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Serology and Virulence of *D. nodosus*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Serology and Virulence of *D. nodosus*" submitted by Sharon M. Gard in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Ovine footrot is regarded as the most economically important, contagious disease of sheep in temperate climates. The causative agent, *D. nodosus (Dichelobacter nodosus)*, is an obligate parasite of the hoof epidermis that functions in synergy with ubiquitous microorganisms.

To date there have been no Canadian studies published that attempt to identify the nature and occurrence of *D. nodosus*. More specifically the proteolytic activity of the organism as an indicator of virulence and serological identity based on the antigenic variation of the fimbriae of *D. nodosus*. This information has application epidemiologically and in disease management and control programs.

The principal conclusions of this study were the identification of hide powder azure assay as a reasonable measurement of proteolytic activity and the presence of a variety of serological strains, as defined by pilus mediated agglutination, including one previously unrecognized serotype, of Alberta and British Columbia isolates of *Dichelobacter nodosus*.

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1. INTRODUCTION

Ovine footrot is a contagious, debilitating disease characterized by bacterial invasion of the epidermis of the interdigital skin and horn of the hoof. Morbidity may exceed fifty percent (Bulgin, 1985). Combined with the increased labor costs and the decrease in weight, wool and lamb production associated with infection, footrot can be economically devastating to the sheep producer.

The disease has been recognized in sheep for more than 250 years (Deane and Jensen, 1955, Youatt, 1837). Its contagious nature was recorded in France in 1813 (Gohier, 1813) and Brown, in 1892, established the transmissibility of the disease using pasture studies (Brown, 1892). The requirement for a specific infective organism was later confirmed by Gregory (1939).

A history of the disease in Europe, U.S.A. and Australia was first reviewed in 1904 (Mohler, 1904). However, it was not until 1941 that the gram negative, obligately anaerobic bacillus, *Fusiformis nodosus*, was recognized as being able to act alone in producing ovine footrot (Beveridge, 1941). Beveridge demonstrated histologically the inflammation and epithelial cell degeneration which results in separation of the hoof. Beveridge's observations were confirmed by Thomas who demonstrated the organism histologically within footrot lesions following experimental infection with a pure culture of the organism (Thomas, 1962).

1.1 Taxonomic status of *Dichelobacter nodosus*

The etiologic agent of ovine footrot is most commonly referred to as *Bacteroides nodosus* (Cato, 1979). Synonyms include *organism K* (Beveridge, 1938), *Fusiformis nodosus* (Beveridge, 1941) and *Ristella nodosus* (Prevot, 1948). However, the taxonomic classification of the organism, historically has been based on phenotypic criteria, and has been uncertain for some time (Johnson and Harich, 1986, Shah and Collins, 1989, Stewart,1977).

Recently the distal (3') portion of a 16S rRNA gene from the type strain A198, ATCC 25549 (Cato, 1979, Krieg, 1984) was cloned, mapped and sequenced (Fontaine *et al.*, 1990). This indicated that the common gnereic status was incorrect and a comparison of the sequence with other bacteria indicated a closer relationship with members of the class *Proteobacteria.* Further study revealed the complete rRNA sequence (Dewhirst *et al.*, 1989). A comparison to the nine beta division proteobacteria and nine gamma division proteobacteria determined the phylogenetic relationship. A proposal has therefore been put forth to transfer the organism to the new genus *Dichelobacter* as *Dichelobacter nodosus* in the new family *Cardiobacteriacae* in the gamma division of *Proteobacteria* (Dewhirst *et al.*, 1989).

1.2. Human infection with *Dichelobacter nodosus*

D. nodosus is not part of the normal microbial flora of humans (Kirby *et al.*, 1980) and is generally not considered a cause of human disease. It has been reportedly isolated from a dental abscess (Chow *et al.*, 1978), female reproductive tract infection (Monif *et al.*,1977), pneumonic lung (Thadepalli *et al.*,1977), meninges (Peterson *et al.*,1979) and surgical debridement material of infected bone (Gristina *et al.*, 1985). Minimal identification information was provided in these reports and a request for further data and/or isolates was unanswered (Gristina, 1990). It is doubtful that these strains compare to the type strain of *D. nodosus* (ATCC 25549). Four human isolates identified as *D. nodosus* and recovered from a pilonidal cyst, rectal fistula, decubitous ulcer, and leg wound respectively were evaluated by Gradin (Krieg, 1984) using a fluorescent antibody conjugate (Burroughs Wellcome, England) and growth on Eugon Agar (BBL Microbiology Systems, Baltimore). None were reactive with the conjugate and the colonial morphologies were not consistent with ovine isolates (Cato *et al.*, 1979). It appears that the reported human isolates of this organism are distinct from the footrot organism (Kirby *et al.*, 1980) and although phenotypically similar are probably not *D. nodosus* (Krieg, 1984).

1.3. Occurrence of *D. nodosus* infection in animals

Footrot in ruminants is widespread in temperate regions (Claxton *et al.*,1986, Forschell *et al.*,1981, Gupta *et al.*, 1986, Hussein *et al.*, 1984, Masalski, 1983, Toussaint *et al.*, 1971). Feedlot and dairy cattle experience a high morbidity due to the disease and producers can suffer severe economic losses (Adams, 1960, Johnson *et al.*, 1969, Weaver, 1971).

Although some controversy exists over the etiological agent of footrot in animals other than sheep, bacteria with the cultural characteristics of *D. nodosus* have been isolated in cattle (Alexander, 1962, Skenman, 1962). In addition *D. nodosus* infection has been transmitted from cattle to sheep with isolation of the organism from the resulting disease condition in sheep (Egerton and Parsonson, 1966). Less invasive footrot than that seen in sheep was also induced in calves by exposure to invasive ovine isolates of *D. nodosus* (Egerton and Parsonson, 1966).

Footrot in goats is reportedly restricted to the interdigital skin regardless of the virulence of the infecting strain of *D. nodosus* (Stewart *et al.*, 1986). Intense tissue inflammation and serous exudate may be evident although underrunning and separation of the horn of the heel are seldom seen (Stewart *et al.*, 1986).

Deer may develop a similar interdigital infection pattern to that of goats and underrunning has been observed (Skerman, 1983).

It is in sheep, however, that the focus of disease control has occurred. They are most affected by potentially invasive strains (Stewart, 1989). The chronic, endemic nature of footrot results in it being the most economically important contagious disease in sheep (Beveridge, 1941). Indeed morbidity can approach 100% if climatic conditions favour transmission (Emery, 1988). Severe extensive necrotic underrunning of the hoof is characterized by lameness and subsequent emaciation as the animal finds it painful to move and graze normally. The animal may use sternal recumbency to compensate and in chronically affected sheep this results in necrosis and ulceration of the sternum. Mortality resulting from combination of emaciation and systemic bacterial infection may occur.

1.4. Definition of ovine footrot

Sheep footrot was a term reserved by researchers for a contagious, chronic condition characterized by inflammation which progresses to lameness, ulceration and separation of the hoof (Mohler *et al.*, 1904). More recently the infection is described as beginning with an area of interdigital hyperemia which develops into necrosis spreading to the heel, sole and wall eventually undermining the corneum (Marsh, 1954).

Four diseases that affect the ovine hoof have been defined (Roberts, 1969). The first, O.I.D. or Ovine Interdigital Dermatitis, is a superficial infection, the etiologies of which include *Fusobacterium necrophorum* and *Actinomyces pyogenes*. A deeper infection caused by these same organisms and affecting the bulb of the heel is termed I.B.N. or Infective Bulbar Necrosis. O.I.D. complicated with a third organism, *D. nodosus*, identifies the other two diseases - benign and virulent footrot. The clinical observation of the degree of penetration of the infection defines the difference between benign and virulent ovine foot rot. Benign strains of *D. nodosus* produce a chronic dermatitis. Virulent strains extend the infection into the epidermal matrix of the hoof (Egerton and Parsonson, 1969, Egerton *et al.*, 1969, Roberts and Egerton, 1969, Thomas, 1962).

1.4.1. Quarantine legislation

The definition of infection becomes an important issue in attempts to legislate control of the spread of ovine footrot. In Australia virulent, but not benign, footrot is a notifiable disease under the Stock Diseases Acts (Egerton *et al.*, 1989). Regulatory control measures serve to restrict exposure of disease free flocks by notification of infection, quarantine and treatment of diagnosed animals. Eradication programs empower stock inspectors to enter suspect properties and organize inspections. Legal provisions provide for the slaughter of contact and infected sheep and the publication of quarantined flock locations.

In Alberta ovine footrot is regulated under the Livestock Diseases Act, Alberta Regulation 383/72 (Alberta Gazette, 1972). Under these regulations the flock owner is required to quarantine affected and exposed sheep. A veterinary inspector has the authorization to effect and terminate quarantine, restrict movement of the footrot infected animals and prescribe treatment. Provisions under the act also allow for inspection and certification of sheep brought into Alberta. Animals exposed to footrot within the preceding six months are denied certification. Due to expanding use of Alberta sheep on summer pasture in the "clearcut" regions of British Columbia logged forests the potential for exposure of local flocks to footrot has increased dramatically (Fenton, 1988).

1.5. Factors affecting natural infection

Infectious ovine footrot, as a disease process, is affected by a number of factors. These include not only characteristics of the bacteria involved but intrinsic and extrinsic factors that affect the host.

1.5.1 Environmental factors

It was once thought that footrot was the direct product of favourable climatic conditions (Beveridge, 1963). It is now known that environmental factors such as warm temperatures, wet soil, lush grazing and improved pasture act only to enhance transmission of the footrot organism (Beveridge, 1963, Egerton *et al.*, 1972, Egerton *et al.*, 1983, Graham *et al.*, 1968). The occurrence of footrot is seasonal with outbreaks coincident with increased temperatures and moisture (Graham *et al.*, 1968). In Alberta this occurs in the spring and early summer. In late summer and in winter the environment becomes drier and transmission rates decrease as the dehydrated interdigital skin provides an effective barrier to invasion (Beveridge, 1963, Fenton, 1988). In addition, a lower atmospheric temperature in the fall and winter inhibits transmission as an ambient temperature of above 10^{o}C is required (Graham, 1968). Also there is less blood flow in the hoof in winter which produces a lowered hoof temperature (Graham, 1968). This inhibits invasion of *D. nodosus* into the surrounding tissue, reducing multiplication and subsequent quantity of infective material capable of environmental contamination (Beveridge, 1963)

D. nodosus is an obligate anaerobe (Beveridge, 1963, Prescott *et al.*, 1986, Egerton *et al.*, 1969) and its oxygen sensitivity limits its survival outside the host tissue. It has been determined that the causative organism, *D. nodosus*, survives a maximum of fourteen days in feces, soil and pasture (Beveridge, 1941). A later study demonstrated the viablility of the organism for a maximum four days in muddy soil (Laing and Egerton, 1981).

1.5.2. Transmission of *D. nodosus*

Sheep are generally infected by direct exposure to the infective organism as it is shed onto the ground from active lesions in infected animals (Beveridge, 1941, Fenton, 1988). The social behaviour of sheep of walking single file and the increasing practice of feedlot production increases the potential for exposure. Reports have implicated mechanical transmission via vehicle tires, human footware, transport vehicle platforms, surface water, wild animals and hoof paring instruments (Beveridge, 1983, Fenton, 1988, Stewart *et al.*, 1982, Stewart *et al.*, 1984).

In addition, trauma to the foot caused by poor pasture or corral conditions will increase an animals susceptibility to infection by providing a portal of entry for the organism (Fenton, 1988). Immune depletion from a variety of stresses, relative incidence of non-footrot disease and bacterial and non-bacterial parasite load are generally accepted contributory factors that lower non-specific host defences (Mimms, 1987).

D. nodosus is an obligate parasite and is not known to proliferate anywhere other than the infected foot (Roberts and Egerton, 1969). The chronically infected animal thus constitutes a major reservoir of infection as the organism is entirely host dependent for its survival. The presence of carrier animals is an important factor in peristence of the disease within a flock and transmission to other animals (Roberts and Egerton, 1969, Fenton, 1988).

1.5.3. Genetic predisposition to footrot

Several reports indicate that crossbred animals were less susceptible to footrot involving virulent strains than purebred animals (Beveridge, 1941, Egerton *et al.*, 1972). This observation was confirmed with pen studies using a virulent challenge strain of *D.nodosus* (Skerman *et al.*, 1982). When the susceptibility to footrot of five breeds was tested in another controlled study (Emery *et al.*, 1964), it was determined that vulnerability was related more to interdigital epidermal integrity than an immune response. Scarification of the interdigital skin, prior to experimental infection, nullified resistance as similarly severe lesions could be produced in all breeds. Humoral immune responses, as measured by leucocytic activity and rate of antibody production were not elevated until stimulated by deeper tissue penetration of the organism in both resistant and susceptible sheep. Emery (Emery and

Stewart, 1984) later observed that the rate of phagocytosis of *D. nodosus* is similar even when the PMN's are derived from sheep breeds of differing footrot susceptibility.

This association was further defined by examining particular MHC Class 1 lymphocyte antigens (Outteridge *et al.*, 1989). It was reported that ovine lymphocyte antigen types SY6 and SY1b were associated with high responsiveness to vaccination and resistance to footrot respectively. Such markers, it was suggested, would be useful in the selection and breeding of footrot resistant sheep (Outteridge *et al.*, 1989).

1.6. Synergistic Mechanisms and Pathogenesis

Microbial synergism plays an important role in naturally acquired footrot infections. Experimentally, *D. nodosus* is capable of producing infection without the aid of other organisms (Beveridge, 1963, Thomas, 1962). However, virulence in pathogenic bacteria is generally a result of a number of factors and gram negative anaerobic bacteria are more often found in polymicrobial infections (Prescott *et al.*, 1986). It has been demonstrated that a combination of *D. nodosus* and *Fusobacterium necrophorum* with or without the action of *Actinomyces pyogenes* is the minimum bacterial requirement to induce naturally acquired footrot. In addition a motile fusiform bacterium and an unidentified anaerobic spirochete are contributing factors to the infectious process (Beveridge, 1963, Egerton *et al.*,1969, Prescott *et al.*, 1986).

Investigation into the mechanisms of synergy in ovine footrot has indicated that an infection begins with the colonization of the stratum corneum with aerobic organisms, mainly corynebacteria and cocci. These organisms function to use available oxygen, decrease Eh and supply catalase which assists in the establishment of a local anaerobic microenvironment and the anaerobic organisms, most importantly *Fusobacterium necrophorum* (Roberts, 1969). By extrapolation from human studies of anerobic, synergistic, infections that have demonstrated growth factor production of isobutyric acid and putrescine (Socransky *et al.*, 1964), it is postulated that the interaction of a corynebacteria and *Fusobacterium spp.* supply growth factors to endogenous spirochetes.

The infection is furthered by the invasive and tissue destructive properties of *F. necrophorum* plus the elaboration of leukocidal toxin either an exotoxin and/or an endotoxin. As described, a leukocidal exotoxin is believed to be produced by *F. necrophorum* which is proteinaceous in nature, heat stable to 60^{o}C and destroyed by proteolytic, but not other degradative, enzymes (Coyle-Dennis and Lauerman, 1978). An endotoxin was described by Fales *et al.*, 1977 that was heat stable to 100^{o}C. Roberts, 1969 reported an exotoxin that was cytotoxic *in vivo.* PMN leukocytes attracted to the infection site are arrested and destroyed by the leukocidal activity of the elaborated toxins of *F. necrophorum.* Inflammatory factors released during cell destruction further tissue damage. While inactivation of the phagocytic process provides protection to other bacteria this is not thought to be significant as there is little opportunity for phagocytosis in the epidermal tissue (Roberts, 1969).

A heat labile, undialyzable macromolecular growth factor supplied by *Actinomyces pyogenes* further accentuates the replication of the anaerobes (Roberts, 1967 II). The resultant tissue damage and hyperkeratosis allows for the colonization and proliferation of *D. nodosus* by providing a suitable microenvironment of vacuoles, cavities and microabscesses (Deane and Jensen, 1955). This chronic state permits deeper penetration with progressive invasion of the horn laminae and supplies a heat stable factor that contributes to the growth of *F. necrophorum.* The factor supplied by *D. nodosus* is heat stable to 100^{o}C (Egerton *et al.,* 1969, Roberts and Egerton, 1969). *D. nodosus* is invasive but its presence in epidermal tissue alone is not associated with inflammation and tissue destruction (Roberts and Egerton, 1969). The growth of *D. nodosus* in tissue is characteristically slow but steady thus sustaining a chronic irritation that precludes healing and maintains the chronic nature of footrot infection, both benign and virulent (Roberts, 1969). *F. necrophorum* while unable to cause virulent footrot by itself is, in turn, responsible for much of the tissue damage. In contrast to *D. nodosus*, *F. necrophorum* growth in tissue is characterized by periods of accelerated invasion by the organism (Roberts, 1969).

A fourth organism, an unidentified motile fusiformic bacterium, can be encountered. It supplies a soluble, heat labile factor known to stimulate the growth of *D. nodosus* (Roberts, 1969).

1.7. Pathogenesis and pathology of ovine footrot

Virulent and benign footrot are differentiated based on the extent of penetration of the infection from the interdigital epidermis to the contiguous epidermal matrix of the foot and by the pathogenic properties of strains of *Dichelobacter nodosus* associated with the infections (Roberts, 1969). Establishment of ovine footrot infection involves the classic disease properties of adherence, colonization, elaboration of virulence factors and proliferation. The pathogenesis of *D. nodosus* has been further defined by Smith and Gradin (Smith and Gradin, 1988) into sequential steps of adherence, colonization, protease production, lysis of hoof, penetration of hoof, secondary invasion, chronic disease and/or spontaneous recovery. *D. nodosus* pili are believed responsible, by extrapolation from similar disease conditions (Emery 1979), for a significant amount of adherence activity although this is has yet to be documented (Stewart, 1975) . Colonization is dependent on the specific site requirements of reduced oxygen with raised ambient temperature and moist conditions further contributing as previously discussed . Protease production by the organism produces lysis of the hoof which progresses to deeper penetration. At this point secondary invasion by a variety of ubiquitous organisms, predominantly *Fusobacterium necrophorum* occurs. As previously discussed a microbial synergism is then achieved which furthers the infection.

Initial studies (Beveridge, 1941) limited active lesions of ovine footrot to the unkeratinized epidermis where inflammatory processes produced detachment from the keratinized horn. Other researchers (Deane and Jensen, 1955) identified the *stratum granulosum* and deeper layer *stratum spinosum* as infection sites and showed that necrosis as being responsible for detachment. The direct relationship of the presence of *D. nodosus* with degeneration, detachment, and enzymatic attack of the partially keratinized *stratum lucidum* and *stratum spinosum* and necrosis in the keratinized horn was identified by investigators (Stewart *et al*, 1982, Thomas, 1962, Thomas, 1964). The animal's response to infection is to accelerate hoof growth and sheep with grossly disfigured feet are easily recognized as infected animals (Fenton, 1988). If sufficient proteolytic activity occurs the rate of underrunning will exceed the rate of hoof growth and the underlying tissue will be exposed as the horn separates.

Histological study consistently identified *D. nodosus* to be present at the point of cleavage at the interface of the *stratum lucidum* and *stratum granulosum* (Egerton *et al.*, 1989).

Circulating antibodies, if present, may be carried into the infected area where disruption of adherence may occur. Leukocytes have been identifed as present at the infection site (Thomas, 1962). This may be counteracted by a leukocidal toxin produced by *F. necrophorum* (Roberts, 1969) the effects of which are partially responsible for tissue destruction as previously described. Seasonal recovery in dry and/or cold conditions is related to the loss of ideal conditions for colonization and penetration.

1.8. Virulence determinants of *Dichelobacter nodosus*

1.8.1. Fimbriae

Investigations of fimbriae of *D. nodosus* demonstrate a primary role in pathogenicity and immunological response in ovine footrot (Every *et al.*, 1982, Emery *et al.*, 1984, Skerman *et al.*, 1981, Stewart, 1978). It has been suggested (Every, 1979) that the *D. nodosus* fimbriae most resemble the non-conjugative pili group which mediate attachment to or locomotion over host cell surfaces (Ottow, 1975). In contrast, the sex pili are involved in the transfer of genetic information (Brinton, 1965). The nonsex pili are defined by polar location and appear to be involved in surface translocation through "twitching motility" (Henrichsen, 1975). Protein sequence studies (Elleman *et al.*, 1986, Marrs *et al.*, 1985, McKern *et al.*, 1983,) have noted the highly conserved nature of the amino-terminal sequence of nonsex versus sex pili notably those exhibiting "twitching motility". The first 30 amino acid residues of this group are virtually identical and the following 25 highly conserved. Pilin molecular size in this group ranges from 16 to 18 kDa and exhibit sequence similarity in the carboxy-terminal region (Elleman *et al*, 1986).

The pilins of *D. nodosus* are of the modified amino-terminal residue N-methylphenylalanine or mePhe group (Elleman, 1988, Elleman and Hoyne, 1984). Characterization studies note their diameter as 5 - 6 nanometers with a length up to 10 microns (Stewart, 1973, Walker *et al.*, 1973, Every, 1979). Each is a homopolymer of the protein pilin of molecular weight 16,000 daltons with integrity maintained by noncovalent forces (Every, 1979). The first 32 residues of the N-terminal of the *D. nodosus* pilin protein are nearly identical with the nonsex pili group, are hydrophobic and closely resemble pilins of bacterial species that exhibit "twitching motility" including *Pseudomonas aeruginosa* (Sastry, 1985), *Moraxella bovis* (Marrs,1985) and *Neisseria gonorrhoea* (Schoolnik, 1984). Twitching motility has been associated with the establishment of ovine footrot infection (Depiazzi and Richards, 1985, Depiazzi *et al.*, 1991).

Fimbriae of these species are usually polar in distribution as are the fimbriae of *D. nodosus* (Elleman, 1988, Gradin *et al.*, 1991). Adherence, as a virulence function of *D. nodosus* fimbriae, could not be demonstrated (Stewart, 1975). However, it is believed to exist through analogy to other bacterial models (Emery, 1984).

1.8.2 Outer Membrane Proteins (OMP)

Six major outer membrane proteins have been extracted from the cell wall of *D. nodosus* (Emery *et al.*, 1984). They include the pilin protein with a molecular weight of 17,500 daltons (Every, 1979, O'Donnell *et al.*, 1983, Emery *et al.* 1984) and five other outer membrane proteins with molecular weights of 75,000, 50,000, 38,000, 34,500 and 26,500 (Emery *et al*, 1984). The 75 kDa OMP most likely functions to anchor the fimbriae in a manner similar to the basal protein of *E. coli* while the remaining four OMP are thought to have a structural function (Emery *et al*, 1984). Similar antigenicity was determined for the OMP and cross-protection *in vivo* was not observed (Emery, 1984, Emery *et al.*, 1985). The outer membrane protein may be a target for bactericidal action of serum (Emery, 1984).

1.8.3. Lipopolysaccharide

D. nodosus has an enterobacterial-type LPS characterized as containing 2-keto-3-deoxyoctulosonate and heptose (Emery *et al.*, 1984, Stewart, 1977). Although endotoxic activity includes pyrogenicity and the production of leucopenia, potency of the LPS endotoxin is low and is not a major virulence factor among clinical isolates (Stewart, 1977). The active release of endotoxin by cell wall blebs as a function of gram negative pathogenicity was not observed in ultrastructure studies of *D. nodosus* (Stewart and Egerton, 1979).

1.8.4 Capsule and diffuse polar material

Stewart and Egerton (1979) observed capsular material in electron microscopic studies of *D. nodosus.* Visualization only with the use of heavy metal salts indicated that they were not predominantly polysaccharide although the chemical composition of the capsule remains to be determined. Gradin *et al.*, 1991 observed a slime layer or capsular material in electron microscopic studies but its production was irregular and varied in thickness.

In a comparative electron microscopic study of virulent and benign strains of *D. nodosus* (Every and Skerman, 1983) diffuse polar material was observed and it was suggested that this layer was associated with virulence and immunoprotection. As *D. nodosus* pili have an unproven adhesive function (Stewart, 1975), it is postulated that the additional layer may have a role in adhesion (Every and Skerman, 1975) as is seen in gram negative rumen bacteria (Costerton *et al.*, 1974).

1.8.5. Proteases

D. nodosus proteinases are serine-type enzymes with an activity similar to that of chymotrypsin requiring calcium ions for activity (Kortt *et al.*, 1982) and stability (Rose, 1972). Inhibitor studies of *D. nodosus* protease activity confirmed that they are chymotrypsin-like serine proteases (Broad and Skerman, 1976, Green, 1985) and such divalent metal ions as calcium, magnesium and manganese but not heavy the metal ions zinc or iron are required for activity. The affinity of *D. nodosus* proteases for the seryl-specific ligand, phenyl borate further demonstrate the serine nature of the enzyme (Gordon *et al.*, 1985). Serine peptidase mechanisms include formation of an ester between the oxygen of serine 195 and the acyl portion of the substrate and the presence of two -NH- groups that become available for hydrogen bonding to the oxygen anion derived from the attacked peptide bond (Dunn, 1989).

Although ovine footrot is restricted to the avascular epidermis (Egerton *et al.*, 1969) infected animals develop antibody titers to a number of *D. nodosus* antigens (Egerton and Merritt, 1973). Specific antibodies associated with the IgG2 and IgG1 immunoglobulins obtained from experimentally footrot infected sheep have the ability to neutralize the proteolytic activity of the organism in vitro (Merritt and Egerton, 1978). Purified proteases as cross-protective antigens proved to be prophylactic *in vivo* by providing broad spectrum protection against a variety of serotypes (Stewart *et al.*, 1986). The recommended treatment of footrot infection with the topical application of 10% zinc sulfate is believed effective due to its bactericidal qualities and the chemicals inactivation of the proteolytic enzymes produced by the organism (Cross,1978).

Protease function of *D. nodosus* was noted during initial *in vitro* investigations of the organism (Beveridge, 1941). It was attributed to keratinase activity and subsequent destruction of the hoof during experimental infection with pure cultures of *D. nodosus* (Thomas, 1962). Further studies (Thomas, 1964) attempted to explain the localization of the infection in the epidermal tissues and its failure to spread into the dermis. Preparations of extracellular enzymes of the organism were able to weakly hydrolyze elastin but not hyaluronic acid, chondroitin sulfate or collagen. Thomas concluded that since all these substrates are contained in the dermis while only elastin exists in the epidermis, the absence of hyaluronidase, chrondroitinase and collagenase and the existence of a weak elastase extracellularly in the organism would account for the localization of infection in the hoof. When studies were undertaken using improved culture techniques, degradative action of a purified protease preparation on fibrin, gelatin, hoof and hide powders was noted (Broad and Skerman, 1976). Proteinase isoenzyme studies of *D. nodosus* strains (Green, 1985) confirmed the hydrolization of casein, elastin, fibrin and haemoglobin noted by others (Broad and Skerman, 1976, Thomas, 1964, Egerton and Parsonson, 1969) but also noted substrate specificity for collagen I, collagen III and alpha keratin when soft keratin, isolated from the *stratum corneum*, rather than hard keratin from wool, was used.

Although the functional significance of the protease in not known, it is thought to be used to harvest nutrients (Emery, 1988).

The molecular weights of the extracellular proteases of *D. nodosus* is a controversial area of research. Kortt *et al.*, 1986 used gel-filtration, nondenaturing gradient polyacrylamide gel electrophoresis, and SDS-polyacrylamide gel electrophoresis to determine a molecular weight of 40 to 42 kilodaltons. Every, 1982 determined the molecular weight of *D. nodosus* extracellular proteases of 70 to 129 kDa using gradient polyacrylamide gels. Every and Green, 1982 purified five proteases from one strain of *D. nodosus* and ascertained their molecular weights as 78, 82, 88, 96 and 107 kDa using gradient polyacrylamide gel electrophoresis. Using gel-filtration other researchers (Depiazzi and Rood, 1984, Gordon *et al.*, 1985) reported molecular weights of 17 to 20 kDa and 8 to 43 kDa. It is suggested that autolysis of the relatively stable proteases may account for the discrepancies and that protease determination can not be used as a virulence determinant (Kortt *et al.*, 1982, Kortt *et al.*, 1983).

1.9. Fimbrial antigen of *Dichelobacter nodosus*

1.9.1. Immune response elicited by fimbrial antigen of *Dichelobacter nodosus*

The humoral immune response to fimbriae of *D. nodosus* was first reported by Walker *et al.*, 1973. Pilus based vaccines have proven useful in both the prevention and therapy of ovine footrot (Bagley *et al.*, 1987, Bulgin *et al.*, 1985, Glenn *et al*, 1985, Lewis *et al*, 1989) especially if vaccines are polyvalent and circulating antibody titres are high (Smith, *et al.*, 1990). The role of various antigenic fractions of *D. nodosus* in eliciting protection against foot-rot in vaccinated sheep was studied by Stewart, 1978. He demonstrated that the protective immunogen was associated with the fimbriae of the organism, being a heat-labile, high molecular weight, surface antigen. Agglutinating antibodies were predominantly IgG_1 and not IgM which are associated with the O not fimbrial antigen.

According to investigators the property of the fimbriae to induce agglutinating antibodies is dependent on an intrachain disulfide bond (Emery *et al.*, 1984). Early studies (Beesley *et al.*, 1984) indicated the existence of more than one antigenic epitope on one individual pilus. Using immunogold labelling (Day *et al.*, 1986) 3 distinct antigenic epitopes were determined on the pili of each of 3 strains. Each strain possessed one unique antigen and one of the other two was shared with a third strain. Thus a total of 6 different pilus antigens were shared among the 3 strains. Immunogold studies further demonstrated that pilus antigens are not localized to separate regions of the pilus. Cross agglutination studies demonstrated at least 4 distinct antigens on the pili of each of the 3 strains with a total of 7 different antigens shared among the 3 strains (Day *et al.*, 1986).

The location and nature of pilus epitopes is of interest in an attempt to provide more comprehensive protection using pilus-based vaccines. Monoclonal antibodies and clones of ovine helper T lymphocytes are being used to define epitopes recognized by B and T lymphocytes. Seventeen monoclonal antibodies were used to analyze the antigenic structure of pilus protein from three serogroups of *D. nodosus* (Young, 1989). Four epitopes on the pilus protein were identified including the agglutinating epitope which was present on the pilus monomer and dependent on the intra-chain disulfide bond.

As little as five micrograms of purified pilus protein incorporated into an oil based adjuvant is sufficient to elicit serotype specific immunity in sheep (Stewart *et al.*, 1986). The ovine footrot vaccine generally in use (*Footvax 10*, Coopers Agropharm, Ontario) contains 10 different serotypes of 8 serogroups A-H of *D. nodosus* (Claxton *et al.*, 1983) of highly piliate cultures of the organism inactivated with formalin and suspended in water-in-oil emulsion (Liadet and Chetwin, 1981). Serotypic classification of *D. nodosus*, as noted herein, has defined 17 serotypes A-H, J-N and O-R (Day *et al.*, 1986, Thorley, 1976). It has been demonstrated that pilus based vaccines containing strains of *D. nodosus* that are homologous to infecting serotypes are more effective (Elleman, 1988, Skerman *et al.*, 1981). This becomes an important point in the continuing control and possible eradication of ovine footrot. As polyvalent vaccines are employed to control homologous strains it then becomes possible for heterologous strains to emerge as dominant infective serotypes. The case would then exist for the isolation, identification and serotyping of contagious, virulent strains of *D. nodosus* and their incorporation into a "custom" homologous vaccine as required. With the current practical limit of eight different serogroups that can be incorporated into a pilus based vaccine such monitoring would identify target strains and correct immunization regimes as needed.

1.9.2. The use of fimbriae in the serotypic classification of *D. nodosus*

The antigenic variation of fimbriae first observed by Beveridge, 1941 can be applied to the serotypic classification of *D. nodosus* (Walker *et al.*, 1973, Short *et al.*, 1976, Claxton, *et al.*, 1983, Anderson, 1984, Elleman, 1988, Smith *et al.*, 1990). Serotypic classification of *D. nodosus* is based on the K-type agglutination reaction with specific antisera (Egerton, 1973). This type of agglutination is pilus mediated (Egerton, 1973).

The first report of the serological classification of *D. nodosus* was of 33 Australian isolates of the organism from clinical cases of benign and virulent footrot (Egerton, 1973). Slide agglutination reactions based on surface or K antigens classified the isolates in 3 serotypes - A, B, and C. Cross tube agglutination tests in Britain later classified local *D. nodosus* isolates into 8 serologically defined groups designated A - H (Thorley, 1976). A subsequent serological survey that included strains from Great Britain and Western Europe expanded the British classification to 17 serotypes: A-H, J-N and O-R (Day *et al.*, 1986). The Australian classification was expanded using slide agglutination by classifying 1,267 isolates into 9 serogroups A - I some of which were divided into subgroups by virtue of cross-tube agglutination testing for a total of 16. (Claxton *et al.*, 1983, Claxton and O'Grady, 1986).

American researchers identified 14 serotypes, designated by Roman numerals as I - XIV (Schmitz and Gradin, 1980). This was updated to a total of 20 serotypes (I - XX) (Smith *et al.*, 1990) and then 21 with the inclusion of XXI (Gradin *et al.*,1992). There have been no reported studies of *Dichelobacter nodosus* isolation and serotypic classification in Canada to date.

The term serotype and serogroup appears to be used interchangeably in the literature. Some British researchers prefer serotype (Day *et al.*, 1986) to serogroup (Claxton *et al.*, 1983) although the latter uses serotype as further refinement of serogroups. American researchers refer to the serogrouping of serotypes with the latter designated by Roman numerals.

Amino acid sequencing of pilins of eight *D. nodosus* serogroups and comparison to each serogroup defined the structural basis of serological variation at the molecular level (Elleman, 1988). Virtual identity was demonstrated between residues 1-32 of serogroups A-H with varying homology in the remaining sequences. Representatives of pilins from serogroups A,B,C,E,F, and G exhibit 60 - 80% identity of sequence and are referred to as A-set pilins (Elleman, 1988). Representatives of serogroups D and H, or D-set pilins, exhibit 68% identity (Elleman, 1988). Comparison of A-set to D-set denotes a less than 40% identity. The disulfide bridge location varies between subsets with a large central loop in A, which has a greater hydrophobicity, while in D two smaller loops are present with a lower relative hydrophobicity.(McKern *et al.*, 1983).

Monoclonal antibody studies of *D. nodosus* have demonstrated several antigenic determinants on the pili and that considerable sharing of these determinants exists between fimbriae types (Gradin *et al*, 1991). Two monoclonal antibodies, 12B10 and JiD3, were used to identify the 2 groups that react with 17 and 4 US serotypes respectively. The epitopes recognized by the monoclonal antibodies encompass all currently recognized *D. nodosus* strains and like the A and D-set may provide a basis for bivalent vaccines (Gradin *et al.*, 1991).

The variation in sequence comparison accounts for low level serological cross reactivity. It is not sufficient to permit cross protection following vaccination as pilus mediated agglutination of bacteria by antibody is believed to be the major mechanism of immunity against footrot (Elleman, 1988). Within serogroups, however, there is sufficient amino acid sequence similarity to provide cross-protection. Serotype A1 and serotype A2 within the serogroup A, exhibit 95% identity. Four serotypes in serogroup B, and 2 in each of A,C,E,F, and G have been identified (Claxton, 1983). In D-set pilins, serogroup D does not have a recognized serological subdivision while serogroup H has 2 serotypes. (Claxton, 1983).

The differing methodologies and designations of *D. nodosus* serotypic classification are confusing. A comparison of classification serotypes was made by Gradin *et al.*, 1992 to resolve some of this. It was demonstrated that at least 12 serologically distinct serogroups of *D. nodosus* exist. Using tube agglutination comparison studies of the 21 US serotypes against the 13 British and Australian prototype strains A - N (there is no I) the US strains were arranged into 11 serogroups. Nine designated as A,B,C,D,E,F,G,H,J, and 2 unique groups (US serotypes IV and XVIII). US serotyype XVIII exhibited a cross-reaction with serogroup B but this was only at a 1:64 titre which was not considered sufficient for assignation. The British/Australian prototypes K,L, and N are all members of serogroup B and the M strain did not react with any of the US ones.

The Canadian study contained herein reports the serotypic classification of clinical isolates of *Dichelobacter nodosus* in both serotype and serogroup designation. That is, the US designation of Roman numerals I - XXI and British A-N for serotypes and the Australian A - I as serogroups.

1.10. Proteolytic Activity as a measurement of virulence

The degree of proteolytic activity of extracellular enzymes expressed by *D. nodosus* on a variety of substrates including casein, elastin and hide-powder has been used to distinquish the virulence of isolates (Depiazzi *et al.*, 1991, Egerton *et al.*, 1969, Gordon *et al.*, 1985, Green, 1985, Kortt, 1982, Kortt *et al.*, 1983, Skerman *et al.*, 1981, Thomas,1964). Immunological designation of virulence using rabbit antibodies produced against electrophoretically separated proteases was not successful (Yong *et al.*, 1986).

Of the insoluble, non-covalent protein-dye complexes in use for the determination of proteolytic activity, hide powder conjugated with Remazolbrilliant Blue has proven an ultrasensitive method for measuring proteolysis in complex environments (Rinderknecht *et al.*, 1968). It was used for determining the activity of six proteases including trypsin, chymotrypsin, elastase, collagenase, fibrinolysin and pepsin (Rinderknecht *et al.*, 1968). Other hide powder-dye complexes including Congo Red and Indigo Carmine have not proven as useful due to the albumin and plasma protein initiated release of acidic dye (Geokas *et al.*, 1968). Azocoll was shown not to be as sensitive as hide powder conjugated with Remazolbrilliant blue in trypsinase determination (Rinderknecht *et al*, 1968).

Hide powder conjugated with azure has been used in the determination of virulence of *Dichelobacter nodosus* (Depiazzi *et al.*, 1979, Kortt *et al.*, 1982). Depiazzi concluded that relative stability and not quantity of proteolytic enzymes formed the basis of the degrading proteinase test and postulated that the net gain of stable enzyme in virulent foot rot infections accounted for the characteristic progressive degeneration of the hoof.

1.11. Objectives

The objective of this research was to identify the nature and occurrence of *Dichelobacter nodosus*, the causative agent of ovine footrot, in locally exposed flocks. As this type of investigation has not been reported in Canada it is hoped that control and eradication methods may benefit and will identify locally important serotypes .

More specifically, the research undertaken included:

1) Collection of tissue samples from sheep in Alberta and British Columbia suspected of infection with *Dichelobacter nodosus*.

2) Isolation and identification of the causative organism, *Dichelobacter nodosus*, from collected samples.

3) Serological classification of identified isolates of *Dichelobacter nodosus*.

4) Characterization of protease as a virulence determinant of identified isolates of *Dichelobacter nodosus*.

2. Materials and Methods

2.1. Sample collection

2.1.1. Use of transport media

Dichelobacter nodosus is a strict anaerobe and as such care must be taken to ensure minimal exposure of the organism to air between field sampling and laboratory culture. Amies transport medium (Difco Laboratories, Detroit Michigan) facilitates transport of pathogenic organims in a viable state by providing a non-nutritive, semisolid, reductive medium. The medium functions to inhibit enzymatic reactions, retard overgrowth of contaminant and prevent oxidation of the sample. The medium was prepared in-house according to manufacturer's instructions and dispensed into five milliliter screw capped sterile vials with minimal air space.

2.1.2. Flock identification

Identification of potentially infected flocks was made by contacting likely producers and agriculture specialists and then visiting locations and collecting samples. It has been suggested by other investigators (Gradin, 1990) that five or six isolates per flock is sufficient to provide information on the serotypes that are active within that population.

2.1.3. Sampling methodology

In the field, samples were collected by first selecting those animals with clinical signs such as limping, holding an affected foot off the ground, reluctance to stand, or abraded sternum or knees. Selected animals were then restrained and tipped back onto their hindquarters until the spine rested along the front of the investigators lower legs. In this position the animal remained relatively cooperative. The feet were then examined for evidence of infection. This ranged from an interdigital dermatitis with or without hyperkeratosis, erythema, erosion, or necrosis to underrunning of the horn progressing to separation. Presence of foul-smelling necrotic exudate was usually distinctive and indicative of an anaerobic infection possibly with *D. nodosus*. Suspect hooves were scraped of debris and pared with hoof trimmers to expose the affected tissue. This sometimes required considerable debridement and care was taken to avoid bleeding as this would compromise the sampling. Two to seven millimeter portions were removed with a sterile scalpel blade from the inner edge of the lesion and placed in transport media. The samples were held on ice and transported as quickly as possible to the laboratory usually within 2 - 6 hours depending on location.

2.2. Culture of *D. nodosus* from clinical samples

2.2.1. Medium Preparation

Field samples were plated onto hoof agar modified from Thorley (Thorley, 1976) and Gradin (Gradin, 1988) by increasing the agar content from 2 to 5% and decreasing the ground sheep hoof from 0.7% to 0.5%. The agar was prepared by combining 0.5% beef extract (Difco Laboratories, Detroit, Michigan), 1% Proteose Peptone (Difco Laboratories, Detroit, Michigan), 0.5% Sodium Chloride (Sigma Chemical Company, St. Louis, MO.), 0.1% Yeast Extract (Difco Laboratories, Detroit, Michigan), 5% Agar (Difco Laboratories, Detroit, Michigan), 1 litre glass distilled water and 0.5% ground sheep hoof. The latter was prepared in-house from abbatoir collected, non-infected hooves which were boiled in tap water for approximately two hours to facilitate removal of the horn of the hoof. The collected material was chopped coarsely with a knife and allowed to dry thoroughly then ground as finely as possible in a single speed blender equipped with a stainless steel blade assembly (Waring Blender Co., Chicago, Illinois). The material was then put through several standard (U.S. series standard sieves, Fisher Scientific, Don Mills, Ontario) wire sieves to achieve a final particle size of 600 micrometers.

Ingredients were placed in a four litre Erlenmeyer flask, heated to boiling temperature and autoclaved at fifteen pounds per square inch pressure for fifteen minutes. The sterile media was cooled to 52 ^{o} C and poured in 20 milliliter amounts into 15 x 100 millimetre sterile disposable petri dishes (Fisher Scientific, Don Mills, Ontario).

2.2.2. Sample inoculation to agar medium and incubation

The clinical samples were removed from the transport media onto a sterile petri dish. They were then cut into approximately one millimeter diameter pieces with a sterile scalpel blade and placed in the top area of the agar plate. The inoculum was streaked for isolation using sterile glass rods.

Plates were placed in a polycarbonate dessicating jar (Canlab, Mississauga, Ontario) with a methylene blue anaerobic indicator (Becton Dickinson Compnay, Cockeysville, Maryland) and reactivated palladium catalyst (Canlab, Mississauga, Ontario) included before closure with metal clips. Utilizing a vacuum pump (Gast Manufacturing Corporation, Detroit, Michigan) the air contained in the sealed jar was removed to a pressure of 25 pounds per square inch. The vacuum was replaced with a mixture of 10% carbon dioxide and 90% hydrogen (Matheson Gas Products, Edmonton, Alberta). The evacuation and replacement procedure was repeated three times to ensure anaerobic conditions.

The hoof agar plates were held in the dessicating jars at 35^{o}C with examination for *D. nodosus* isolates at 2, 4 and 7 days.

2.2.3. Identification of *D. nodosus*

Initial identification of the causative organism of ovine footrot was based on colony morphology. Using a 2.5x magnifying glass, the plates were examined under diffuse angled light for characteristic flat, spreading colonies 1 - 4 millimeters in diameter with a fine granular fimbriate edge surrounding a clearer central zone. Suspect colonies were subcultured to hoof agar for isolation of pure cultures.

Samples from pure cultures were suspended in sterile distilled water on sterile 25 x 75 millimeter glass slides for microscopic examination. Using both original and pure culture slide preparations, one set of glass slides was stained with Gram's stain (Carter, 1979) and examined at 1000x with oil immersion for large gram negative straight or slightly curved bacilli with lengths varying from 3 to 10 microns and width 0.6 to 1 micron. Characteristic terminal enlargments and presence of radially disposed gram negative bacilli were also noted.

The other set of glass slide preparations was fixed in 50:50 acetone/methanol for indirect fluorescent antibody testing using polyclonal rabbit antisera to *D. nodosus* prepared in-house as described herein and FITC conjugate goat anti-rabbit IgG (Product No. F-0511 Sigma Chemical Company, St. Louis, MO). Several drops of antiserum diluted 1:1000 with phosphate buffered saline pH 7.2, prepared by dissolving 150 mM each of sodium phosphate and sodium chloride in 100 ml glass distilled water, were applied to the fixed smears and spread evenly. The slides were held in a humid chamber for 35 minutes at room temperature then washed in phosphate buffered saline pH 7.2 for 5 minutes with 3 changes of solution and then drained. Goat anti-rabbit IgG conjugated with fluorescein isothyanate (product # F-0511, Sigma Chemical Company, St. Louis, MO) was applied to the slides which were returned to the room temperature humid chamber for 20 minutes. The buffer wash was repeated and the slides drained and blotted dry. Mounting fluid pH 7.2 prepared from 9 parts glycerol and 1 part phosphate buffered saline was applied to the slide and a 24 x 40 millimeter coverslip placed over the smear. Fluorescent slide preparations were read at 400x using a Zeiss standard microscope equipped with an HBO 50 watt super pressure mercury short arc lamp and filters KP490, KP500, Rfl510 and LP528 (Carl Zeiss Canada, Toronto, Ontario).

The presence of large strongly fluorescent organisms with characteristically stronger polar emittance was considered diagnostic.

2.3. Antiserum production

Antiserum to *D. nodosus* was prepared in mature New Zealand White rabbits using nonviable, unwashed whole bacterial cells from 5 day pure hoof agar cultures of the organism. The surface growth was harvested by flooding the agar surface with phosphate buffered saline pH 7.2 using a bent glass rod to lightly scrape the surface and release the cells. The density was adjusted photometrically to contain 4.0×10^9 bacterial cells per milliliter and the preparation preserved with 0.25% formalin.

On Day 1 one milliliter of cell suspension was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). One milliliter was injected intra-muscularly into each hind limb. On Day 15 the antigen was mixed with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI) and 1 milliliter injected sub-cutaneously between the scapulas. This injection was repeated on Day 29. Harvesting of the antiserum was on Day 39 when the animal was completely exsanguinated by cardiac puncture while under halothane anesthesia. The whole blood was aliquoted into sterile centrifuge tubes and allowed to clot. Tubes were then centrifuged at 3,000 x g for 10 minutes and the serum drawn off. Aliquots of the serum were held at -70°C.

2.4. Serological Classification of Alberta and British Columbia Isolates of *D. nodosus*.

The serological classification of the *D. nodosus* isolates was performed according to the methods of Schmitz (Schmitz, 1980) and Egerton (Egerton, 1973) with the assistance of Dr. J.L. Gradin and Anita Sonn, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon.

Serial dilutions of rabbit antiserum raised, as previously described, against serogroup/serotype specific recognized British/Australian-New Zealand and U.S. prototypes were used. These included U.S. I - XXI and British/Australian-New Zealand A - N. Agglutination tests were performed in 13 x 100 millimeter clean glass tubes containing 0.5 milliliters of antiserum serially diluted in 0.5% formalinized phosphate buffered saline pH 7.2 starting at 1:2 to a maximum 1:2048. The antigen was prepared from *D. nodosus* whole cells harvested from agar cultures and suspended in formalinized phosphate buffered saline pH 7.2 to a photometrically read density of 5×10^8 cells per millilitre. An equal volume of bacterial suspension was added to each tube, briefly vortexed and incubated 4 hours in a 35^{o}C waterbath.

The tubes were observed for K or surface type agglutination as described by Kauffman (Kauffman, 1951) and Namioka (Namioka, 1970) where rapid formation of coarse aggregates usually occurs within 30 - 60 minutes. The aggregates settle leaving a clear supernatant that can be dispersed by shaking but reforms on standing. Agglutination was graded macroscopically - negative or no clumping, 1+ or questionable, 2+ or slight, 3+ or moderate and 4+ or heavy clumping. The highest dilution yielding a 2+ was considered sufficient assignation. For a designated identity it was necessary for the clinical isolate to be identical or within one dilution of the prototype and its homologous antisera.

An isolate of undetermined serological identity was first screened against prototype antiserum beginning at 1:64 dilution using U.S. I - XXI and British/Australian A-N as described in 1.9.1.. If an isolate agglutinated a prototype at 1:64 antiserum dilution the endpoint titres,using doubling dilutions of the antiserum, for each prototype agglutination was then determined. To be assigned to a specific serogroup or serotype the isolate had to ideally be within one dilution of the homologous culture and its prototype antiserum reaction. The agglutination titres of the isolates and prototype antiserum were recorded and the highest titre determined the designation. Also taken into account were the cross-reactions determined by Gradin *et al.*, 1992 of U.S. against British/Australian serotypes and serogroups as discussed in 1.9.1.. In some cases the agglutination titre of the clinical isolates was atypically high for the designated group.

2.5. Measurement of proteolytic activity by hide powder azure assay

The spectrophotometric measurement of the release of azure dye from its complex with hide powder by the enzymatic cleavage of peptide linkages was chosen to demonstrate the virulence of field and prototype cultures of *D. nodosus*. As noted in 1.10. hide powder conjugated with Remaxol Brilliant Blue R is an ultrasensitive general proteolytic substrate for the colorimeteric determination of proteolytic activity (Rinderknecht *et al.*, 1968). Studies of the proteases of *D. nodosus* have confirmed substrate specificity for chymotrypsin, collagen, elastin, and fibrin (Thomas, 1964, Egerton and Parsonson, 1969, Broad and Skerman, 1976, Kortt *et al.*, 1982, Green, 1985) all of which can be determined colormetrically by the use of Hide Powder Azure. (Rinderknecht *et al.*, 1968). The use of Azocoll, a dye impregnated hide powder (Sigma Chemical Co., Mississauga, product # A9409) was also considered for use as a non-specific protease substrate. However, it produced erroneous results when tested due to the instability of the substrate with variable colorimetric readings observed without the inclusion of proteases.

Isolates tested included type strains (Table 7) obtained from researchers in Australia (Dr. D. Liu, Department of Agriculture and Rural Affairs, Hamilton, Victoria, Dr. A.A. Kortt, CSIRO, Parkville, Victoria), Oregon State University (Dr. J.L. Gradin, Corvallis, Oregon) and the American Type Culture Collection (Atlanta, Georgia) strains of *D. nodosus*. Representative clinical isolates collected from Alberta and British Columbia flocks (Table 8) were also tested. Prototype strains had been given their designation according to elastase testing methodology (Stewart, 1979) as supplied by the contributing investigators (Liu, 1990, Vaughan, 1990, Gradin, 1990) and published reports (Stewart, 1979). The designated virulence of field isolates is noted as clinical as no other protease testing was done to determine activity. Clinical isolates can be described as virulent, intermediate or benign according to their hide powder assay pattern (Depiazzi and Richards, 1979, Depiazzi *et al.*, 1991). Isolates exhibiting a high value within the first 2 days that increases and is maintained a relatively longer length of time are considered as virulent. Benign strains are relatively slower to exhibit proteolysis and this value increases slightly then declines rapidly reaching unreadable values within 9 days. Intermediate strains fall between these two patterns with an initial value quicker to appear than that for benign strains, and increasing to a peak relatively early on with a slower decline.

The isolates were grown anaerobically in 10% carbon dioxide, 90% Hydrogen at 35^{o}C for 3 days on modified hoof agar as described and transferred to labelled, individual 16 x 100 millimeter capped tubes containing 10 millilitre TAS

(Trypticase Arginine Serine) broth (Thorley, 1976). The inoculated tubes were incubated anaerobically for 24 hours to achieve active growth of the organism. Sufficient inoculum was removed to another set of TAS containing tubes to achieve a density of 9.0 x 10^8 CFU per millilitre. An uninoculated tube of TAS was included as a media control to determine if any proteolytic activity could be related to the growth medium alone. The inoculated amd medium control tubes were incubated anaerobically as described. On Days 0, 1, 2, 5, 9, 13 and 20 the anaerobic jar was opened briefly and one millilitre of each culture transferred to a labelled 13 x 100 millimetre capped tube containing 15 milligrams of Hide Powder Azure #H-6268, lot 60H3852 (Sigma Chemical Co., St Louis. MO.) held in an ice bath. Inoculated hide powder tubes including *Pronase* standards as described below, were placed synchronously into a 37°C shaking water bath (Blue M Magni Whirl, Blue M Electric Co., Blue Island, Illinois) for 45 minutes at a speed of 50 cycles per minute. All tubes were then removed once again to an ice bath to halt the degradation of the hide powder. Using glass wool (Fisher Scientific, Ottawa, Ontario) plugged nine inch, long tip, disposable borosilicate glass Pasteur Pipettes (Fisher Scientific, Ottawa, Ontario), the contents of each tube were filtered into the upper portion of the pipet. Filtered contents were removed by a second long tip pipet to a labelled 13 x 100 millimeter tube for spectrophotometric reading using an Ultraspec LKB K 4053 (Fisher Scientific

Canada, Ottawa, Ontario) set at a wavelength of 595 nanometers.

Pronase is a nonspecific protease isolated from *Streptomyces griseus*. One unit (PUK or proteolytic unit) is defined as the amount of enzyme that liberates a digestion product equivalent to 25 ug of Tyr per minute at 40 ^{o} C, pH 7.02 (Calbiochem, 1992). Six *Pronase* protease standards #53702, lot 102613 (Calbiochem, San Diego, Calif.) with an activity of 45,000 PUK per gram plus a blank standard were run in triplicate to achieve a linear curve. These were prepared by diluting the *Pronase* in Tris buffer (Tris (hydroxymethyl) aminomethane), 0.05M Trizma Base) pH 7.02 (Sigma Chemical Co., St. Louis, MO.) to achieve concentrations of 0, 0.006, 0.0013, 0.0025, 0.0050, 0.100 and 0.200 PUK per millilitre. Aliquots of each of the seven standards were prepared in advance and held at minus 20^{o}C until required. Reaction of the hide powder azure with the standards was as described for the TAS inocula.

3. Results

3.1. Location of sampling in Western Canada

In Alberta (Figure 1) a total of 15 different flock locations were visited 11 of which were culture positive for *D. nodosus* as indicated by (+) and 5 were culture negative for *D. nodosus* as indicated by (-). A total of 93 samples were collected with location, sample identification and isolation results recorded (Table 1). Culture positive flocks, from 11 locations in Alberta, were designated A-K and yielded 42 identified isolates of *Dichelobacter nodosus* as indicated (Table 2).

In British Columbia 6 flock locations were visited in the lower mainland area 5 of which were culture positive for *D. nodosus* as indicated (Figure 2). A total of 113 samples were collected from the 6 different locations with primary sample identification noted (Table 3). Twenty three isolates of *D. nodosus* were cultured from 5 of the 6 locations investigated and the flocks designated L - P with double flock designation (L and M) at one location which was a slaughter house receiving from multiple locations (Table 4).

3.2. Serological Classification of *D. nodosus* isolates

A total of 66 isolates of *Dichelobacter nodosus* were serologically classified (Table 5, Table 6). In Alberta, 42 isolates from 11 flocks A - K were classified into 4 serotypes - N, I, XIV,XV and 4 serogroups A,B,D and H (Table 5). The titres demonstrate the cross-reactivity of strains with prototype anti-serum as described by Gradin *et al.*, 1992 and discussed in 1.9.1 and expressed as titre reactions with the non-classified serotypes or serogroups (Table 5).

Figure 1: Alberta *D. nodosus* sampling locations

(+) isolation of *D. nodosus*

(-) no isolation of *D. nodosus*

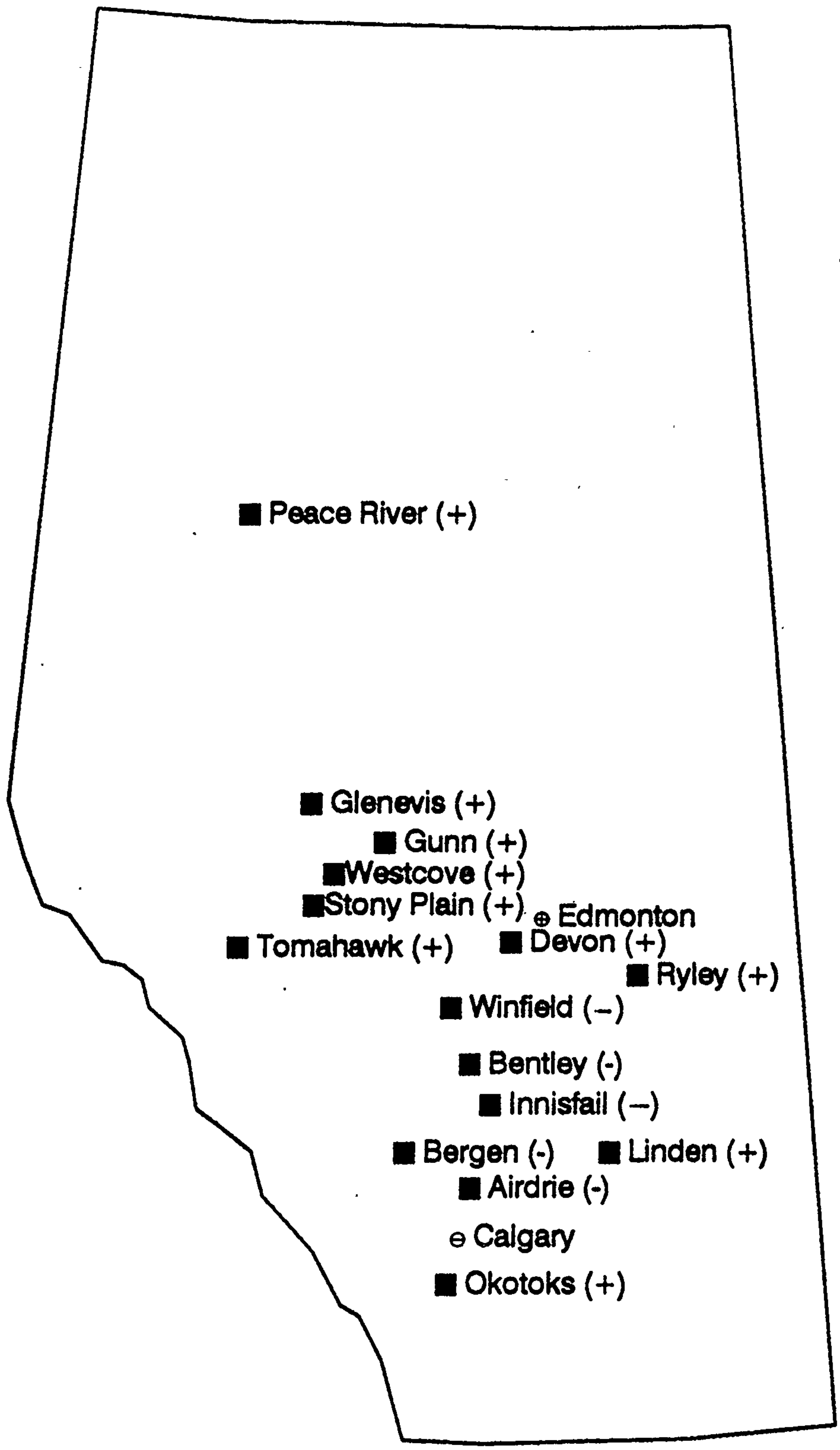

Peace River (+)
Glenevis (+)
Gunn (+)
Westcove (+)
Stony Plain (+)
Edmonton
Tomahawk (+)
Devon (+)
Ryley (+)
Winfield (–)
Bentley (-)
Innisfail (–)
Bergen (-)
Linden (+)
Airdrie (-)
Calgary
Okotoks (+)

Table 1: Alberta sampling location, sample identification and isolation result

Location	Sample Identification	Isolation of *D.nodosus*
Bergen	1 - 2	negative
Gunn	3 - 8	positive
Glenevis	9 - 13	positive
Ryley	14 - 18	positive
Devon	19 - 26	positive
Westcove	27 - 31	positive
Innisfail	32 - 34	negative
Bentley	35 - 36	negative
Peace River	37 - 43	positive
Winfield	44 - 49	negative
Airdrie	50 - 59	negative
Winfield	60 - 69	positive
Tomahawk	70 - 78	positive
Stoney Plain	79 - 89	positive
Linden	1220 - 1221	positive
Okotoks	6445	positive

Table 2: Alberta isolates of *D. nodosus* by flock designation A - G, H - K, sample identification and location

Flock	Identification	Location
A	90 - 3	Gunn
B	90 - 8	Glenevis
B	90 - 9	Glenevis
B	90 - 10	Glenevis
B	90 - 11	Glenevis
B	90 - 12	Glenevis
C	90 - 14	Ryley
C	90 - 15	Ryley
C	90 - 15:2	Ryley
C	90 - 16	Ryley
C	90 - 18	Ryley
D	90 - 19	Devon
D	90 - 20	Devon
D	90 - 21	Devon
D	90 - 22	Devon
D	90 - 23	Devon
D	90 - 25	Devon
E	90 - 27	Westcove
E	90 - 29	Westcove
F	90 - 38	Peace River

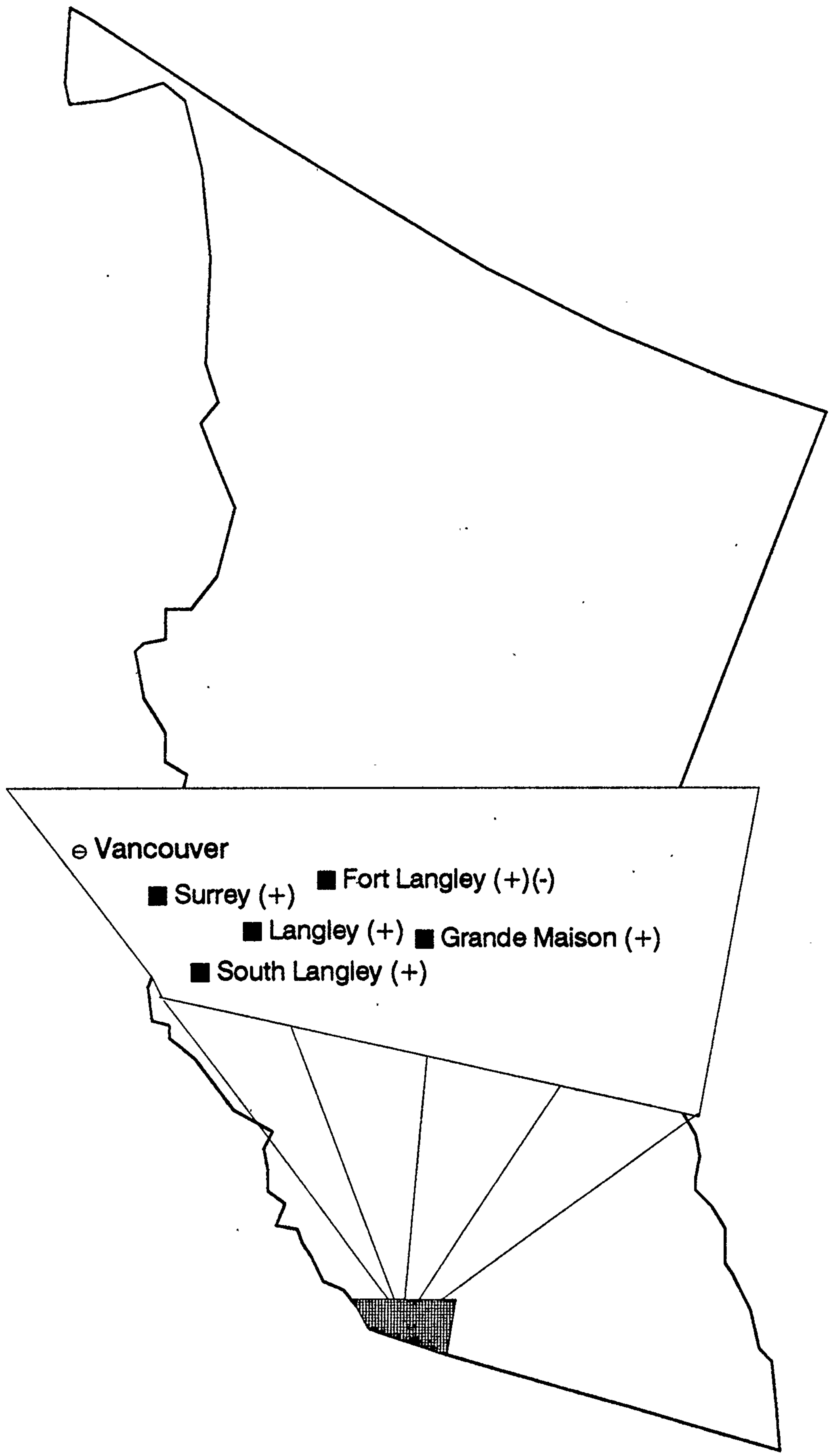
Flock	Identification	Location
G	90 - 62	Winfield
G	90 - 63	Winfield
H	90 - 70	Tomahawk
H	90 - 71	Tomahawk
H	90 - 72	Tomahawk
H	90 - 73	Tomahawk
H	90 - 74	Tomahawk
H	90 - 75	Tomahawk
H	90 - 77	Tomahawk
H	90 - 78	Tomahawk
I	90 - 79	Stoney Plain
I	90 - 80	Stoney Plain
I	90 - 81	Stoney Plain
I	90 - 82	Stoney Plain
I	90 - 83	Stoney Plain
I	90 - 84	Stoney Plain
I	90 - 85	Stoney Plain
I	90 - 86	Stoney Plain
I	90 - 89	Stoney Plain
J	1220	Linden
J	1221	Linden
K	6445	Okotoks

Figure 2: British Columbia *D. nodosus* sampling locations

(+) isolation of *D. nodosus*

(-) no isolation of *D. nodosus*

(+)(-) adjacent farms, one *D. nodosus* isolated


⊖ Vancouver
■ Surrey (+)
■ Fort Langley (+)(-)
■ Langley (+)
■ Grande Maison (+)
■ South Langley (+)

Also the unique titres as previously discussed in 2.4 are noted in parenthesis (Table 5).

In British Columbia 6 serotypes - I, IV, XIV, XV, XX, XXI and 4 serogroups - B,D,E and H were defined (Table 6). Flock N (Table 6) harboured 3 serologically distinct strains - serotypes I, IV and XX, with serogroup designation B, D and 1 unclassified. Flock Q (Table 6) harboured 2 serotypically distinct strains - XIV and XXI classified into the A and E serogroups (Table 6). Of particular interest is the isolation in Flock P (Table 6) of a serologically unique strain. It failed to react with any of the prototype antiserum. As noted, in the Alberta isolates, is the presence of serological cross-reactivity and unique serotype titres expressed by many of the strains with the latter noted by parenthesis around the designation (Table 6). This highlights the observation that, although a serotype was classified according to Gradin *et al.*, 1992, the titre of the clinical isolate was unusually high for that prototype.

3.3. Measurement Of Proteolytic Activity

Both prototypic and clinical isolates of *D. nodosus* were tested for proteolytic activity using the spectrophotometric measurement of the release of azure dye from its complex with hide powder as described in 2.5.. Eleven prototypic strains including 4 designated benign, 2

Table 3: British Columbia sampling locations, sample identification and isolation result

Location	Sample Identification	Isolation of *D. nodosus*
Grande Maison	1 - 78	positive
Ft.Langley	79 - 89	positive
Ft.Langley	90	negative
Langley	91 - 95	positive
South Langley	96 - 98	positive
Surrey	99 - 103	positive

Table 4: British Columbia isolates of *D. nodosus* by flock designation L - Q, sample identification and location

Flock	Identification	Location
L	BC 29	Grande Maison
M	BC 46	Grande Maison
N	BC 79E	Fort Langley
N	BC 79int	Fort Langley
N	BC 81	Fort Langley
N	BC 81h	Fort Langley
N	BC 82	Fort Langley
N	BC 82tip	Fort Langley
N	BC 83at	Fort Langley
N	BC 85hd	Fort Langley
N	BC 88	Fort Langley
O	BC 91-2	Langley
O	BC 92	Langley
O	BC 95	Langley
P	BC 97	South Langley
P	BC 98	South Langley
Q	BC 99	Surrey
Q	BC 100	Surrey
Q	BC 100-2	Surrey
Q	BC 101	Surrey
Q	BC 101-3	Surrey
Q	BC 102	Surrey
Q	BC 103	Surrey

Table 5: Serotype/serogroup classification of field isolates of *D. nodosus* from Alberta - Flocks A-G, H-K, endpoint titres recorded for reactive anti-serum

Ident.	Classified type	Classified group	Titres
A 90-3	N	B	512N, 128XVI, 64XII, 64XIII
B 90-8	N	B	512N, 256XII, 256XVI, 128XIII, 64IX
B 90-9	N	B	512N, 256XII, 256XVI, 128XIII, 64IX
B 90-10	N	B	512N, 256XII, 256XVI, 128XIII, 64IX
B 90-11	N	B	1024N,256XII, 256XVI, 128XIII, 64IX
B 90-12	N	B	512N, 256XII, 256XVI, 128XIII, 64IX
C 90-14	I	D	512I
C 90-15	I	D	512I
C 90-15:2	I	D	512I
C 90-16	I	D	512I
C 90-18	I	D	512I
D 90-19	(XV)	H	1024XV, 256XI, 256XIX
D 90-20	(XV)	H	1024XV, 256XI, 256XIX
D 90-21	(XV)	H	1024XV, 256XI, 256XIX
D 90-22	N	B	1024N, 256XII,256XVI, 64IX, 64XXI
D 90-23	(XV)	H	1024XV, 256XI, 256XIX
D 90-25	(XV)	H	1024XV, 256XI, 256XIX
E 90-27	N	B	1024N, 256XII,256XVI, 128XIII, 64IX
E 90-29	N	B	512N, 256XII,256XVI, 128XIII, 64IX
F 90-38	(XIV)	A	4096XIV, 256VII, 256A
G 90-62	(XV)	H	1024XV, 256XIX, 128XI, 128H
G 90-63	(XV)	H	1024XV, 256XIX, 128XI, 128H

Ident.	Classified type	Classified group	Titres
H 90-70	N	B	512N, 256XII, 256XVI, 64IX, 64XIII
H 90-71	N	B	512N, 256XII, 256XVI, 64IX, 64XIII
H 90-72	N	B	512N, 256XII, 256XVI, 64IX, 64XIII
H 90-73	N	B	512N, 256XII, 256XVI, 64IX, 64XIII
H 90-74	N	B	512N, 256XII, 256XVI, 64IX, 64XIII
H 90-75	N	B	512N, 256XII, 256XVI, 64IX, 64XIII
H 90-77	N	B	512N, 256XII, 256XVI, 64IX, 64XIII
H 90-78	N	B	512N, 256XII, 256XVI, 64IX, 64XIII
I 90-79	(XV)	H	1024XV, 512XI, 256XIX
I 90-80	(XV)	H	1024XV, 512XI, 256XIX
I 90-81	(XV)	H	1024XV, 256XI, 256XIX
I 90-82	(XV)	H	1024XV, 512XI, 256XIX
I 90-83	(XV)	H	1024XV, 256XI, 256XIX
I 90-84	(XV)	H	1024XV, 512XI, 256XIX
I 90-85	(XV)	H	1024XV, 256XI, 256XIX
I 90-86	(XV)	H	1024XV, 256XI, 256XIX
I 90-89	(XV)	H	1024XV, 256XI, 256XIX,
J 1220	I	D	512I, 128XV
J 1221	I	D	512I, 128XV
K 6445	(XV)	H	2048XV, 512XIX, 256XI

Table 6: Serotype/serogroup classification of field isolates of *D. nodosus* from British Columbia - Flocks L-Q, endpoint titres recorded for reactive anti-serum

Ident.	Classified type	Classified group	Titres
L BC29	(XV)	H	1024XV, 256XIX, 128XI
M BC46	(XX)	B	2048XX,256IX,256XII,256XVI,256XVIII
N BC79E	IV	-	8192IV
N BC79int	(XX)	B	4096XX, 256XVIII
N BC81	(XX)	B	4096XX, 256XVIII
N BC81h	(XX)	B	4096XX, 256XVIII
N BC81int	(XX)	B	4096XX, 256XVIII
N BC82	I	D	512I
N BC82tip	I	D	512I
N BC83at	I	D	512I
N BC85hd	(XX)	B	4096XX, 256XVIII
N BC88	(XX)	B	4096XX, 256XVIII
O BC91-2	(XXI)	E	2048XXI, 512E
O BC92	(XXI)	E	2048XXI, 512E
O BC95	(XXI)	E	2048XXI, 512E
P BC97	-	-	unreactive I-XXI, A-N
P BC98	-	-	inreactive I-XXI, A-N
Q BC99	(XXI)	E	2048XXI, 512E
Q BC100	(XXI)	E	2048XXI, 512E
Q BC100-2	(XXI)	E	2048XXI, 512E
Q BC101	(XXI)	E	2048XXI, 512E
Q BC101-3	(XXI)	E	2048XXI, 512E
Q BC102	(XXI)	E	2048XXI, 512E
Q BC103	(XIV)	A	4096XIV, 256X

intermediate and 5 virulent from a variety of sources were used (Table 7). Twenty one strains labelled 12 - 32 were randomly chosen for proteolytic assay as representative of clinical isolate locations (Table 8).

The readings of the hide powder proteolytic assay (HPA) taken at days 0, 1, 2, 3, 5, 9, 13 and 20 of both prototypic and clinical strains of *D. nodosus* plus a media control expressed as PUK (proteolytic units) per millilitre x 10^{-4} is recorded in Table 9.

3.3.1. Virulent prototype strain activity

The results of the hide powder azure assay (HPA) results are illustrated in Table 9. The values for the prototypic strains (Table 7) are in predictable ranges as the strains were classified by the contributors by elastase testing discussed in 2.5.. All of the virulent strains demonstrate proteolytic activity on day 1 with recorded values of between 3 and 35. For the virulent strains these values increase to reach a high on day 3 for #7, 10 and 11 of 50, 40 and 43 PUK/ml x 10^{-4} respectively. Virulent strains #8, and 9 reach a peak on day 5 of 58 and 49 respectively. Although strain #7 does have a low value of 3 on day 1 the proteolytic activity increases rapidly and to a high of 50 on day 3. The relatively high values are then maintained which is characteristic of virulent strains. By day 20

Table 7: Prototypic strains of *D. nodosus* tested for proteolytic activity measured by hide powder azure assay.

#	Identification	Designation	Source
1	A291	benign	Hamilton, Australia
2	A654	benign	Hamilton, Australia
3	OSU 72	benign	Corvallis, Oregon
4	OSU 473	benign	Corvallis, Oregon
5	A393	intermediate	Hamilton, Australia
6	A397	intermediate	Hamilton, Australia
7	S198	virulent	Parkville,Australia
8	A459	virulent	Hamilton, Australia
9	A378	virulent	Hamilton, Australia
10	ATCC 27521	virulent	Atlanta, Georgia
11	ATCC 25549	virulent	Atlanta, Georgia

Table 8: Clinical strains of *D. nodosus* tested for proteolytic activity measured by hide powder assay.

#	Identification	Designation	Source
12	90-12	clinical	Glenevis, Alberta
13	BC 91-2	clinical	Langley, B.C.
14	BC 79 int	clinical	Ft. Langley, B.C.
15	90-3	clinical	Gunn, Alberta
16	90-16	clinical	Ryley, Alberta
17	90-19	clinical	Devon, Alberta
18	90-22	clinical	Devon, Alberta
19	90-29	clinical	Westcove, Alberta
20	90-38	clinical	Peace River, Alberta
21	90-62	clinical	Medicine Lake, Alta.
22	90-72	clinical	Tomahawk, Alberta
23	90-80	clinical	Stoney Plain, Alta.
24	BC 29	clinical	Grande Maison, B.C.
25	BC 46	clinical	Grande Maison, B.C.
26	BC 79E	clinical	Ft. Langley, B.C.
27	BC 82 tip	clinical	Ft. Langley, B.C.
28	BC 98	clinical	South.Langley, B.C.
29	BC 100	clinical	Surrey, B.C.
30	BC 103	clinical	Surrey, B.C.
31	1220	clinical	Bentley, Alberta
32	6445	clinical	Sundre, Alberta

Table 9: Hide powder azure assay of prototypic and field strains of *D. nodosus*. Units expressed in PUK (proteolytic units) per millilitre x 10^{-4}

#	Designation	0	1	2	3	5	9	13	20
		Day Sampled							
PROTOTYPE STRAINS									
1	benign	0	9	44	27	18	0	0	0
2	benign	0	0	8	6	0	0	0	0
3	benign	0	0	4	9	4	0	0	0
4	benign	0	0	8	5	9	0	0	0
5	intermediate	0	6	9	9	4	0	0	0
6	intermediate	0	10	29	23	12	0	0	0
7	virulent	0	3	44	50	45	41	35	28
8	virulent	0	24	50	51	58	33	42	19
9	virulent	0	11	51	46	49	35	35	10
10	virulent	0	26	42	40	36	22	25	20
11	virulent	0	35	46	43	32	28	26	8
FIELD STRAINS									
12	field	0	18	34	34	37	18	21	9
13	field	0	47	59	51	48	35	34	18
14	field	0	26	47	50	44	32	33	7
15	field	0	0	44	38	28	26	29	4
16	field	0	35	43	43	35	22	23	8
17	field	3	28	46	38	42	32	41	7
18	field	0	0	36	42	41	37	33	8
19	field	0	0	32	41	45	31	23	9
20	field	0	0	44	46	36	27	27	13
21	field	0	0	12	16	7	0	0	0
22	field	0	0	34	31	29	22	20	10
23	field	0	8	31	35	26	27	19	2
24	field	0	17	40	36	42	35	35	7
25	field	0	0	51	66	69	61	59	19
26	field	0	0	27	14	8	0	0	0
27	field	0	0	15	31	37	29	28	10
28	field	0	0	57	51	53	47	52	23
29	field	0	0	23	73	86	65	67	37
30	field	0	10	47	41	45	42	38	10
31	field	0	0	8	35	47	35	30	12
32	field	0	4	28	41	32	22	15	7
Media control									
33	TAS	0	0	0	0	0	0	0	0

0 = less than minimum of 6 PUK/ml x 10^{-4}

there is still measureable proteolytic activity in all virulent strains ranging from values of 8 to 28.

3.3.2. Intermediate prototype activity

There were 2 intermediate strains received from contributors #5 and #6 (Table 7). They demonstrated (Table 9) the characteristics of intermediate strains - proteolytic activity that develops rapidly with values of 6 and 10 on day 1 for strains 5 and 6 respectively. Strain 5 exhibited a slow rise in activity level to 9 on days 2 and 3, declining to 4 on day 5 and 0 on days 9, 13, and 20. Strain 6 activity level peaked on day 2 with a value of 29 that declines steadily to a 0 value on days 9, 13 and 20. These values are characteristic of intermediate strains - a moderate proteolytic activity is demonstrated on the first day with a moderate increase that rapidly declines on the third day to immeasurable values by day 9.

3.3.3. Benign prototype strain activity

The benign strains #'s 2, 3 and 4 (Table 7) did not have a measureable proteolytic value until day 2 (Table 9). The activity did not increase appreciably and is immeasureable by day 9. This was characteristic of benign strains. However strain #1 did not exhibit these characteristics. Proteolytic activity had a value of 9 on day 1 which rapidly increased to 44 on day 2 and thereafter declined to a value of 0 by day 9. These values were more characteristic of an intermediate strain using hide powder assay.

3.3.4. Clinical isolates activity

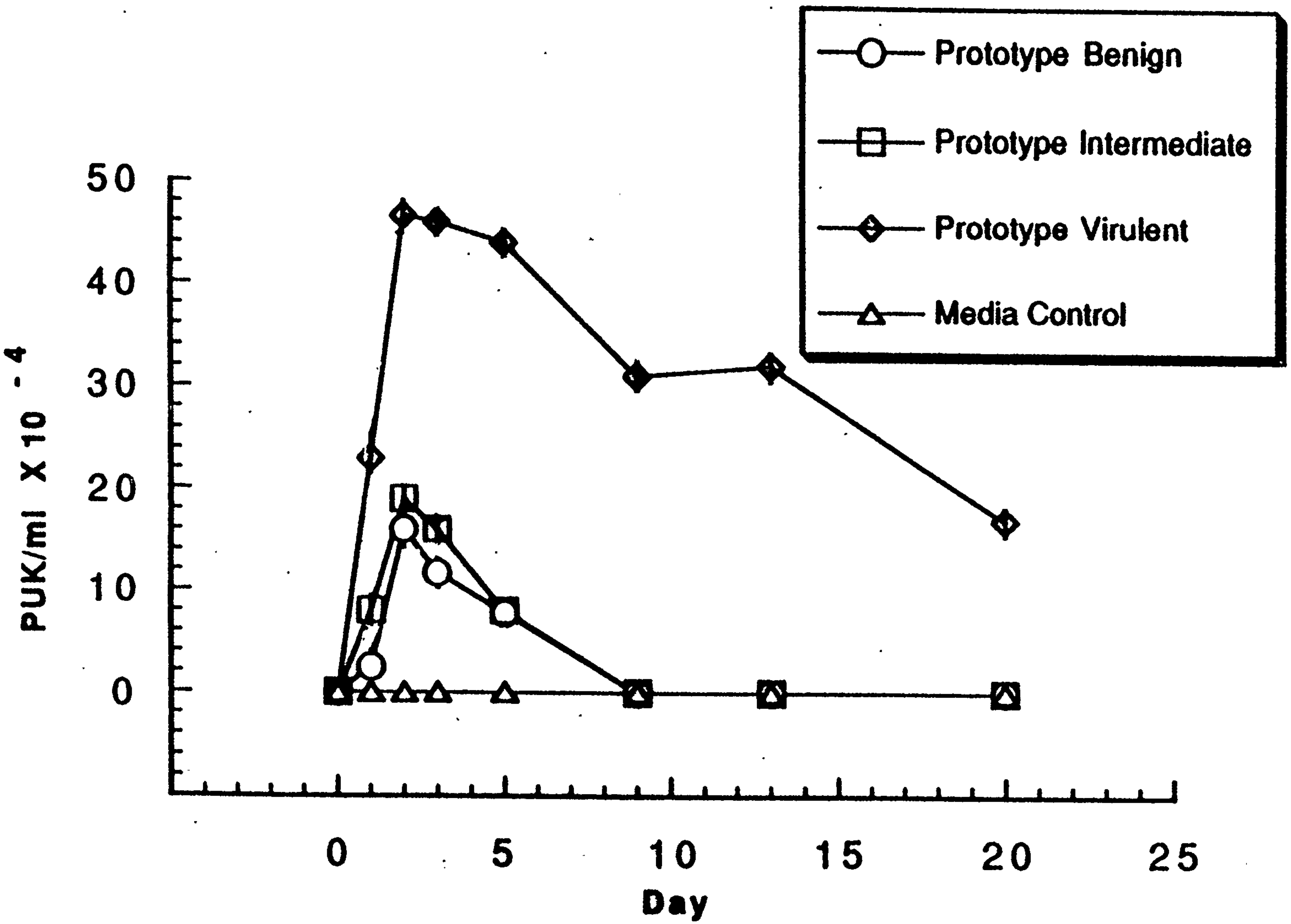
The twenty one clinical isolate strains (Table 8) were assessed using the same parameters for virulent, intermediate and benign designation as described in 2.5. with comparison of their hide powder assay results (Table 9). It was determined that #'s 12, 13, 14, 16, 17, 24, 30 and 32 fit the profile of virulent strains. Clincial isolates #'s 15, 18, 19, 20, 22, 25, 27, 28, 29 and 31 are slower to begin proteolytic activity as none is measureable until day 2 as compared to day 1 with the previous set. However, the high values that are maintained until day 20 categorize the latter set into the virulent patterning. Field isolate #21 can be classified as benign as the proteolytic activity is not measureable until day 2 with a slightly higher maximum value on day 3. The activity then rapidly declines to 0 on day 9. Isolates #'s 23 and 26 have been designated intermediate strains. Isolate 23 has a low activity of 8 on day 1 which peaks on day 3 to 35 declines to 26 on day 5, 19 on day 13 and 2 on day 20. Although the activity is comparable to virulent strains the measurement of rapidly decreasing activity to a low of 2 on day 20 is characteristic of intermediate strains. Isolate 26 does not exhibit any activity until day 2 when a sudden rise to 27 is followed by a rapid decline to 0 on day 9.

3.3.5. Analysis of the activity of the prototypic and clinical strains

The means of the hide powder assay of the prototype benign, intermediate and virulent strains with comparison to the media control are plotted in Figure 3. This illustrates the characteristics of each - the virulent strains achieve the highest value in the shortest time and maintain high values, intermediate strains demonstrate more proteolytic activity in less time than benign strains but not as great as virulent. Benign strains lose proteolytic activity more readily than intermediate. The media control did not have any measureable proteolytic activity.

The means of the proteolytic activity of the 18 virulent, 2 intermediate and 1 benign clinical strains as assayed using hide powder are plotted in Figure 4. The virulence patterning with high, quickly achieved activity that slowly declines to still measurable levels is illustrated. The intermediate strains exhibited lower initial activity that declined more quickly than the virulent strains. The benign clinical isolate exhibited a lower proteolytic activity that rapidly declined to

Figure 3: Hide powder azure assay of prototypic strains of *D. nodosus* and media control. Values are mean of PUK/milliliter x 10^{-4}.



Prototype Benign
Prototype Intermediate
Prototype Virulent
Media Control
50
40
30
20
10
0
PUK/ml X 10 - 4
0
5
10
15
20
25
Day

immeasurable values. The media controls exhibited no measureable proteolytic activity.

As illustrated in Figure 5, the mean of the prototype and field virulent strain activity exhibited similarity that is characteristic of the virulent category. The mean of field and prototype intermediate strains illustrated characteristics of this designation although the field strain activity reached a higher level and was slower to decline than the prototype. The mean of the prototype benign achieved the same level of activity as the field benign although this was measurable earlier in the prototype. The prototype benign and intermediate and field benign achieved similar activity levels on day 5 with a rapid decline to 0 by day 9.

Figure 4: Hide powder azure assay of field isolates of *D. nodosus* and media control. Values are mean of PUK/milliliter x 10^{-4}

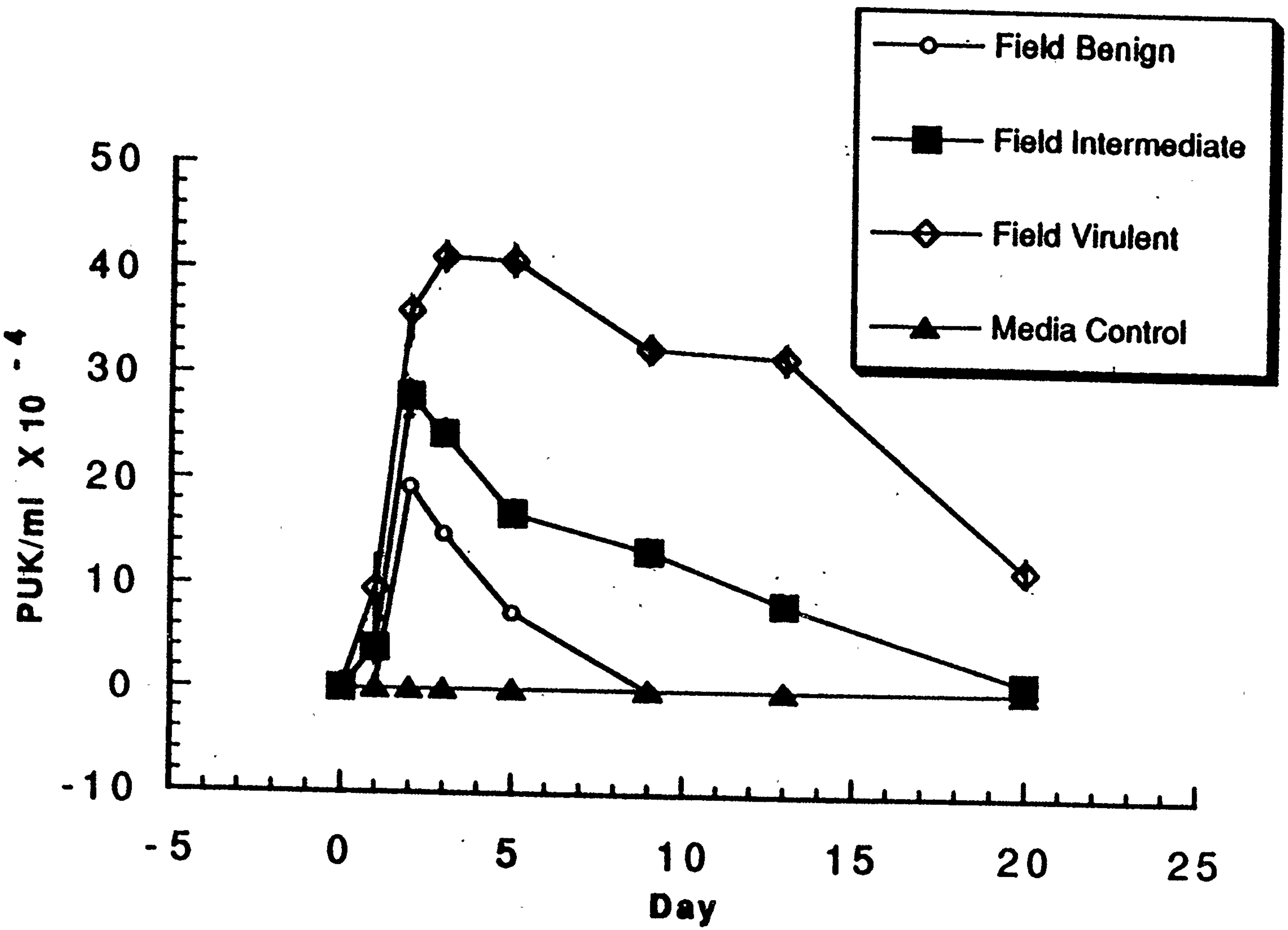
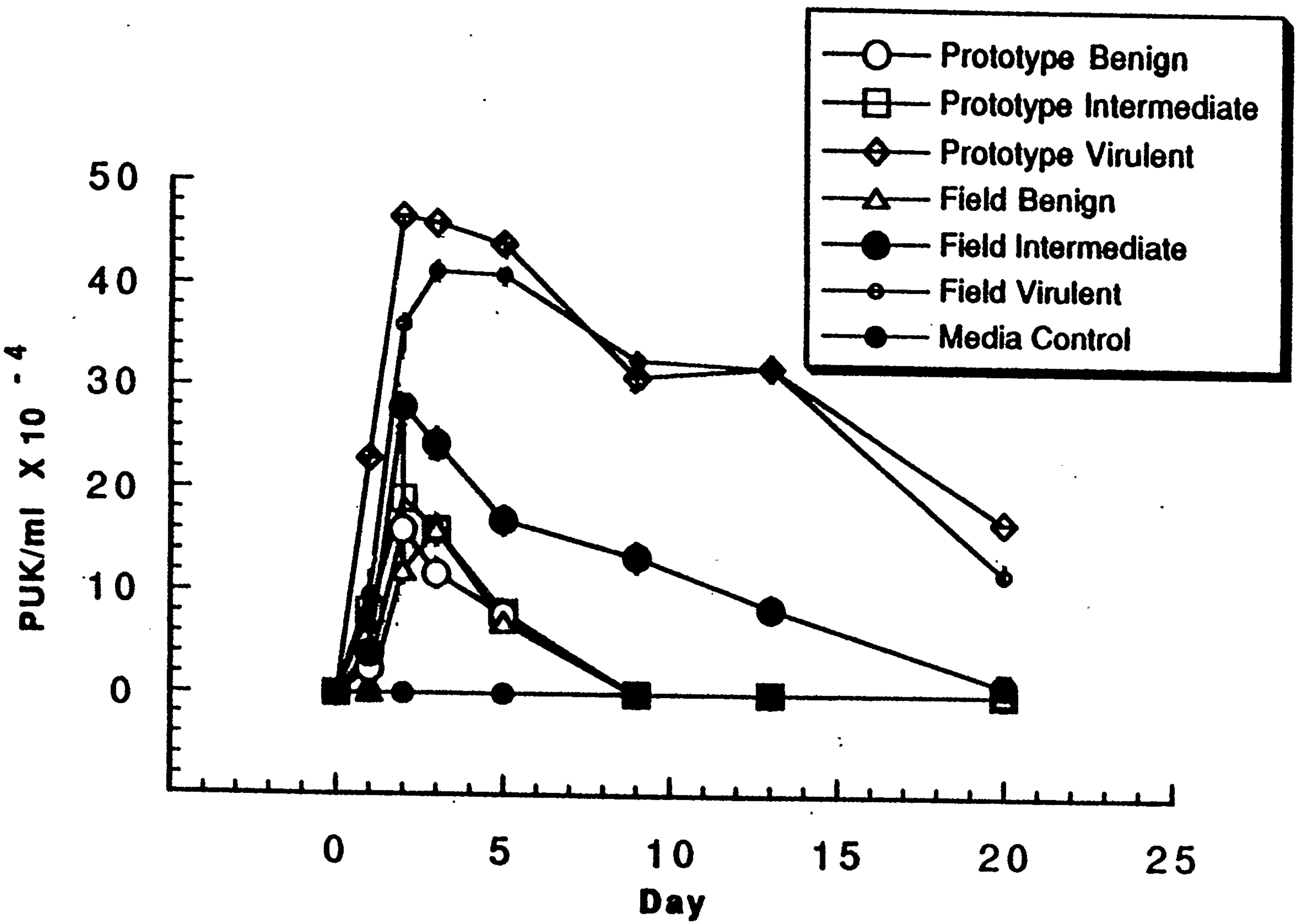

Field Benign
Field Intermediate
Field Virulent
Media Control
50
40
30
20
10
0
-10
PUK/ml X 10 -4
-5
0
5
10
15
20
25
Day

Figure 5: Comparison of the means of hide powder azure assay of prototypic and field strains of *D. nodosus.* Values are means of PUK/milliliter x 10^{-4}.


Prototype Benign
Prototype Intermediate
Prototype Virulent
Field Benign
Field Intermediate
Field Virulent
Media Control
PUK/ml X 10 - 4
50
40
30
20
10
0
0
5
10
15
20
25
Day

4. Discussion

4.1. Isolation of *D. nodosus* from field samples

Field sampling was limited by the reportable disease (Livestock Diseases Act, 1972) status of ovine footrot in Alberta. There is both a social stigma and economic effect to quarantine and this fact made it more difficult to make contact with producers. However, most of the province of Alberta was represented in the sampling achieved (Figure 1). Table 5 demonstrates the serological variety of *Dichelobacter nodosus* in locally exposed flocks especially in consideration of the cross border exchange of Alberta sheep being grazed on logged areas of British Columbia under contract. While the sampling undertaken in this study was not meant as a survey of infected flocks throughout the province, it does substantiate the accepted premise that clinical footrot in naturally drier areas south of Calgary is rare.

Time and information constraints restricted the British Columbia sampling to the lower mainland (Figure 2). However, a variety of serogroups were isolated (Table 6). It was thought that perhaps slaughter house collected material would be of value and would circumvent the legislation problem. In the British Columbia sampling (Table 3) the first 78 samples collected were from the Grande Maison slaughter house. Of these only 2 isolates of *D. nodosus* were cultured. Compared to the other location percentages of isolates this was very low. The low culture success rate was attributed to the fact that there was minimal compliance of sample collection recommendations by industry staff. As inspection and sampling of live slaughter animals was not permitted, suspect hooves were to be kept frozen at -20°C for prompt collection. However, there was ample opportunity for freeze and thaw in the facility which no doubt compromised the samples. Also there was no way of correlating clinical signs with sampling to limit collection to suspect animals. In hindsight, this method, although potentially promising due to the large sampling possible, proved a poor one in comparison to the more controlled and productive method of direct producer contact and sample collection.

4.2. Serological Classification

As noted in Table 5 there was a predominance of serotype N in Alberta with 5 out of 11 flocks harboring this serotype. This may have been due to carrier animals being distributed by a single producer to several new owners. It is also noted that flock D in Table 5 harbored 2 serotypes, (XV) and N, of *D. nodosus*. This was not unexpected as the flock source for many producers, including this one, is the auction market. Such serogroup diversity within a flock further illustrates the challenge of vaccine efficacy in mixed infections.

The uniqueness of the Alberta isolates as indicated by the parenthesis around the serogroup in flocks D, F, G, I and K, Table 5 further exemplifies the local diversity of this organism. The fact that these isolates do not more closely compare with the prototype strains illustrates the potential antigen variety on the pilus. A more antigenically similar isolate would be within the standard (Gradin, 1992) 1 or 2 dilutions of the prototype and its homologous antiserum.

Also of note is that 4 different serogroups were isolated from the 11 positive flocks and that nearly half of the flocks could account for their strain from a single source. Thus the potential for strain diversity is again evident even from a relatively small sampling.

Of particular interest is the isolation from British Columbia flock P of a probable new serogroup (Table 6). This flock had recently been brought home from the "Clearcut" grazing reserve in the interior of British Columbia where it probably acquired and transmitted the infection through necrotic, *D. nodosus* infected hoof material deposited on the pasture. The producer had vaccinated (Footvax 10, Coopers Agropharm Inc., Ontario) the animals prior to their use on the reserve and was having a difficult time clearing the infection although the herd was well managed. This again illustrates the challenge to the vaccine to protect against heterologous strains.

The British Columbia isolates demonstrate a greater diversity of serogroups with a total of 6 different strains isolated from a total of 6 flocks (Table 6). Unique titres are again illustrated in 3 flocks and mixed infections are noted in flock N which harbors 3 different serogroups and flock Q which contained 2. Unlike the Alberta flocks with mixed infections that obtained animals from auction markets the B.C. flocks were relatively closed flocks with minimal importation. Chronic carriers within the flocks may have perpetuated the mixed infection.

Of note also is the presence of 2 different serogroups in one animal (Table 6). Flock N, sample BC 79E and BC 79i were sampled from the same animal, the same hoof except one specimen was taken from the outer edge and the other interdigitally.

The challenge for control and eradication measures would be to overcome the mixed, carrier states of the infection.

4.3. Measurement of Proteolytic Activity

The proteolytic degradation of hide powder conjugated with azure has been suggested as a clinical tool in the determination of virulence (Depiazzi *et al.*, 1979, Kortt *et al*, 1982). However, it was proposed that relative thermal stability and not quantity was the virulence determinant. In this research the direct measurement of the level of proteolytic activity of prototypic and clinical isolates of *D. nodosus* over time was employed. The hide powder assay method reported herein proved valid in identifying prototypic strains and by comparison clinical isolates were designated benign, intermediate and virulent (Table 9).

The results of the hide powder assay are compiled in Table 9 and Fiqures 3 - 5. The assay was consistent in confirming 3 of the prototype benign strains as low in enzyme activity as defined previously. Strain number 1 (A291) would be defined by the hide powder assay as an intermediate rather than benign. This would appear to conflict with the negative elastase test results supplied by the contributor (Liu, 1990). However, it may serve to indicate, instead, the presence of other virulence factors not recognized in the elastase test or that the hide powder assay is a more sensitive indicator of activity.

Intermediate strain number 5 (A393) exhibits an enzyme activity closer to benign than intermediate (Table 9). This would correlate with the supplied lengthy elastase reading of 21 days (Liu, 1990).

The 5 prototype virulent strains had hide powder assay readings consistent with their designation.

Of the 21 field strains tested all but 3 would be defined by the assay as virulent. This is recognized by a rapid rise to a high value of activity which was then maintained after cell death between 3 and 5 days.

As evidenced by a slow rise and fast decline to immeasurable enzyme activity, 90-62 is seen as a benign strain and BC79E an intermediate due to an initially higher value at day 2. Number 90-80 is seen as an intermediate strain because of the slower decline in activity as compared to the benign strains.

E. Conclusions

The nature and occurrence of *Dichelobacter nodosus*, the causative agent of ovine footrot, was further defined by this research. The sampling, culture and identification methods were applied to determine the extent of strain diversification in locally exposed flocks. It was demonstrated that there is a great deal of serological diversity in isolates to the extent that both unique titres and novel serotypes/serogroups are present in local flocks.

It was also determined that enzyme activity, using hide powder azure, can be measured and correlated with virulence. This has application in prevention, treatment and eradication programs. By recognizing specific serological types present in a flock, treatment programs involving either commercial or expanded antigen preparations can be more wisely applied. Quarantine restrictions become more credible when direct knowledge of the level of enzyme activity, as a function of virulence, is known.

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