a further 1 mL of antigen, followed by incubation, is repeated twice. A final centrifugation and collection of supernatant results in 4.0 mL of 1/40 serum which has been absorbed four times with antigen. The absorbed serum should always be checked to determine that no agglutinating activity to the absorbing antigen remains.

(b) Formalised, piliated cells of *D. nodosus* are isoelectrically precipitated at pH 4.5 and the suspension is centrifuged at maximum speed in an Eppendorf microfuge. Each Eppendorf tube contains a sufficient pellet of cells for absorption of one serum, using three sequential absorptions for 45 min at 37°C and a cell volume to serum ratio of 1:3–1:6 (Day *et al.*, 1986). All sera are routinely absorbed with cells of two heterologous serotypes and tested for specificity in the slide agglutination test. A further absorption step is carried out if cross reactions are still present (Thorley and Day, 1986).

9.10.5 Agglutination Tests for Serogrouping

9.10.5.1. Slide agglutination test

These tests are carried out on clean, 75 x 25 mm glass microscope slides. The appropriate undiluted high titred rabbit or sheep serum is dispensed using a 2.0 mm flamed nichrome wire loop, ensuring that a full loop is used.

Preferably two pools of antiserums representative of serogroups A, B, C, D, E, and F, G, H, and I, respectively, should be used. If a positive reaction is obtained with one of the pools, then individual antisera represented in that pool can be tested using four drops per slide. The antigen (usually plate antigen) at a concentration of about 5 x 10^9 bacteria/mL is dispensed with a 3.0 mm loop and thoroughly mixed with the serum on the slide. It is essential that a full loop of antigen is used to ensure consistent results.

The slide is rocked back and forth several times and examined. A positive reaction is recorded when heavy floccular agglutination occurs within five seconds of mixing the serum and antigen. Reactions occurring after five seconds are finer agglutinations and are not serogroup-specific.

Sheep antisera should be diluted 1/10 before use in the slide agglutination test to remove low-titre cross-reactions and thereby increase the serogroup specificity of the antiserum.

9.10.5.2. Tube agglutination test

This test is performed essentially as described by Egerton (1973). The sera are dispensed in 0.1 mL volumes into 10 x 87 mm clean rimless glass tubes, following which 0.9 mL of 0.15 mol/L *saline* is added, to give an initial dilution of 1/10. Doubling dilutions in 0.5 mL volumes in *saline* are then prepared.

Antigen prepared from the appropriate strain of *D. nodosus* and containing about 10^8 bacteria/mL is then added to each tube. The final serum dilution in the first tube of each series is, therefore, 1/20.

Tests are held in an incubator for four hours at 37°C before reading or they may be incubated overnight. Before recording the results, the tests should be stood at room temperature for about an hour. The titre of the serum is recorded as the reciprocal of the highest dilution in which typical, loose, floccular 'K' type agglutination is visible. A reference serum with known titre should be included in each batch of tests.

9.10.5.3. Microtitre plate agglutination test

The procedure described was adapted from that originally developed by D.L. Emery (unpublished data, 1986).

9.10.5.3.1. Materials.

(a) PBS tablets (Oxoid). Dissolve 10 tablets in 1 L of distilled water.

(b) Titertek multichannel pipettes (eight channels), 50–200 µL with tip ejector. (Cat. No. 7785700; formerly Flow Laboratories, now ICN Biomedicals, PO Box 187, Seven Hills, NSW).

(c) Titertek brand, non-sterile pipette tips, bands of four (100 per box) (Cat. No. 7789605).

(d) Agglutination trays (microtitre plates).

(e) Removawell polystyrene rigid strip holders to accommodate eight strips of 12 wells (Dynatech Laboratories; Cat. No. DY011-010-6604).
(f) Immulon 1 & 2 strips of 12 flat-bottom wells (disposable) (Dynatech Laboratories; Cat. No. DY011-011-6301). Pacific Diagnostics Pty Ltd, PO Box 658, Archerfield, Qld 4108, Australia.
(g) One hundred watt (blue coloured daylight) bulbs (not UV) to facilitate easier reading of positive reactions, although good reactions should still be clearly visible to the naked eye.

9.10.5.3.2. Spot serogrouping test. To microtitre plate add the following.

(a) PBS (100 µL) to every well.
(b) Stock antiserum (100 µL) (diluted 1/20 with PBS) to the first well, mix, then transfer 100 µL of the diluted antiserum from the first well to the second well, mix, then discard 100 µL from second well. This gives 1/40 and 1/80 dilutions of antisera. The microtitre trays have eight rows labelled A–H with 12 wells in each row. Thus on one microtitre plate eight antigens can be tested against six antisera.
(c) To each row add 100 µL of the appropriate antigen which has been diluted to a standard concentration of 10^8 organisms/mL with PBS. After adding the antigen, the final dilutions will be 1/80 and 1/160, respectively.
(d) The microtitre plates are then carefully wrapped in clear plastic (Cling Wrap, Alcan Foil Products) and incubated at 37°C overnight.
(e) When read against a blue light, positive (+) responses have distinct cotton wool-like precipitate in the well, while negative responses are clear. Sometimes the wells need to be gently rocked to disturb settled precipitate from the bottom of the well. There may also be very tiny precipitates floating in the well which are usually taken as being negative. Poor antigens with few pili can produce this type of reaction.

9.10.5.3.3. Full serogrouping test. A full test is usually only done on an antigen which has shown a positive response in both the 1/80 and 1/160 wells.

(a) Add 100 µL PBS to every well.
(b) Add 100 µL of stock antiserum (diluted 1/20) to the first well, mixed well and serially diluted across the row from well 1–12, producing a range of dilutions from 1/40 to 1/81 920.
(c) Then 100 µL of the appropriate antigen is added to each well in the row of antiserum dilutions. After adding the antigen, the dilutions are now from 1/80 to 1/163 840.
(d) The microtitre plates are incubated and read in the same manner as the spot test plates.

9.10.6. Agglutination Test for Serology of Vaccinated Sheep

Usually the lower dilutions of 1/20 to 1/40 960 are used with the initial bleeds, up to the second vaccination when antibody production is expected to be within these limits. Thereafter, dilutions of 1/200 to 1/204 800 are used.

9.10.6.1. Low antibody testing

(a) Add 100 µL PBS to every well, then add a further 80 µL PBS to only the first well of each row.
(b) Add 20 µL of stock antiserum to the first well, mix well then serially transfer 100 µL down the row. Discard the excess 100 µL, once well 12 has been diluted and mixed.
(c) Add 100 µL of the appropriate standardised antigen to every well.
(d) Incubate and read as previously described. Final dilutions will be 1/20 to 1/40 960.

9.10.6.2. High antibody testing

(a) Add 100 µL PBS to every well, then add 80 µL PBS to the first and second well of each row.
(b) Add 20 µL of stock antiserum to the first well, *mix*, transfer 20 µL to the second well and mix. Transfer 100 µL from the second to the third well and serially dilute down the row. Discard the excess 100 µL once well 12 has been diluted and mixed.
(c) Add 100 µL of the appropriate standardised antigen to wells 2–12.
(d) Incubate and read as previously described. Final dilutions will be 1/200 to 1/204 800.

9.11. Appendix 11 — Suppliers

Amrad Pharmacia Biotech. Unit A, 25–27 Paul St, (PO Box 1775) North Ryde, NSW 2113. Tel. (02) 367 4200, (008) 252 265; Fax (02) 367 4251.

BBL. Becton Dickinson Pty Ltd, 2A, 15 Orion Road, Lane Cove, NSW 2066. Tel. (02) 418 6166; Fax (02) 418 6881.

BDH Merck. 207 Colchester Rd, Kilsyth, Vic. 3137. Tel. (03) 728 5855; Fax (03) 728 1351.

Biorad. Biorad Laboratories , 96 Camberwell Rd, East Hawthorn, Vic. 3123. Tel. (03) 882 8391, (008) 224 354; Fax (03) 805 1920.

Calbiochem. Calbiochem, PO Box 140, Alexandria, NSW 2137. Tel. (02) 318 0322; Fax (02) 319 2944.

CSL Ltd. 45 Poplar Rd, Parkville, Vic. 3052. Tel. (03) 389 1911, (008) 032 675; Fax (03) 389 1434.

Difco. FSE Pty Ltd, Unit 3, 149 Arthur St, Homebush, NSW 2140. Tel. (02) 746 1122; Fax (02) 273 7122.

Dynatech. Pacific Diagnostics, PO Box 185, Seven Hills, NSW 2147. Tel. (02) 674 3800; Fax (02) 273 7122.

Gallay Scientific. 145 Errol St, North Melbourne, Vic. 3051. Tel. (03) 329 8584; Fax (03) 329 8985.

Gibco. Gibco BRL Division, PO Box 366, Glen Waverley, Vic. 3150. Tel. (03) 562 8245,

(008) 331 627; Fax (03) 543 7542.

Hardie Health Care. 2 Eskay Rd, Oakleigh, Vic. 3166. Tel. (03) 579 4055; Fax (03) 579 4853.

ICN Biomedicals. PO Box 187, Seven Hills, NSW 2147. Tel. (02) 838 7422; Fax (02) 838 7390.

Mancor. Bleakley Fine Chemicals, 24 Fariola St, Silverwater, NSW 2141. Tel. (02) 648 3222; Fax (02) 647 1009.

Oxoid. Oxoid Australia. PO Box 220, Heidelberg West, Vic. 3081. Tel. (03) 458 1311, (008) 331 163; Fax (03) 458 4759.

Pacific Diagnostics. PO Box 658, Archfield, Qld 4108. Tel. (07) 273 7111, (008) 777 713; Fax (07) 273 7122.

Sigma. Sigma–Aldrich, Unit 2, 10 Anella Ave., Castle Hill, NSW 2154. Tel. (008) 800 097; Fax (008) 800 096.