Molecular epidemiology, clonality and virulence of *Dichelobacter nodosus*, the agent of ovine footrot

Presented by

Nicky Buller

Submitted in total fulfilment of the requirements of the degree of

Doctor of Philosophy

School of Veterinary and Biomedical Sciences

Murdoch University

June 2005

Declaration

The experiments in this thesis constitute work carried out by the candidate unless otherwise stated. The thesis is less than 100,000 words in length, exclusive of tables, figures, bibliography and appendices, and complies with the stipulations set out for the degree of Doctor of Philosophy for Nicky Buller.

School of Veterinary and Biomedical Sciences

Murdoch University;

Murdoch, Western Australia 6150

Australia

Thesis abstract

Dichelobacter nodosus, an anaerobic bacterium, is the major transmissible agent of ovine footrot. The disease expresses as a virulent or benign lesion in the hoof. Virulence is related to the production of serine proteases, particularly a thermostable protease. Isolates of *D. nodosus* are characterised according to the type of protease produced (either heat-stable or heat-labile) and the electrophoretogram (zymogram) of the protease. This study reports on the use of the DNA-based typing techniques Pulsed-Field Gel Electrophoresis (PFGE) and Infrequent-Restriction-Site-PCR (IRS-PCR) to investigate the molecular epidemiology of *D. nodosus*, including a consideration of the relationship between genetic type, zymogram patterns and whole cell protein profiles.

The aim of the project was to obtain a better understanding of *D. nodosus* strain diversity and dissemination in Australia and its relationship to virulence within the population. The overall intention was to use this information to assist in the long-term control of virulent footrot.

Field isolates of *D. nodosus* from Western Australia (n = 735), New South Wales (n = 16), Victoria (n = 24) and South Australia (n = 21) were obtained and analysed. Both typing techniques that were used offered good differentiation between isolates for epidemiological purposes, and the results were in general agreement. PFGE provided slightly better discrimination between isolates, with 214 PFGE types (181 from Western Australia) compared to 94 IrsT types (77 from Western Australia). Within this diverse range of molecular types clonality was observed - with clones being defined as clusters of isolates having closely related PFGE types. The strains were categorised as genetically diverse, genetically similar or identified as the same strain. This diversity of genetic types was found overall, within flocks of sheep on a farm and within a single hoof where, on a number of occasions, multiple molecular types and zymogram types were found colonising a single hoof. One isolate that was experimentally inoculated into a flock of sheep produced six different genetic types when tested 12 months after the initial infection. This indicates that *D. nodosus* undergoes rapid genetic change, which means that follow-up epidemiological investigation of disease outbreaks and trace-backs need to be done as soon after infection as possible. The genetic differences appeared to be due to large insertions or deletions of DNA.

Amongst sheep on some properties, isolates that had a different protease expression and virulence expression were found to have the same molecular type. Investigation of these isolates by SDS-PAGE showed that they also had the same whole cell protein profiles. Isolates from the same clonal groups also had the same protein profile, whereas genetically diverse isolates had different protein profiles. The lack of protein differences between isolates of the same molecular type, or within a clonal group, suggests that the differences in protease thermostability may be due to conformational changes in the protein, rather than to overall detectable genetic change and/or expression of different proteins. These results demonstrate that PFGE typing can be useful in predicting likely phenotypic expression of whole cell proteins. Further work is required to elucidate differences between virulent and benign strains of *D. nodosus*.

General background and introduction to the Thesis

Ovine footrot is a disease of considerable economic importance in several southern Australian States. It results from infection with virulent strains of the anaerobic bacterium *Dichelobacter nodosus*. In 1974 a policy to eradicate virulent ovine footrot from Western Australia (WA) was introduced. The eradication program is now in its final stages, with the aim of WA being declared free of virulent footrot in 2007. At June 2004 there were still 77 properties in quarantine. With these last few properties in quarantine it is vital to the success of the eradication program that outbreaks of infection with virulent *D. nodosus* can be rapidly traced, so as to prevent a resurgence of the disease to levels seen before the introduction of the eradication scheme.

There is a requirement that *D. nodosus* strains be differentiated from each other so that isolates can be identified as either originating from the outbreak strain or not. Similarly, there is a requirement to determine if a strain is known in WA, or whether it is likely to have come from a source outside the State. Many of the highly virulent strains that were present in the early stages of the program and before the eradication policy was introduced, are no longer found in WA. Therefore, methods needed to be established that would assist in determining if an outbreak of infection was a result of the re-emergence of these highly virulent strains, or were from the milder strains that are currently found on farms in WA. The molecular epidemiology of many different pathogens has been studied in both the medical and veterinary fields. Molecular methods were investigated as part of this research to determine their suitability for strain typing of *D. nodosus* and use in the eradication program. The investigation of these different methods for the suitability for molecular epidemiology of *D. nodosus*, and the optimisation of the successful techniques is presented in **chapter 3**. The pulsed field gel electrophoresis (PFGE) method was used because studies have shown that it is very sensitive to detecting genetic difference between isolates, and is considered a ‘gold standard’ for epidemiological typing of pathogenic bacteria. A second method, the infrequent restriction site polymerase chain reaction (IRS-PCR) also was investigated. Molecular typing of isolates by two methods is recommended so as to improve the accuracy of establishing the relationship of an isolate to an outbreak strain. The optimised methodology of these two techniques is presented under general materials and methods in **chapter 2**. The application of PFGE and IRS-PCR to isolates from WA and a small selection of isolates from other Australian States is described in **chapter 4**.

WA isolates from various years throughout the eradication program were investigated, and the data was analysed to determine the genetic diversity of the organism, the numbers of molecular types, the recognition of clonal groups, and the persistence over time of certain clonal groups.

In WA, virulent and benign *D. nodosus* strains are routinely identified by protease thermostability testing and protease isoenzyme profiles. These methods are very reliable. However, they are not sensitive enough for differentiation beyond the 13 profiles generated by these techniques, and therefore have a limited application in disease trace-back. Also, they are limited in their application to epidemiological studies. A major aim of this research was to develop reliable and discriminatory methods that could be used for trace-backs, and could also be applied to study the epidemiology of the organism to gain more insight into the natural history of the disease. The results of case studies of molecular epidemiological investigation of disease outbreaks are presented in **chapter 5**.

In WA, virulent strains are identified as those that produce a heat-stable protease, and it is these strains that are targeted in the eradication policy. However, there is a very small percentage of strains (less than 1% of isolates between 1997 and 2003) that produce a virulent lesion in the hoof, yet do not produce the heat stable protease. These strains tested virulent using *in vivo* pen trial tests. They are identified as U5 by the protease and zymogram methods. Only a small subset of U5 strains are capable of producing virulent lesions. These strains required examination by molecular methods to enable further differentiation between those responsible for either the virulent or benign forms of the disease, and to determine whether molecular typing could replace *in vivo* pen testing as a measure of virulence potential. A decision on their potential virulence is required for the eradication program, so as to determine whether or not a farm should be quarantined.

Another two groups of strains needed investigation for eradication and epidemiological purposes. These are strains that have either a U6 or a ‘T’ profile in the protease and zymogram tests. Although they only cause benign lesions, cases have occurred where virulent footrot has re-emerged on a farm following a successful eradication of the disease. The only sheep left on the property were those that harboured benign strains, U6 strains and/or T strains. These strains needed investigation to

determine their relationship to virulent strains, and therefore their impact on the eradication program. The relationship of protease thermostability and their isoenzyme profiles compared to their molecular types is presented in **chapter 6**.

Of prime importance when investigating an outbreak of infection, is to know how long the isolates recovered during an outbreak will remain genetically similar to the source strain before genetic changes render them distinctly different. The rate of genetic change for *D. nodosus* needed to be understood so that limitations of the methods and time of investigation for trace-backs could be determined. Bacteria undergo genetic change through a number of different mechanisms such as the activity of genetically mobile elements, plasmid exchange, transposons, recombinations, and mutations. For some pathogenic bacteria the mechanisms that lead to genetic diversity may also affect virulence of the organism. The rate of genetic change of selected strains of *D. nodosus* was investigated to determine possible mechanisms responsible for this diversity, and if there was an effect on virulence as defined by protease thermostability. This work forms the basis of **chapter 7**.

Footrot was first recognised in France in the 18th century and since then much research, debate and controversy about the disease, its cause, and definition of virulence has occurred. A review of this information is presented in **chapter 1** and leads to the aims and objectives of the research presented in this thesis.

Summary of thesis aims

The aims of this thesis were to develop and apply molecular typing methods that could be used to investigate disease outbreaks, to improve the knowledge of epidemiology of *D. nodosus* and to determine if there was a relationship between virulence and observable genetic type in this bacterial species.

TABLE OF CONTENTS

LIST of TABLES

List of Figures

Acknowledgments

My sincere thanks to Professor David Hampson (Murdoch University) my principal supervisor, for all his encouragement and wisdom, and for his patience when I diverted my PhD time into writing a reference book on a different subject!

I would like to acknowledge the management of the Animal Health Laboratories, Department of Western Australia and the Footrot Research Program for providing the opportunity to participate in this area of research, which in turn gave me the opportunity to study for this PhD. My thanks to Barry Richards, my co-supervisor at the Department of Agriculture Western Australia, Bob Mitchell (Footrot Coordinator and Program Manager), and all the other members of the Footrot Research Team at the Department for their enthusiasm for footrot research. The Footrot Program provided the funds for this research and also funded the purchase of the Chef DR III Pulsed Field Gel Electrophoresis machine and the gel electrophoresis equipment used for the Infrequent Restriction Site PCR. To Dr Laurie Depiazzi, thank you for lending me many of the early references on footrot published between 1905 to 1980.

I would like to acknowledge Mike Palmer and David Pitman at the Australian Reference Laboratory for Ovine Footrot, Albany, WA who do all the diagnostic isolations, and maintain a collection of over 8,000 *D. nodosus* strains, and who readily reconstituted particular strains when I asked for them. They were responsible for the protease thermostability and zymogram profiles of the strains used in this thesis. They also provided much of the culture media for strains tested from 2000 onwards. Thanks also to technical officers Paul Ashley and Julia Carson who prepared buffers for the DNA extraction and to Paul who undertook the PFGE typing of isolates cultured after 2001.

Thankyou to Dr Darren Trott, a fellow PhD student in Professor Hampson's group, who demonstrated the multilocus enzyme electrophoresis technique to me.

Thankyou to Dr Bruce Allworth for offering the electronic versions of chapters 3, 4 and 5 of his thesis, titled "Investigations of the Eradication of Footrot" (Allworth, 1995). These chapters contained results on the use of RFLP in the typing of *D. nodosus*.

The bulk of the work undertaken for this PhD was done between 1996 and 2001, and I was responsible for optimising the PFGE, IRS-PCR and SDS-PAGE methods for application to *D. nodosus*. This PhD was done on a part-time basis while I was fully employed at the Department of Agriculture Western Australia.

And to my family and friends, who when they came to stay or visit, had to step around piles of papers in the laundry and dinning room, thankyou for your patience and encouragement.

Publications

Publications arising from this thesis

Book Chapter

Hampson D. J., T. G. Nagaraja and **N. B. Buller**. (2004). Gram-negative Anaerobes. Chapter 27 In: Pathogenesis of Bacterial Infections in Animals, 3rd Edition. Carlton Gyles, Charles Thoen, John Prescott and Glenn Songer (eds). Iowa State University Press. pp. 373-383.

Publications

Collighan R. J., R. D. Naylor, P. K. Martin, B. A. Cooley, **N. B. Buller** and M. J. Woodward. (2000). A spirochete isolated from a case of severe virulent ovine foot disease is closely related to a Treponeme isolated from human periodontitis and bovine digital dermatitis. Veterinary Microbiology 74: 249-257.

Conference Proceedings

Buller N. B., M. A. Palmer, D. R. Pitman, R. B. Richards and D. J. Hampson. (2000) Molecular epidemiology of *Dichelobacter nodosus* isolates from ovine footrot in Western Australia. B. Besier and R. Woodgate (eds) Proceedings of the Australian Sheep Veterinary Society, Indooroopilly, Queensland. pp. 171-175.

Buller N. B., M. A. Palmer, D. R. Pitman, R. B. Richards and D. J. Hampson. (2000) Determination of genetic diversity of *Dichelobacter nodosus* isolates from ovine footrot using pulsed field gel electrophoresis and infrequent-restriction-site PCR. Microbiology Australia 21:A88.

Conference Presentations

This work has been presented at the following conferences:

Buller N. B., P. Ashley, M. Palmer, D. Pitman, R. Mitchell, R. Richards and D. Hampson. (2003) Molecular epidemiology: relationship between virulence and clonal strains of *Dichelobacter nodosus* in ovine footrot. Presented at the Animal Health Laboratories, Department of Agriculture Conference, held at AQWA, Perth, June 24-25th June 2003.

Buller N. B., P. Ashley, D. Roberts, M. Palmer, D. Pitman, R. Mitchell, L. Depiazzi, R. Richards and D. Hampson. (2001). Applications of Molecular Fingerprinting to *Dichelobacter nodosus* isolates from Western Australia. Invited Proffered Paper in a symposium and proffered paper session dedicated to Ovine Footrot at the Australian Society for Microbiology Conference, Perth, Western Australia, October 2001.

Buller N. B., M. A. Palmer, D. R. Pitman, R. B. Richards and D. J. Hampson. (2000). Determination of genetic diversity of *Dichelobacter nodosus* isolates from ovine footrot using Pulsed-Field Gel Electrophoresis and Infrequent-Restriction-Site PCR. Proffered Paper at Australian Society for Microbiology Conference, Cairns, QLD July 2000.

Buller N. B., M. A. Palmer, D. R. Pitman, R. B. Richards and D. J. Hampson. (2000). Molecular epidemiology of *Dichelobacter nodosus* isolates from ovine footrot in Western Australia. Presentation at the Australian Veterinary Association Conference, Perth, Western Australia. June 2000.

Poster Presentations

Buller N. B., R. B. Richards and D. J. Hampson. (2003). Relationship of molecular type to protein fingerprint. Poster Presentation at the Australian Society for Microbiology Conference, Auckland, New Zealand Oct 2003.

Buller N. B., R. B. Richards, D. J. Hampson. (1996). Direct detection by PCR of *D. nodosus* from footrot lesion material. Poster Presentation at the Australian Society for

Microbiology Conference, Christchurch, New Zealand Oct 1996.

Sections of the PhD were presented at the Murdoch Post-Graduates student poster days in 2001, 1999, and 1997.

Prizes

Buller N. B., R. B. Richards and D. J. Hampson. 2001. Relationship of Molecular Type to Protein fingerprint. For Poster at Murdoch University Post Graduate Poster Day, Nov 2001. Winner of the Third Year PhD student award.

Other publications whilst working on the PhD

Book

Buller N. B. 2004. Bacteria from fish and other aquatic animals: A practical identification manual". Centre for Agriculture and Biosciences International Publishers (CABI), UK.
This 354 page book details phenotypic and molecular methods for the detection, isolation and identification of bacteria from fish and other aquatic animals. Writing of the book began in May 2000 with the publication of the book in the UK in February 2004, followed by publication in the USA in April 2004. Writing of this book during my PhD contributed significantly to prolonging the time taken to complete the thesis.

Work experience in other laboratories

During this PhD I undertook a three month work experience with Professor Martin Woodward, at the Molecular Genetics Unit, Central Veterinary Laboratories, United Kingdom in 1998. This work involved cloning and sequencing DNA from a spirochaete isolated from an ovine hoof with digital dermatitis. This work contributed to the results presented in the publication, Collighan *et al*. 2000.

Glossary

Table 1-1. Glossary

Abbreviation	Name
AC	Bacterial culture number designated by the Albany regional laboratory
ADH	Arginine dihydrolase
AFLP	Amplified fragment length polymorphism
AWI	Australian Wool Innovation
bp	Base pairs
BOX	Highly conserved repeated DNA element found on the chromosome of Gram-positive organisms. This region is termed BOX
°C	Degrees Celsius
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Dendrogram	A tree diagram showing taxonomic relationships (Oxford dictionary)
DNA	Deoxyribonucleic acid
EDTA	EDTA di-sodium salt
Electrophoretogram	The densitometric or colourimetric pattern obtained from filter paper or similar porous strips on which substances have been separated by electrophoresis; may also refer to the strips themselves (Medical dictionary definition)
ESP	Buffer for DNA extraction in PFGE (EDTA, Sarkosyl, Proteinase K)
g	Gram
h	Hour
HEPES sodium salt	Synonyms: 1-Piperazineethane sulfonic acid, 4-(2-hydroxyethyl)- monosodium salt Chemical Formula: C8H18N2O4S.Na
IRS-PCR	Infrequent Restriction Site Polymerase Chain Reaction
IrsG	Prefix for molecular type using the method of IRS-PCR and primer PXG
IrsT	Prefix for molecular type using the method of IRS-PCR and primer PXT
KDa	Kilodalton
Kg	Kilogram

Glossary continued

Abbreviation	Name
l	Litre
mg	Milligram
MGC	Mitsubishi Gas Company
min	Minute
ml	Millilitre
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
mm	Millimetre
mM	Millimolar
MW	Molecular weight
NCC	National culture collection
NSW	New South Wales
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFA	Prefix for molecular type using the method of PFGE and restriction with *Apa*I enzyme
PFGE	Pulsed field gel electrophoresis
PFX	Prefix for molecular type using the method of PFGE and restriction with *Xba*I enzyme
pmol	picomole
PMSF	Phenylmethylsulfonyl fluoride
Population biology	The study of changes in a population
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
rpm	Revolutions per minute
S	Stable protease (thermostable)
SA	South Australia
TAS	Trypticase Arginine Serine agar - growth medium for *D. nodosus*
TASH-IM	Trypticase Arginine Serine agar Isolation Medium: contains 3.0% powdered ovine hoof horn and 4.0% agar

Glossary continued

Abbreviation	Name
TASH-MM	Trypticase Arginine Serine agar Maintenance Medium: contains 1.5% powdered ovine hoof horn and 1.5% agar
TBE	Tris Borate EDTA buffer used in gel electrophoresis
TE	Tris EDTA buffer
Temporal	Relating to time (Oxford Dictionary)
U	Unstable protease
μg	Microgram
μl	Microlitre
UV	Ultraviolet
v	Volt
VIC	Victoria
v/v	Volume per volume
WA	Western Australia
w/v	Weight per volume
yr	year

Chapter 1

Literature Review

CHAPTER 1

1. Literature Review

1.1 Introduction

Footrot is a contagious disease affecting the hoof of sheep and sometimes other cloven-hoofed animals such as goats and cattle. The major infectious agent is the anaerobic bacterium, *Dichelobacter nodosus*. It is rarely the dominant organism in the lesion, which contains other bacterial flora such as *Fusobacterium necrophorum*, *Bacteroides* species, *Arcanobacterium pyogenes*, *Staphylococcus* species and occasionally spirochaetes. The disease is characterised by lameness, ulceration and sometimes separation of the hoof from the foot. Two main forms are recognised; benign and virulent, with an intermediate classification recognised by some veterinarians. The classification of the lesion into virulent or benign is based on clinical expression, and a grading system has been recommended. The expression of lesion severity is complicated by the effect of the environment, as a lesion may not show full expression of virulence potential in a dry environment. The classification of the *D. nodosus* organism into virulent or benign is based on the production of thermostable protease (virulent) or heat labile protease (benign). The disease occurs seasonally during the late spring and early summer months. It occurs in nearly every country of the world where a temperate climate exists.

Footrot is considered to be a chronic, endemic disease that causes substantial economic loss through loss of body weight, condition and wool growth, decreased lambing percentages, and reduced value at sale of the affected sheep. Other economic costs associated with the disease are; the cost of treatments and eradication that include cost of labour, cost of treatment chemicals, vaccines, upgrading of fences, and possible decreased income potential due to sale of infected sheep to abattoir rather than to the market.

1.2 Nomenclature

In 1941, W. I. B. Beveridge first named the main causative organism of ovine footrot as *Fusiformis nodosus*. It was later transferred to the genus *Bacteroides* as

Bacteroides nodosus and later in 1990, following sequencing of the 16S rRNA gene, given its own genus, *Dichelobacter nodosus* (Dewhirst *et al.*, 1990). The name Dichelobacter relates to the fact that it colonises cloven-hoofed animals with two digits per foot.

1.3 History of footrot

The history of footrot was reviewed in 1905 by Mohler and Washburn, and again in 1941 by Beveridge (Mohler and Washburn, 1905; Beveridge, 1941, 1981).

The first recorded report of footrot was in 1791, in the French region north of the Pyrenees along the Gironde and Lower Medoe rivers (Chabert *et al.*, 1791). Footrot was again reported in France in 1813, this time in central France. The disease was suggested to be contagious following the observation that footrot appeared in a flock of sheep after the introduction of two rams (Gohier, 1813-1816). This observation was further strengthened following a report in 1821 stating that footrot always followed the introduction of lame sheep into a healthy herd (de Gasparin, 1821). In one respect our knowledge of footrot has changed little since these first observations.

The disease was reported in Italy and in Germany in 1815, where it was introduced through the purchase of French Merinos. Footrot spread to Great Britain and was reported in 1837, although it was hypothesized that it existed there well before that time (Youatt, 1837).

Footrot was well established in flocks of sheep in Maryland and Virginia by 1832, and it was thought that it was introduced into North America as early as 1797 via the importation of sheep from Europe to the regions of Ohio, Indiana and Illinios. Interestingly, it was noted that although Spanish Merino sheep were imported, Spain was not considered to be a country of origin for footrot, particularly because of its dry climate and the fact that footrot had not been reported from there. It was suggested that the Spanish Merinos had spent time in some other country (where footrot occurred) before the transportation of these subsequently-infected sheep to America (Mohler and Washburn, 1905).

Footrot is thought to have been introduced into Australia soon after the arrival of the first sheep flocks, as a report in 1802 suggested that "sheep with footrot died when they were not well cared for" (Robertson, 1932).

Control measures have changed little in two centuries, as the French in 1818 recommended quarantining affected flocks, and we still employ this basic measure today. In America in the 19th century control measures were also established and the transportation of sheep by rail was recommended, rather than driving them on foot. High risk areas, such as railway stock yards, had Federal inspectors present who prevented the mixing of healthy and infected sheep (Mohler and Washburn, 1905).

Treatment also has remained much the same for over a century. Scientists in the late 18th and 19th centuries recommended the treatment of paring of the hoof and footbathing, where the sheep walk through a trough containing an appropriate solution. Copper sulphate, carbolic acid, and chloride of lime were all recommended (Mohler and Washburn, 1905). Today, the solution recommended for use in the trough is zinc sulphate (Beveridge, 1941; Cross and Parker, 1981; Malecki and Coffey, 1987).

For almost two centuries controversy raged as to whether the disease was contagious or not. It was often confused with foot and mouth disease and other diseases of the hoof. In 1821, de Gasparin suggested that the disease was due to a contagious agent and discussed experimental methods for reproducing the disease. In these experiments no disease was reproduced until a piece of diseased hoof was applied to a pared, healthy hoof. No disease was produced when pus was applied, or a diseased hoof was rubbed against a healthy one (de Gasparin, 1821). However, it was not until the early 1900s that - "it was proved that footrot could be produced at will in healthy sheep not only by spreading a little purulent matter from a diseased hoof upon shaven interdigital skin of sound feet, but quite as readily when bouillon culture inoculated with some discharge from an affected foot were applied in a like manner…" (Mohler and Washburn, 1905). This was an almost exact replica of the method suggested by de Gasparin, and finally the scientific community agreed that the disease was due to an infectious agent and not a result of damage from pasture. Today, in experiments to reproduced the disease, a culture of *D. nodosus* is applied to a lightly scarified, pared hoof and the culture held in place by bandages for 3 days during which time the organism is able to penetrate the skin.

Once it was accepted that an infectious agent was involved in footrot, scientists set out to isolate it and reproduce the disease using pure cultures. In the early 1900s the causative organism was thought to be an anaerobic, long filamentous, pleomorphic rod identified as *Bacillus necrophorus*. *B. necrophorus* (later renamed to *Fusiformis necrophorus*) was recognised as causing a number of conditions in animals where there was necrotic destruction of tissue (see under 1.9.1 - other hoof diseases).

Reports from France, and supported by some American researchers, suggested the causative organism was a spirochaete named *Treponema podovis* (Blaizot and Blaizot, 1928, 1929). The organism could not be cultivated, and Australian scientists (Murnane, Carne and Beveridge) doubted that the organism played a major role in the disease, and suggested that it was a secondary invader (Murnane, 1933).

The next organism named as having a role in footrot, despite its inability to reproduce the disease from pure cultures, was another anaerobic rod identified as a *Spirochaeta penortha* (Beveridge, 1936). Finally, in 1938, an organism that had been seen in relatively small numbers in infected material was isolated when 25% serum was added to the medium. This was an anaerobic, non-motile, Gram-negative rod that showed bipolar staining. Footrot infection was produced from pure cultures of this organism, initially called organism K and thought to belong to the genus *Bacteroides* (Beveridge, 1938). It was later named *Fusiformis nodosus* and proved to be the causative and primary organism involved in footrot (Beveridge, 1941). The organism has undergone two more name changes since then, to *Bacteroides nodosus* (as originally thought by Beveridge), and to its own genus *Dichelobacter nodosus* (Dewhirst *et al.*, 1990).

Since the first reports there have been many publications about the disease, the research conducted into the disease, the causes, treatments and control measures. All publications carry a similar description of the disease in the hoof, its two major forms (benign and virulent), how it can be controlled, and how it can be treated. The major element that has evolved over this time concerns the nature and identification of the causative agent.

1.4 Geographical distribution

Footrot has a world-wide distribution and has been reported from countries including Australia (Beveridge, 1941), Britain (Naylor *et al.*, 1998), Canada (Olson *et al.*, 1998), Denmark (Jessen *et al.*, 1993), France (Popoff, 1991), Holland (Toussaint and Cornelisse, 1971), India (Wani *et al.*, 2004), Malaysia (Zakaria *et al.*, 1998), Nepal (Egerton *et al.*, 1994; Ghimire *et al.*, 1998), New Zealand (Zhou and Hickford, 2000b), Portugal (Jiménez *et al.*, 2003), Spain (Piriz *et al.*, 1990) and the USA (Gradin, 1993).

A number of aspects of the environment (climate, temperature, rainfall) have an influence on the distribution, clinical expression, and survival of footrot. In Australia footrot occurs in the temperate rainfall areas of Western Australia (WA), South Australia (SA), Victoria (Vic), Tasmania (Tas) and New South Wales (NSW). Its occurrence in Queensland is sporadic (Graham, 1870; Beveridge, 1941).

1.5 Effect of environment on disease expression

One of the major factors involved in expression of virulence is the environment. Moisture and temperature are key factors involved in the clinical expression of footrot. A mean ambient temperature above 10°C is required together with adequate moisture (Beveridge, 1941; Graham and Egerton, 1968). Because of its dependence on temperature and moisture, footrot is a seasonal disease. In Australia, the most severe clinical expression of footrot occurs in late spring and early summer.

The importance of the effect of environment on the expression of virulence was demonstrated when a virulent strain that was inoculated into sheep that were then grazed at five different geographical locations in WA, produced footrot that showed variation in clinical expression. This expression appeared to be related to the amount of moisture contained in the pasture and the ability of the soil type to retain moisture (Depiazzi *et al.*, 1998). It is possible that pasture type may also have an inadvertent role by the damage it causes to the skin of the interdigital horn. *D. nodosus* cannot penetrate unbroken skin and it is only after scarification of the skin that infection occurs. Other organisms (especially *Fusobacterium necrophorum*) present on the hoof skin and in the soil and pasture may also have a role to play in the initial damage of the interdigital skin of the hoof.

Therefore, a sole reliance on clinical expression to diagnose footrot as benign may lead to a false-negative diagnosis unless appropriate environmental conditions are present.

1.6 Survival of *D. nodosus* in the environment

The survival of the causative organism of footrot was first tested in 1892 when experiments showed that the agent survived in muddy soil for two days (Brown, 1892). It was also noted that pastures left for 12 months did not subsequently transmit footrot (Mohler and Washburn, 1905). No infection could be detected when the soil in the pens was allowed to dry for 15 days or after 30 days if the soil was kept continually wet. However, it had been noted that the organism appeared to survive in an "attenuated form" for nine months in a swamp (Marsh and Tunnicliff, 1934). In another experiment, soil was used to successfully produce the disease, however this same soil was unable to produce the disease in sheep when it had been left in a Petri dish on the bench for seven days (Beveridge, 1935). Extensive experimentation using sheep in yards that contained contaminated material indicated that *D. nodosus* could survive in the environment for no more than two weeks (Beveridge, 1941).

Researchers frequently have postulated that the disease may go into a latent state, where the organism resides deep in the hoof with no outward sign of infection. When conditions occur that are conducive to footrot, the organism ceases its dormant state and infection is seen (Mohler and Washburn, 1905; Beveridge, 1935). This was also indicated in a trial where sheep that had been experimentally infected with a virulent strain of footrot were grazed in a dry climatic area for two years. Apart from the initial lesion which subsequently healed there were no signs of lesions, nor could the organism be isolated from the hoof. However, on removal to a geographical location favourable to footrot, the sheep developed classical lesions and the organism was re-isolated (Depiazzi *et al.*, 1998).

It remains to be established whether *D. nodosus* is capable of surviving in the environment in a "viable but non-culturable stage" (VNC), as has been demonstrated with other organisms such as the *Vibrio* species and *Campylobacter jejuni* and *C. coli* (Rollins and Colwell, 1986; Oliver, 1999).

1.7 Host range and susceptibility

Footrot affects mainly ruminants including sheep, goats, cattle and deer, but also has been reported in pigs. Sheep and goats may develop virulent or severe forms of the disease, whereas cattle and deer develop a mild or benign form of the disease. The strains isolated from cattle and deer do not tend to cause virulent lesions in sheep or goats, and therefore cattle and deer are not considered to be a reservoir of infection for small ruminants (Beveridge, 1941; Egerton and Laing, 1978/1979). A case of footrot in WA in 2004 indicated that a cattle strain infected sheep with virulent footrot, and that the molecular type of the isolates was identical from both sheep and cattle (T. Higgs, Department of Agriculture, WA, unpublished data, 2004).

In cattle, footrot generally presents as a milder form of the disease than that expressed in sheep. Lameness and severe lesions rarely occur. Lesions in the bovine hoof are seen as an interdigital dermatitis that may express as small discrete circular ulcers or progress to a severe lesion that involves the entire interdigital space. There may also be necrosis, a foul smelling exudate and severe underrunning can occur (Toussaint and Cornelisse, 1971; Laing and Egerton, 1978; Richards *et al.*, 1980). A number of breeds of cattle have been reported to be infected with footrot and these include dairy cattle, especially Fresians, Herefords, *Bos taurus* and *Bos indicus* breed types such as Australian Illawarra Short-horn, Charolais cross, Brahman, and Sahiwal (Toussaint and Cornelisse, 1971; Frisch, 1976; Laing and Egerton, 1978; Richards *et al.*, 1980).

D. nodosus has been isolated from pigs in Spain, where the associated condition is know as ‘bush foot’. The disease appears as a white line lesion at the heel of the hoof. The infection begins at the posterior part of the hoof and moves toward the horn/skin junction to the coronary band. The predominant serotype in a study in Spain was serotype B; a predominant serotype in ovine cases in Australia (Claxton *et al.*, 1983; Píriz *et al.*, 1996).

1.7.1 Natural and artificial immunity, host response and relationship to pathogenesis

Genetic factors, including host susceptibility, may play a role in determining the extent of the lesion that develops. The immune response to natural and experimental

infection varies among breeds of sheep and within an infected flock. All ages of sheep are susceptible, although the lesions in lambs are less severe than those in adult sheep.

The coarse-woolled British breeds of sheep are generally considered to be more resistant to natural infection with *D. nodosus* than Merino sheep, as lesions are benign and of short duration. However, in experimental infections where the organism was applied directly to the hoof, all breeds expressed severe lesions (Emery *et al.*, 1984).

Natural resistance manifests as mild lesions, delayed infection and self-curing lesions. Research suggests that there is a paternally inherited genetic basis to natural resistance to footrot that involves the alleles in the ovine major histocompatibility complex (MHC) class II region (Escayg *et al.*, 1997). An involvement in the SY6 and SY1b Class I ovine lymphocyte antigens also suggests a contribution towards disease resistance by producing a greater antibody response after vaccination (Outteridge *et al.*, 1989).

The bacterial invasion in the hoof lesion is restricted to the avascular epidermis (the stratum spinosum) (Egerton *et al.*, 1969), however, when sheep are challenged with different antigenic fractions from *D. nodosus* a weak immune response is elicited. Pilus antigens generate a greater immune response than do cell envelope antigens, lipopolysaccharides or extracellular protease antigens. The primary and anamnestic humoral immune response, as detected by antibody level, is highest when lesions are severe and the infection prolonged (Whittington and Nicholls, 1995b). Host response is associated with severity of lesions in the early stages of an infection, whereas after five to six weeks of an infection the duration of the severe lesions influences the immune response (Whittington and Nicholls, 1995b).

Based on pilus antigens, there are ten serogroups (A-I, M) of *D. nodosus*, and protection is serogroup specific (Claxton *et al*., 1983; Chetwin *et al*., 1991). Infection with multiple serogroups occurs within a flock and within a hoof (Claxton, 1989). Unfortunately, when using a multivalent vaccine, antigenic competition or inhibition of the immune response occurs such that the immune response is lower than that elicited when a monovalent vaccine is used (Schwartzkoff *et al.*, 1993).

1.8 Economic affects of the disease

The main effect of the disease, apart from the animal ethics issues, is economic due to the cost of mortality (rare), loss of condition of affected animals, the cost of treatments and the cost of eradication and re-stocking. Trading of live animals is also prevented. As a results of the lameness caused by the severe or virulent lesions, sheep show inappetence and inability to feed, which results in a loss of body condition, wool growth and wool quality (Murnane, 1933; Marshall *et al.*, 1991).

1.9 Diagnosis of footrot

In Australia, footrot is a notifiable disease in WA, SA and parts of NSW and Vic (Stewart and Claxton, 1993). Diagnosis of the disease is based on clinical examination and culture of the causative organism. In WA, where there is an eradication program for virulent footrot, infected farms are placed in quarantine. The identification of virulent footrot is dependent upon clinical diagnosis in conjunction with laboratory tests that identify virulent strains.

1.9.1 Other hoof diseases

The differential diagnosis of hoof diseases in sheep and goats is not always easy to define. Similar diseases include infectious pododermatitis, digital dermatitis, and strawberry footrot, all of which have at some time been referred to as footrot. Strawberry footrot, or proliferative dermatitis, is caused by *Dermatophilus congolensis*, and has been reported from Scotland (Harriss, 1948). Affected animals are not lame, and the disease affects the limb from the coronet to the hock with scabs that when removed cause areas of bleeding that resemble a strawberry. The primary lesion is rarely seen in the interdigital cleft. Infectious pododermatitis (interdigital necrobacillosis, foul in the foot, fouls) affects mainly cattle, but sometimes sheep, and is caused by infection with *Fusobacterium necrophorum*. *Bacteroides melaninogenicus* is a secondary invader, but is not considered to have a primary role in the disease (Clark *et al.*, 1985).

Foot abscess is due to infection with *Fusobacterium necrophorum*, usually with *Arcanobacterium* (*Corynebacterium*) *pyogenes* present, and is characterised by lameness and thick pus expressed from the sinuses around the coronet (Gregory, 1939).

The disease is also referred to as infective bulbar necrosis (Roberts *et al.*, 1968), digital suppuration (Gregory, 1939; Thomas, 1962a), and known internationally as *Phlegmona interdigitalis*. This disease can occur in both sheep and cattle. Foot scald, also called benign footrot or non-progressive footrot, is a condition caused by benign strains of *D. nodosus*. There is no underrunning of the horn, no smell and only mild lameness (Egerton and Parsonson, 1969).

Ovine interdigital dermatitis (OID) is caused by invasion of the epidermis with *Fusobacterium necrophorum* (Murnane, 1933: Beveridge, 1941) and these lesions can assist *D. nodosus* in the invasion of the hoof to produce footrot (Egerton *et al.*, 1966; Parsonson *et al.*, 1967). In OID the animals are rarely lame and usually recover.

Recently, a disease termed severe virulent ovine footrot (SVOFR) was described in the United Kingdom (Harwood *et al.*, 1997; Naylor *et al.*, 1998; Collighan *et al.*, 2000; Demirkan *et al.*, 2001). This disease appears to differ from classical footrot by the type and appearance of the lesion in the hoof, and the organisms cultured. In SVOFR the lesion begins around the coronary band, the feet are red and swollen and the disease is aggressive. *D. nodosus* was not isolated from the lesions in these cases, nor seen on microscopic examination. However, in two separate SVOFR cases in the UK, *Treponema* species were cultured and identified by 16S rRNA gene sequencing. The treponeme isolated from the second case of SVOFR had 98.6% similarity to a spirochaete isolated in the USA from a case of bovine digital dermatitis (Walker *et al.*, 1995) and 95.1% similarity to *T. denticola* (Demirkan *et al.*, 2001). It had 90.5% similarity to the treponeme in the first SVOFR case in the UK, which was identified as *T. vincentii*-like (Collighan *et al.*, 2000).

Spirochaetes are often observed in footrot lesions, and in early studies on the causative organism of footrot infection two species were identified, *T. podovis* (Blaizot and Blaizot, 1928, 1929) and *T.* (*Spirochaeta*) *penortha* (Beveridge, 1936). However, footrot could not be reproduced following inoculation with pure cultures of these organisms (Murnane, 1933; Beveridge, 1936). Further work needs to be done to clarify the identity of the recently isolated spirochaetes, their involvement in footrot lesions, pathogenicity, and the status of the disease as separate from classical footrot.

1.9.2 Field diagnosis and clinical expression

An initial diagnosis of footrot is made by examining the hoof for the presence of a lesion that is characteristic of footrot. A lesion in the hoof begins with an inflammation of the interdigital skin, and may progress to invasion of the hoof matrix that separates the soft tissue of the foot from the hard horn. The severity of the invasion classifies the lesion as benign or virulent.

A lesion that does not progress beyond the mild stage of invasion is referred to as benign footrot, shown in Figure 1-2, compared to a healthy hoof in Figure 1-1. There is no underrunning of the hoof and minimal exudation. The benign lesion cannot be distinguished from ovine interdigital dermatitis. Lameness is either absent or mild, a large number of the flock may be affected and there is a minimal economic effect (Thomas, 1962a; Parsonson *et al.*, 1967; Egerton *et al.*, 1989).

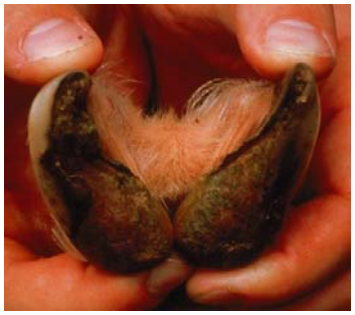


Figure 1-1. Healthy hoof

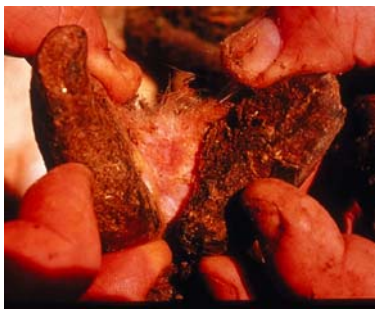


Figure 1-2. Benign footrot lesion

A lesion that progresses beyond a mild invasion to a lesion that shows severe erosion of the hoof matrix, and causes separation or underrunning of the keratin matrix, is termed virulent footrot, as seen in Figure 1-3 (Beveridge, 1941). Moist, white-grey, necrotic material is usually present between the separated layers, and this has a

distinctive foetid smell. Usually both digits and more than one hoof are affected, and sheep become lame. Sheep tend not to move because of the lameness and pain associated with the severe underrunning lesions. Food intake is reduced, and this leads to loss of body condition and ultimately affects wool growth.

Figure 1-3. Hoof showing severe lesion of virulent footrot

Unfortunately, classification of lesions into two classes, and thereby determining virulent or benign footrot, is not straightforward. A range of lesion severity is seen in the field. This problem has resulted in a number of lesion-scoring systems where lesions are graded according to the amount of invasion and subsequent damage of the soft tissue and hard horn. They follow a basic grading of the lesion into 0 for no lesion, score 1 and 2 for mild lesions where damage is confined to the interdigital skin, followed by scores of 3a, 3b, 3c, 4 and 5 for increasing underrunning of the horn (Stewart and Claxton, 1993). In an attempt to obtain a consensus method, these scoring systems were analysed for their relationship to host humoral immune response to the organism. The method that most accurately aligned with the immune response was a Total Weighted Footscore (sum of the weighted lesion scores of the four feet) (Whittington and Nicholls, 1995a). A standardised method assists in determining virulent and benign lesions in diagnosis of the disease in the field, in research for effectiveness of vaccines, and in assessment of virulence factors. Unfortunately, mild or low scoring lesions may not necessarily rule out infection with a virulent strain as other factors are also involved in virulence.

Defining and identifying a severe lesion as virulent is easy, but defining a less severe lesion presents problems. Not all strains of *D. nodosus* that are capable of producing a severe lesion do so, as environmental conditions and other unknown factors impact on lesion expression. Therefore, a strain that is not expressing its maximum potential for lesion development is difficult to classify as virulent. The definition of virulent and benign is contentious, and scientists cannot agree on whether the definition should be based on clinical observation, laboratory tests on the bacterium or a

combination of both. Also, some scientists recognise an intermediate category of lesion expression, thus compounding the problem. Intermediate footrot is defined as a milder disease, with only a small percentage of sheep in a flock presenting with lesions that extend to the abaxial wall, but rarely underrun the hoof (Egerton *et al.*, 1989). There is no reliable laboratory test that distinguishes *D. nodosus* strains of intermediate virulence.

1.9.3 Laboratory diagnosis

1.9.3.1 Histopathology of the hoof lesion

Dichelobacter nodosus, although the primary transmissible agent of footrot, is rarely the predominant organism in the infected hoof environment. It is often present in low numbers compared to other Gram-negative rods such as members of the *Fusobacterium* genus, *Bacteroides* genus, and Gram-positive organisms such as *Corynebacterium* species and *Staphylococcus* species. Spirochaetes may also be seen. Some of these organisms are still considered to play a role in the development of the footrot lesion, particularly fusiform bacteria and spirochaetes. Certainly, *Fusobacterium necrophorum* (previously *Bacillus necrophorus*) is capable of causing ulcerated lesions that produce a foul smelling discharge and abundant yellow pus (Mohler and Washburn, 1905; Beveridge, 1935).

Another organism that is occasionally seen in smears from footrot lesion is the so-called “Beveridge organism” (Figure 1-4). This is a small Gram-negative rod that clumps around a single cell of *D. nodosus* and was first noted by Beveridge (Beveridge, 1941, 1956). It has never been formally identified nor has its role in the footrot lesion or the nature of its association with *D. nodosus* been established.

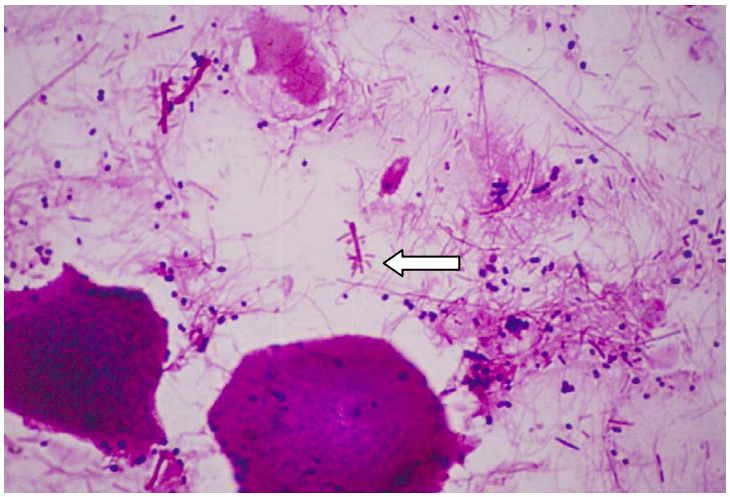


Figure 1-4. Lesion smear showing *D. nodosus* surrounded by the "Beveridge organism"

Lesions of footrot are limited to the integument. In early lesions only the interdigital skin and axial wall are affected. The cells of the epithelium, particularly the stratum granulosum and superficial stratum spinosum, swell, show liquefaction, and become necrotic with vacuoles present in the cytoplasm. Leucocytes infiltrate the vacuoles and also the papillae of the corium, which becomes oedematous. In advanced lesions these changes extend laterally through the stratum spinosum. Necrosis may be seen without the vacuolation, and the epithelial cytoplasm becomes granular. This lateral, spreading necrosis results in separation of the stratum corneum from the underlying tissues (Deane and Jensen, 1955).

D. nodosus is not the organism that penetrates furthest into the tissues in the hoof. A dense, felted mass of rods and filaments can be seen penetrating the fissures of the epidermis, and these generally are considered to include *F. necrophorum,* an unidentified motile fusiform and *Spirochaeta penortha.* These three organisms are almost always present in a footrot lesion together with *D. nodosus,* although it is *D. nodosus* that is essential in reproducing the disease. Although it is more commonly seen near the superficial region of the lesion, *D. nodosus* has been found penetrating the epidermis together with either the motile fusiform or with *S. penortha* (Beveridge, 1941). However, although bacteria are seen on the surface of the area of separation, some researchers have not seen them where the lesion advances into healthy tissue (Deane and Jensen, 1955). Now that more is known about *D. nodosus* it would be interesting to determine if there is any difference in the penetrating abilities of the different strains.

1.9.3.2 Microscopic appearance of *D. nodosus*

Dichelobacter nodosus is a strict anaerobe, and is a large Gram-negative rod with enlarged ends (Figure 1-5). In the lesion the organism measures 0.6–0.8 μm wide by 3–10 μm in length (Beveridge, 1941).

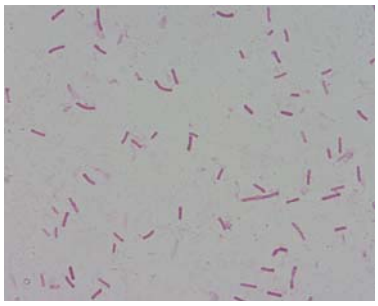


Figure 1-5. Gram smear of *D. nodosus*

In culture, the organism can be pleomorphic, especially if the conditions are not optimal for its growth. In these instances, the organism can become quite long, convoluted and almost unrecognisable as *D. nodosus* (personal observation). Coccoid forms may also be seen (Beveridge, 1941; and personal observation).

1.9.3.3 Culture of *D. nodosus*

Dichelobacter nodosus requires anaerobic conditions and has particular growth requirements. It is routinely cultured onto TASH agar plates that contain trypticase, arginine, serine, yeast extract, peptone and ground hoof (Skerman, 1975; Depiazzi and Richards, 1984/85; Pitman *et al.*, 1994).

The culture of *D. nodosus* or the identification of the bacterium in smears of lesion material is indicative of footrot infection, however in WA the program for eradication of virulent strains demands that isolates are tested for virulence.

1.9.3.4 *In vitro* identification of virulent and benign isolates

In WA, a laboratory classification of virulence is currently based on the production of a thermostable protease that is detected using the gelatin-gel test. Thermostable strains retain their ability to hydrolyse gelatin after heating to 68°C, whereas benign strains produce a protease that is susceptible to heating and does not retain gelatinase activity (Depiazzi and Richards, 1979; Green, 1985a; Palmer, 1993).

Strains are further subdivided based on the isoenzyme profile of their extracellular protease (gelatinase), as detected in the zymogram method (Pitman *et al.*, 1994). These tests are discussed in more detail in the next section.

1.10 Virulence factors of *Dichelobacter nodosus*

One of the major problems in differentiating between a virulent and a benign strain is the definition of virulence. The problem is that there appears to be no single virulence factor, and there has been a great deal of controversy about defining virulence for *D. nodosus*.

Ever since *D. nodosus* was detected and identified, researchers have been trying to find a definitive virulence factor that distinguishes strains that have the potential to produce a severe lesion from those that only cause a mild inflammatory response under all circumstances. Knowledge of virulence factors is important for control and eradication programs, and vaccine production. A number of characteristics of *D. nodosus* have been investigated, and continue to be investigated, to determine if they are virulence factors in their own right, or contribute to enhancement of virulence in some way. These characteristics include colony morphology (Short *et al.*, 1976; Depiazzi *et al.*, 1990), growth inhibition by brilliant green, the presence of pili (Walker *et al.*, 1973; Every, 1979; Lee *et al.*, 1983; Elleman, 1988; Gradin *et al.*, 1991), twitching motility (Depiazzi and Richards, 1984/85), the presence of an additional layer outside the outer membrane proteins and diffuse polar material (Every and Skerman, 1980), and protease production and type.

Dichelobacter nodosus produces a number of different proteases that have been investigated for virulence potential including gelatinase, fibrinogenase, collegenase, caseinase and elastase (Beveridge, 1941; Thomas, 1962a, 1964a; Egerton *et al.*, 1969; Skerman, 1975; Broad and Skerman, 1976; Depiazzi and Richards, 1979; Stewart, 1979; Every, 1982; Kortt *et al.*, 1982; Green, 1985a, b; Kortt *et al.*, 1994a). The organism possesses type IV pili that are involved in twitching motility and adhesion (Walker *et al.*, 1973). However, to date, the main virulence factor that enables a distinction between an isolate capable of producing a virulent lesion and an isolate that will only produce a benign lesion even under ideal conditions, is the detection of heat stable protease (Depiazzi and Richards, 1979; Green, 1985a; Palmer, 1993). Research

on the proteases produced by *D. nodosus* has spanned more than fifty years, yet there are still questions that need to be answered on the relationship between proteases and virulence.

1.10.1 Proteases – general background

Proteases are enzymes that catalyze the hydrolysis of peptide bonds in proteins or peptides. They can be classified into exopeptidases or endopeptidases according to their mode of action on a protein. Exopeptidases act on the carboxyl termini, and endopeptidases act on the internal peptide bonds of proteins. The proteases produced by microbes are mostly extracellular, and these can be divided into five groups (aspartate; cysteine; thiol; metalloproteases; serine) according to the catalytic residue at their active site. Serine proteases are so characterized because they have a serine residue at the active site. They are further classified according to their structure and mechanism of action. The two main types of serine proteases are chromotrypsin and subtilisin.

Proteases from *D. nodosus* were first reported in 1941, when it was noted that meat particles in a broth culture were digested and tyrosine crystals were produced, indicating that proteolysis had occurred (Beveridge, 1941). Further study detected two peaks of enzyme activity; one was associated with the growing phase and the other with bacterial cell lysis. Both enzyme preparations digested casein, haemoglobin, fibrin and gelatin, where the proteolytic activity was highest in alkaline conditions. It was surmised that proteolytic activity accounted for the types of lesions seen in the hoof (Thomas, 1962b, 1964a, b). Further testing on these enzyme preparations showed that no hydrolysis occurred with chondroitin sulphate B (a measure of mucopolysaccharide degradation), collagen, hyaluronic acid or polysaccharides in the hoof, however there was weak activity against elastase. These latter proteins are constituents of the dermis, that part which is unaffected in the footrot lesion.

In contrast, the disease does affect the epidermis. The clinical lesion presents as a separation of the keratinized horn from the foot followed by degeneration of the horn. Keratin is composed of helical and fibrous strands of protein called intermediate filaments or microfibrils. This protein is composed of amino acids, in particular cysteine, which forms disulphide bonds to give a rigid structure (Fraser *et al.*, 1959). When horn and the individual constituents, both the amorphous and fibrous proteins,

were tested against extracellular enzyme, all were digested. Optimal enzyme activity occurred at two alkaline pH values. Cysteine with 4M urea enhanced activity for horn degradation, whereas urea (a substance that denatures proteins) used alone inhibited activity. It was surmised that rather than the cysteine having an effect on the enzyme itself, its benefit was due to sulphydryl transfer that exposed the proteins in the hoof to the enzymes (Thomas, 1964a). When keratinized hoof is broken down by dry grinding, peptides are thought to be released and these peptides are then reduced by the proteases of *D. nodosus* (Thomas, 1964a). Also, keratin has a high content of arginine, an amino acid that is degraded by *D. nodosus* cells, and appears to be a major energy source (Skerman, 1975).

These tests were also confirmed when frozen sections of hoof were incubated in the enzyme preparations. The epidermis separated from the dermis along the basement membrane of the epidermis. Ulceration of the epidermis in a hoof was achieved when the enzyme preparations were injected into the skin of the axilla in a sheep (Thomas, 1964a).

In contrast to the findings and interpretation of Thomas (1964a), no evidence was found that the proteases of *D. nodosus* degraded ovine hoof (Broad and Skerman, 1976). However, although both groups used the same method for purification of the protease, Broad and Skerman carried their tests out at 25°C rather than at 37°C as used by Thomas. In addition, they tested their protease preparation on wool keratin, not hoof as used by Thomas. Also they did not add cysteine with 4M urea to the reaction, and this was found to improve the activity of the enzyme (Thomas, 1964b). Thomas clearly stated that without the addition of cysteine, hoof was not digested (Thomas, 1964b).

The extracellular proteases were found to be inhibited by EDTA, but activity was restored after the addition of $CoCl_2$, $MnCl_2$ (100% restoration), $CaCl_2$ and $MgCl_2$ (70% restoration) (Broad and Skerman, 1976). Activity was not affected by trypsin or chymotrypsin. However, in contrast to previous research (Thomas, 1964a), no affect on the activity of the proteases was found using sulphydryl reagents such as ascorbic acid, cysteine, iodoacetamide, 2-mercaptoethanol, sodium formaldehyde sulphoxylate, sodium thioglycollate, and thioglycollic acid, whereas dithiothreitol was found to increase the caseinolytic activity of the enzymes.

In 1979, a test that measured the relative degradation of protease activity was used to differentiate between virulent and benign isolates of *D. nodosus* (Depiazzi and Richards, 1979). Isolates were grown in TAS broth to termination, and protease activity measured by the method of Rinderknecht, where hide-powder azure was used as the substrate. Hide powder-azure is an insoluble chromogenic substrate used to detect proteolytic enzymes (Rinderknecht *et al.*, 1968). Although the protease activity of both virulent and benign isolates was similar during the growth phase, isolates from virulent lesions retained enzyme activity for a longer time after cell death than did isolates from benign footrot. It was hypothesized that virulent isolates caused more severe lesions because they caused more prolonged proteolysis than benign isolates (Depiazzi and Richards, 1979). These results concurred with the findings of Thomas (1964a,b), who also found protease activity upon cell lysis, yet did not make the connection between virulent and benign isolates.

The degrading protease test (Depiazzi and Richards, 1979) was improved by heating the cultures to 55°C. Isolates from virulent lesions retained significant enzyme activity against casein and hide-powder-azure when heated to 55°C for 30 minutes, whereas isolates that were benign lost activity on heating (Kortt *et al.*, 1982). As with earlier studies (Thomas, 1964a), it was found that different culture media affected the enzyme's ability to retain activity. The proteases retained activity when stored at 4°C at pH 8 in the presence of 5 mM $CaCl_2$. The protease activity was inhibited by phenylmethylsulfonyl fluoride (PMSF) and other compounds such as N-Tosyl-L-phenylalanyl chloromethylketone (TPCK), which is an inhibitor of α-chymotrypsin, whilst iodoacetate inhibited 20 to 30% of activity. EDTA, a metal-ion chelator, inhibited protease activity, whereas 1,10-phenanthroline did not, and this indicates that divalent ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} are required for activity - thus identifying the proteases as serine proteases. There was no digestion of native haemoglobin, native bovine serum albumin or fine wool keratin, α-*N*-benzoyl-L-arginine ethyl ester, or *N*-benzoyl-L-tyrosine ethyl ester. Proteases digested casein, hide-powder-azure, and elastin, and denatured haemoglobin (Kortt *et al.*, 1982).

Later, the protease degrading test was modified and the test time reduced to four hours (Green, 1985a). The test was carried out at 60°C. Virulent isolates were more

stable at 60°C than benign isolates. Isoenzyme forms of the protease were also tested for heat stability and found to retain activity after heating (Green, 1985b).

The protease test to differentiate virulent and benign strains has continued to be improved. $CaCl_2$ improves the stability of the protease enzymes, and so its incorporation into the test medium means that the thermostability test is performed at its optimum reagent concentrations (Depiazzi and Rood, 1984). The test was optimised at a temperature of 68°C, using gelatin to detect the protease activity rather than hide azure, and the end result was a good diagnostic test that enabled the differentiation of strains into virulent or benign (Palmer, 1993). Subsequently, it took a number of years for this procedure to be generally accepted as being a reliable test for the differentiation of virulent and benign strains.

1.10.2 Proteases – isoenzymes

The extracellular proteases have different isoenzyme forms, and these can be detected in a zymogram gel. The isoenzyme profiles are characteristic and can differentiate benign and virulent isolates from ovine footrot (Every, 1982; Kortt *et al.*, 1982; Gordon *et al.*, 1985; Green, 1985b; Depiazzi *et al.*, 1991). Isoenzymes from thermostable protease isolates are a relatively homogenous group. Four isoenzyme patterns are recognised (S1, S2, S3, S4), with the majority (99% as tested in WA) giving the isoenzyme pattern S1. Heat-labile isolates have one of nine isoenzyme profiles (U1, U2, U3, U4, U5, U6, U7, U8 and T), with the majority having the U1 profile (Palmer, 1993; M. Palmer, Australian Reference Laboratory for Ovine Footrot, unpublished data, 2003). This classification now forms the basis of strain typing and identification of virulent types in WA (Pitman *et al.*, 1994).

The individual isoenzyme bands have activity against a range of substrates such as collagen I and III, casein, elastin, α-elastin, fibrinogen, gelatin, haemoglobin and α-keratin (Green, 1985b). Overall, the isoenzymes from virulent isolates have more proteolytic activity than those from benign isolates. Individual isoenzymes within an isolate have a range of activities against different substrates. Some of the highest proteolytic activity occurs with fibrinogen, casein, gelatin and α-elastin (Green, 1985b).

Individual isoenzymes are inhibited to different degrees by EDTA, TPCK (L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone), and PMSF (phenylmethylsulphonyl fluoride). Activity is not inhibited by soyabean or ovomucoid trypsin, iodoacetamide (a thiol inhibitor), ε-aminocaproic acid (a fibrinolytic inhibitor), or phenanthroline (a heavy metal chelator) (Green, 1985b). The isoenzymes are serine proteases and chymotrypsin-like enzymes (Kortt *et al.*, 1982 ; Green, 1985b). Metal ions affect the isoenzymes differently. There is variable inhibition with Sr^{++}, Cr^{+++}, Mn^{++}, Se^{++++}, or Pb^{++}, whereas all isoenzymes are inhibited by Ni^{++}, Cu^{++}, Cd^{++}, Hg^{++} and Fe^{++}. Zinc ions completely inhibit all isoenzymes. (Zinc sulphate is used in footbathing solutions for treatment of the infection (Cross and Parker, 1981)). Some of the isoenzyme bands are activated by Ca^{++}, Mg^{++}, Cr^{+++} and Se^{++++}. Only one isoenzyme band from the benign isolates that were tested was activated by some of these ions (Green, 1985b). The activation or inhibition of isoenzymes by the various metal ions could not be used to differentiate benign from virulent strains. It is worth noting that one of the critical components in the gelatin-gel test for the differentiation of virulent and benign strains is the addition of $CaCl_2$ into the growth medium (Depiazzi and Rood, 1984; Depiazzi *et al.*, 1990). At high concentrations of $CaCl_2$, the protease from benign strains is more thermostable than at low Ca^{++} concentrations, whereas Ca^{++} has minimal effect on the thermostability of protease from virulent strains.

Features that are the same for isoenzymes from both benign and virulent isolates are their pH optima (pH 6 to pH 9), reaction with inhibitors and their molecular weight range. Factors that are different for benign and virulent isolates are thermal stability, pH stability, reaction to metal ions and their rates of hydrolysis of various proteinaceous substrates (Green, 1985b).

Approximately 15 isoenzymes with molecular weights ranging from 70,000 to 129,000 daltons may be produced from culture supernatants of *D. nodosus* (Every, 1982). This range varies according to the method used, and other results have suggested the proteases range from 8,000 to 43,000 daltons (Gordon *et al.*, 1985). Purification of extracellular proteases from strain A198 using phenyl boronate to select serine proteases only, identified a major electrophoresis product of 38 kilodaltons using Coomassie Blue staining and a denaturing gel, with a number of minor bands detected by silver staining. More protease bands are detected in a non-denaturing system (Moses *et al.*, 1989). In

the routine zymogram test that uses a gelatin overlay, only the prominent bands are included in the analysis for determining isoenzyme type (Palmer, 1993; Pitman *et al.*, 1994). (See Figure 2-3, chapter 2). When all bands are compared there are some bands in common, but many bands are different between virulent and benign isolates (Every, 1982). All strains of *D. nodosus* have a fast moving isoenzyme band in common, and this is heat labile. Only the slower moving bands are thermostable (Gordon *et al.*, 1985; Green, 1985b). The fact that different molecular forms of the isoenzymes are produced indicates that they are genetically distinct (Every, 1982).

1.10.3 Elastases

Elastase has been investigated as a potential virulence factor, as there is a correlation between the production of elastase and production of clinically virulent lesions (Stewart, 1979). However, the correlation did not hold for the majority of isolates, and was not absolute in pen tests for virulence. Other studies found that benign isolates also hydrolysed elastin, although in general, virulent isolates had higher levels of activity. However, the differences in the activity levels were not as marked as those seen for hydrolysis of casein or hide-powder-azure (Kortt *et al.*, 1982).

1.10.4 Pili

Dichelobacter nodosus contains large numbers of pili on its cell surface (Short *et al.*, 1976; Cooper, 1977) and these are composed of one type of polypeptide subunit, pilin (Every, 1979). These pili or fimbriae have been identified as type IV because they have a highly conserved N-terminal amino acid region containing an N-methylphenylalanine residue. The N-terminal 24 residue sequence of *D. nodosus* is very hydrophobic, and is highly homologous with pilins from *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, and *Moraxella nonliquefaciens* (McKern *et al.*, 1983). The type IV pili are implicated in virulence because of their adhesive qualities, and involvement in twitching motility, extracellular protein secretion, and antigenic and phase variation (Strom and Lory, 1993). *D. nodosus* exhibits twitching motility common to that of other bacteria with type IV pili (Depiazzi and Richards, 1984/85). It is thought that the highly conserved hydrophobic region of type IV pili may facilitate transport of extracellular proteins through the cell membrane, and promote interactions between the subunits to form filamentous structures (McKern *et al.*, 1983).

The pili are easily lost on serial subcultures, and cells with low numbers of pili have a different colony morphology on artificial media compared to those cells that are heavily piliated (Short *et al.*, 1976; Thorley, 1976; Cooper, 1977). Type IV pili are highly immunogenic for sheep, and when purified pili were inoculated into sheep they induced protection against infection (Stewart, 1978). Agglutination reactions with the pili result in K-type agglutination, which is described as "a distinctive agglutination that is characterised by the rapid formation of coarse, flocculent granules, and subsequently by a loosely coherent sediment at the base of the test tubes" (Egerton, 1973). Ten serogroups (A, B, C, D, E, F, G, H, I and M) (Claxton *et al.*, 1983; Chetwin *et al.*, 1991) are recognised based on reactions obtained from antisera raised against prototype strains of *D. nodosus*. These serogroups are further divided into 18 subgroups (also referred to as serotypes), (A1, A2, B1, B2, B3, B4, C, D, E1, E2, F1, F2, G1, G2, H1, H2) according to cross-absorption tests (Claxton *et al.*, 1983; McKern *et al.*, 1989).

Other serotyping schemes for *D. nodosus* have been proposed. A system in North America identified 21 serotypes (I-XXI) using tube agglutination with unabsorbed serum (Gradin, 1993), whereas a British system identified 17 serotypes (A-H, J-N, O-R) using absorbed cells (Day *et al.*, 1986; Thorley and Day, 1986). In the Australian system, the serogroups are identified by slide agglutination using unabsorbed antisera and subdivided into serotypes using tube agglutination. The serogroups correlate with cross-protection following vaccination with appropriate serogroups (Ghimire *et al.*, 1998). Detailed methodology for preparation of antisera, slide and tube agglutination tests are presented in the Australian Standard Diagnostic Techniques for Animal Diseases (Stewart and Claxton, 1993).

1.11 Genetics of *D. nodosus* and molecular basis for virulence

Much research over the last ten years has concentrated on finding a genetic basis for virulence in *D. nodosus*, and a number of regions on the genome appear to be significant. These include the fimbrial genes (*fim*A), basic protease (*bpr*V), acidic protease (*apr*V5), a virulence related locus (*vrl*), and virulence associated proteins (*vap*).

1.11.1 Fimbrial genes

Recent research confirms the importance of the type IV pili in the pathogenesis of *D. nodosus* infection. Inactivation of the encoding gene, *fim*A, by allelic exchange produced mutants that did not exhibit twitching motility, had lower levels of extracellular protease and did not cause infection when inoculated into sheep hoof (Kennan *et al.*, 2001). Genetic recombination appears to explain the diversity that results in the many different serogroups. Fimbrial subunits, which are responsible for the serogroups, can be divided into two classes based on the organisation and nucleotide sequences of their encoding genes. Class I subunits are responsible for serogroups A, B, C, E, F, G, I and M, whereas Class II subunits give rise to serogroups D and H (Mattick *et al.*, 1991; Ghimire *et al.*, 1998). The two Classes are common in the leader sequence and the conserved hydrophobic amino-terminal sequence, but contain a variable region that contains areas of insertions and deletions. It is this highly variable region of the pili that is responsible for the serogroups. There is an absence of silent codon changes, which indicates the genes may be involved in genetic exchange through recombination (Mattick *et al.*, 1991). It was suggested that the Class II variable regions evolved in another species that produces type IV pili, and that the genetic information was exchanged by lateral transfer and is responsible for the diversity of serotypes (Hobbs *et al.*, 1991). Genetic recombination in the fimbrial genes may also explain two novel amplicons produced when the *fim*A gene was amplified by PCR. Sequencing of the novel PCR products suggested recombination between two different serotypes (Zhou and Hickford, 2000a). The ability of *D. nodosus* to undergo natural transformation and homologous transformation was identified when a serogroup I strain was seroconverted to a serogroup G strain *in vitro* (Kennan *et al.*, 2003).

1.11.2 Protease genes

A number of protease genes have been identified, sequenced and related back to protease isoenzymes. These genes are *bprV* (Lilley *et al.*, 1992), *aprV5*, *aprB5* (Kortt *et al.*, 1993; Riffkin *et al.*, 1993) and *aprV2*, *aprB2* (Riffkin *et al.*, 1995). Virulent isolates of *D. nodosus* produce four extracellular acidic serine proteases (V1-V3, V5) and a basic protease (*bprV*) (Lilley *et al.*, 1992; Riffkin *et al.*, 1993). Benign isolates produce five acidic proteases (B1-B5) and a basic protease *bprB* (Kortt *et al.*, 1994b; Lilley *et al.*, 1995). In the benign strain 305 the basic protease with a pI of ~8.6 has a genetic

sequence (*bprB)* that is highly homologous to the gene sequence (*bprV*) of the basic protease (pI ~9.8) from strain A198 (Lilley *et al.*, 1995).

The *prvA* (designated for protease virulent) gene was identified from the screening of a recombinant library constructed from *Bam*HI-digested fragments from strain A198. The recombinant plasmid containing the *prvA* gene was identified by immunoreaction in a Western blot using rabbit antisera raised to a protease of 38 kDa. The protein product from *prvA* produced an immunoreactive protease of 60 kDa instead of the expected 38 kDa product. It was suggested that the discrepancy in molecular size indicated that the protease was expressed initially as a proenzyme (Moses *et al.*, 1989).

1.11.3 Virulence associated gene regions

A number of studies have been undertaken to find the genetic basis for virulence in *D. nodosus*. In the course of these studies different genomic regions have been identified and sequenced, and reported to be responsible for virulence of the organism. Further research has disputed or modified these claims. In all cases the genetic region that is said to be responsible for virulence is found in a small percentage of benign isolates as well as predominating in virulent strains. The issue of the genetic basis of virulence in *D. nodosus* remains unresolved.

Initially, the genetic regions responsible for virulence in *D. nodosus* were suggested to be located on pathogenicity islands. Pathogenicity islands refer to large genomic regions on the bacterial chromosome where virulence genes and regulatory genes involved in pathogenicity are located. These gene clusters include many virulence genes that are selectively present in pathogenic strains but not in benign strains. These areas typically may differ from the rest of the genome in G+C content. Pathogenicity islands contain distinct genetic units that may be flanked by direct repeats, and are associated with transfer ribosomal RNA, and contain integrase genes and mobile genetic elements such as insertion sequences and transposons (Hacker *et al.*, 1997). A non-pathogenic strain may become pathogenic by acquisition of a pathogenicity island. The insertion, deletion or rearrangement of such large amounts of DNA allows the organism to generate new variants more rapidly. For bacteria that exhibit a natural competence, gene transfer may occur by transformation. For other non-competent bacteria, gene transfer

occurs through plasmids or bacteriophages, which can overcome genetic barriers between species and transfer DNA between them by conjugation and transduction.

Pathogenicity islands were suggested to be present in *D. nodosus*, however the indicated virulence regions so far defined on the genome do not entirely fit the definition for pathogenicity islands because they are scattered throughout the genome (La Fontaine and Rood, 1997; Billington *et al.*, 1999). Regions on the genome of *D. nodosus* known as the virulence-associated proteins (*vap*) (Katz *et al.*, 1992) and the virulence-related locus (*vrl*) (Haring *et al.*, 1995) were suggested to relate to virulence because they occurred more frequently in virulent strains than in benign strains. These two areas were identified following differential hybridization experiments between the virulent strain A198 and the benign strain C305. Three recombinant plasmids (pJIR318, pJIR313 and pJIR314b) were identified that hybridized with DNA from strain A198, but not with DNA from C305. When tested against 29 virulent isolates, 36 intermediate isolates, and 36 benign isolates, the recombinant plasmid pJIR318 occurred in 100% of the virulent isolates, 94% of intermediate and 33% of benign isolates. The other two chromosomal genetic regions inserted into the recombinant plasmids both occurred in 36% of intermediate and 6% of benign strains. The recombinant plasmid pJIR313 was found in 94% of virulent strains and pJIR314B was found in 100% of virulent strains (Katz *et al.*, 1991). Sequencing of pJIR318, which contained 2.26 kb of genomic DNA from strain A198, revealed four open reading frames and these genes were designated as virulence associated genes, *vapABCD*. The putative protein encoded by *vapA* has sequence similarity to an unknown open reading frame (ORF) in *Synechococcus* species (Cheetham *et al.*, 1995). Putative VapB and VapC had 34% and 41% similarity, respectively, with a protein encoded by the *trbH* region of the F plasmid of *E. coli*. The VapD protein had an amino acid sequence similar to a cryptic plasmid from *Neisseria gonorrhoeae* (Katz *et al.*, 1992), and ORFs from plasmids in *Actinobacillus actinomycetemcomitans*, and *Treponema denticola* (Katz *et al.*, 1994). The function of these genes is unknown. The *vap* region varies in size by about 500 bp depending on the presence of the *b* region, which is present in weakly virulent and benign strains. It was suggested that the virulence of *D. nodosus* might have evolved from rearrangements of the various gene regions through duplication and deletion (Liu *et al.*, 1995).

Sequencing of a further 9.8 kb of the *vap* region on the left-hand side of the genomic area contained in recombinant plasmid JIR318 revealed a putative ORF

designated *intA*. This gene encodes a region that is similar to integrases found in bacteriophages ϕR73 (retronphage), P4 and Sf6, and in all cases the *intA* is adjacent to the tRNA genes. This indicates that *D. nodosus* may acquire genetic information via bacteriophage or plasmids. In *D. nodosus* it appears that duplication of several *vap* genes has occurred after the integration of the bacteriophage or plasmid. Other *vap* genes, *vapE*, *vapG* and *vapH* were also identified. The *vap* region thus far identified is 11.9 kb (Cheetham *et al.*, 1995). A second putative integrase gene (*intB*) was also reported. PCR primers were developed for the *vap* genes A, B, C, D, E, G, H, and the putative integrase genes *intA* and *intB*. When these primers were tested on six different strains of *D. nodosus* (four virulent and two benign strains) no consistent results were seen between benign and virulent strains (Bloomfield *et al.*, 1997). The estimated amino acid sequences for the proteins coded by these genes does not have significant homology to proteins known to be involved in virulence in either *D. nodosus* or other bacteria. VapA has a 22% similarity in amino acid sequence to a HigA protein from a killer plasmid Rts1, and this protein has now been designated ToxA (Bloomfield *et al.*, 1997), although its toxic properties are unproven. It is postulated that the *vap* regions were acquired by integration of a genetic element (*intA*) into transfer RNA genes (Bloomfield *et al.*, 1997).

Two more integrated elements, *intC and intD* have been reported within the virulence associated areas in *D. nodosus* (Whittle *et al.*, 1999). These were found in the benign strain C305 and in most benign and virulent strains, except for the type virulent strain A198. It has been postulated that these integrated genetic elements can affect the virulence of an organism depending on where they insert into the genome. If they insert in an area that alters the expression of two regulatory genes, the putative global repressor gene A (*glpA*) or the polynucleotide phosphorylase (*pnpA*) gene, then there can be an effect on the *tRNA-ser*$_{GCU}$ gene, *tRNA-ser*$_{GGA}$, or the aspartokinase (*askA*) gene. It is postulated that this in turn affects the level of thermostable protease, which may be reduced through an effect on mRNA stability, tRNA availability or homoserine lactone (HSL) levels (Whittle *et al.*, 1999; Cheetham *et al.*, 2004). The model for virulence that is proposed states that a virulent isolate would have the *intA* element next to the *pnpA* gene, and the *intC* or *intA* next to the *glpA* gene. These studies have been tested on 16 strains of *D. nodosus*, and a larger number of well-characterised strains need to be tested before this hypothesis can be verified.

The DNA inserts in the recombinant plasmids pJIR313 and pJIR314B (found in 6% of benign strains and respectively in 94% and 100% of virulent strains) were found to be derived from a 27 kb genetic locus that was designated a virulence-related locus, *vrl*. This region was found to code for several proteins of molecular weight between 12 to 40 kDa, but no proteins were expressed in *E. coli* (Haring *et al.*, 1995). A total of 22 open reading frames (ORF) were identified in the *vrl* locus, and the proteins encoded by these genes were thought to be cytoplasmic proteins. Western blots using rabbit antisera to whole cells of strain A198 did not detect any *vrl*-encoded proteins. It was surmised that the *vrl* locus has arisen from a horizontal transfer of exogenous DNA that introduced the DNA via a site-specific recombination event, which resulted from the insertion of a mobile genetic element such as a bacteriophage or plasmid (Billington *et al.*, 1999). There has been only one report of a plasmid (pDn1) in one isolate of *D. nodosus*, isolate 1311 (Whittle *et al.*, 2000).

The recombinant plasmids pJIR313, pJIR314B and pJIR318 that formed the basis for the designation of the *vrl* and *vap* regions were used to develop gene probes in an attempt to design a diagnostic test that would differentiate virulent and benign isolates (Rood *et al.*, 1996). The gene probes were tested on 771 isolates and the results compared to the protease thermostability, elastase stability and colony morphology. Category 1 isolates were those that reacted with all three probes (*vrl* and *vap*), category 2 were those that hybridized with pJIR318 (*vap*) only, and category 3 were those isolates that did not hybridize with any of the probes. A fourth category was discovered during the course of the project, because some isolates hybridized to pJIR318 and pJIR314B, but not to pJIR313 (Rood *et al.*, 1996). The presence of these putative virulence associated genetic regions (*vap* and *vrl*) did not correlate well with protease thermostability. The majority (95%) of protease thermostable (S) isolates from Victoria belonged to category 1, but the same was not seen for S isolates from Western Australia. No protease heat labile (U) isolates from WA were in category 1, but a low percentage of strains from Victoria were found in this category. A variable number of both S and U isolates were found in category 2 and 3 for isolates from both Victoria and WA. If the probe categories were related to protease thermostability, then all benign isolates should have been found in category 3, with no S strains present in this category.

The virulence associated regions are scattered throughout the genome, suggesting that virulence is an evolving trait and not due to a single event, although horizontal

exchange of DNA appears to be a common event (Billington *et al.*, 1996; La Fontaine and Rood, 1997; Billington *et al.*, 1999). Protein expression studies on these gene regions do not appear to relate to known phenotypic virulence factors. Conclusive proof that these regions are directly involved in virulence is still to be obtained (Billington *et al.*, 1996).

1.12 Molecular methods used for studying the epidemiology of microorganisms

Epidemiology includes the study and identification of risk factors that contribute to a disease occurring in an individual or a population (Monis and Andrews, 1998). Such risk factors include transmissibility of the organism, host susceptibility, environmental factors, phenotype of the organism, and virulence of the organism (particularly if different strains have different virulence capabilities). By understanding the risk factors for disease transmission, guidelines for disease prevention and control can be implemented.

Molecular epidemiology combines the areas of molecular biology, epidemiology and biostatistics, and allows greater information about the organism to be included in the list of risk factors. Characterisation of bacterial strains into genotypes may allow particular genotypes to be linked to relevant epidemiological phenotypes such as strain virulence and transmissibility. Identification of bacterial strains by DNA fingerprinting facilitates epidemiological studies and improves the capacity for disease control.

Molecular techniques detect nucleotide variations in the chromosomal DNA of an organism. A DNA-based method for typing of microorganisms must have a high power of discrimination between strains. In a disease outbreak there is usually one common source of the infection or an aetiological agent, and therefore the organisms involved in the outbreak are clonally related. Organisms that are clonally related have the same virulence factors, phenotype, biochemical characteristics and the same genetic traits. Over the course of the outbreak the subsequent generations of organisms arising from the single source begin to show diversity. A discriminative typing system can demonstrate this diversity, such that organisms isolated at different times, from different geographical locations and from different hosts can be differentiated or classified into subtypes or strains (Olive and Bean, 1999).

There are a number of methods that are currently employed for molecular epidemiology and identifying variation in bacterial strains. Such methods include Pulsed Field Gel Electrophoresis (PFGE) (Arbeit *et al.*, 1990; Cameron *et al.*, 1994; Feizabadi *et al.*, 1996), Restriction Fragment Length Polymorphisms (RFLP) (Grimont and Grimont, 1986), Amplified Fragment Length Polymorphism (AFLP) (Valsangiacomo *et al.*, 1995; Lin *et al.*, 1996), Infrequent Restriction Site PCR (Mazurek *et al.*, 1996), Random Amplification of Polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al.*, 1990), BOX (based on conserved repeat elements) (van Belkum *et al.*, 1996), and enterobacterial repetitive intergenic consensus sequences (ERIC) (Hulton *et al.*, 1991; Versalovic *et al.*, 1991; Martin *et al.*, 1992; Rivera *et al.*, 1995).

The method selected for typing or epidemiological investigations must be discriminatory and reproducible. Pulsed field gel electrophoresis (PFGE) is considered the “gold standard” of molecular typing techniques as it is reliable, reproducible and highly discriminatory. The sensitivity of the technique is such that it can detect recent evolutionary divergence as seen in a disease outbreak, and thus is well suited to molecular epidemiology (Arbeit *et al.*, 1990).

Other techniques such as the multilocus enzyme electrophoresis (MLEE) method and its molecular counterpart, multilocus sequence typing (MLST) measure genetic changes that occur in stable parts of the genome. Genetic changes occurring in these parts of the genome occur at a slow rate, as opposed to the high rate of change seen in less stable areas on the chromosome that are detected by methods such as PFGE. Information obtained from observing slow rates of genetic change can provide data on evolutionary linkages, which precede the changes occurring from rapid change. Thus, this information provides an overall look at the evolution of, for example, a virulent clone (Brueggemann *et al.*, 2003; Feil and Enright, 2004). These methods do not provide the fine differentiation needed for tracing a disease outbreak, but they do provide the linkages between genetic types and their common ancestor that may not be apparent from the differences seen from tracing rapid genetic change. Thus, these two different approaches that detect either slow rate of genetic change (MLEE, MLST) or the rapid rate of genetic change (PFGE, IRS-PCR) provide complementary results.

The PFGE, IRS-PCR, AFLP and RAPD methods were investigated in this thesis and so they will be discussed in further detail. Multilocus enzyme electrophoresis (MLEE) was attempted, but because of technical difficulties, complete analysis of a large range of results was not achieved. There was insufficient time to investigate the molecular version of the MLEE technique; multilocus sequence typing (MLST). However, because a complete analysis of a bacterial species should be performed using a range of techniques, the potential of these methods will be discussed briefly in relation to their capability for measuring genetic variation and how the information generated from MLST complements the information generated from other molecular techniques.

1.12.1 Pulsed field gel electrophoresis (PFGE)

The PFGE technique is generally considered to be one of the most powerful for genetic analysis, is extremely sensitive to strain variation, is highly reproducible and is the most widely used epidemiological analysis tool for bacteria (Grundmann *et al.*, 1995). PFGE was first developed in the 1980s, and was described as allowing the separation of large molecular weight DNA fragments in agarose gels (Schwartz *et al.*, 1983). In the electrophoresis tank, a hexagonal array of 24 electrodes is positioned, or contoured, around the gel, and specific voltages are used to produce an almost homogeneous electric field inside the contour. The separation of the large fragments of DNA, that otherwise would not show separation on a conventional horizontal gel bed, is achieved when the electric field has an alternating orientation, thus causing the DNA fragments to move backwards and forwards in the gel. The duration of the pulses can be varied to achieve an optimal separation of fragments (Chu *et al.*, 1986). This system is known as the contour-clamped homogeneous electric field, or CHEF. Conventional electrophoresis is only capable of separating fragments of less than 50 kb, whereas PFGE can separate fragments of greater than 40 kb. For PFGE, the chromosomal DNA is digested with a low frequency cutting enzyme, usually with a six to eight base pair recognition site. This means that an entire genome of up to 15 Megabase can be digested and all restriction fragments examined on a gel. Using this system, the size of the genome of an organism can be estimated.

The restriction fragments generated from PFGE reflect the polymorphisms seen when alterations occur to nucleotides in the DNA. These changes may be due to a number of events that include: mutations in the coding or non-coding regions that create

or destroy sequence recognised by the restriction enzyme; alteration of a restriction site caused by inversions or transpositions; and duplications that are large enough to disrupt and change the molecular weight and hence migration rate of a fragment (Arbeit *et al.*, 1990).

The applications applied to PFGE include: genome mapping and the study of gene structure and organisation; the detection of translocation and re-arrangements in the genome; detection of extra-chromosomal fragments; and species identification and strain variation, or genotyping, in bacteria (Monaco, 1995). Detecting and identifying strain variation in bacteria by PFGE has great application for epidemiology, and several studies have shown that this method provides the highest discriminatory power for differentiating between species (Saulnier *et al.*, 1993; Schlichting *et al.*, 1993; Skov *et al.*, 1995a).

1.12.2 Amplified fragment length polymorphism (AFLP)

The AFLP method was developed initially based on the restriction fragment length method where the DNA was cut with an enzyme that had a four-base recognition site (Valsangiacomo *et al.*, 1995; Lin *et al.*, 1996). This method generated many fragments making interpretation difficult, especially when a large number of isolates were analysed. The AFLP method was modified to generate a subset of fragments that could be amplified by the PCR. The DNA is restricted and adaptors are ligated to the restriction fragments. The adaptors are designed so that one oligonucleotide has a longer sequence (21 mer) than the second oligonucleotide (14 mer), with the second oligonucleotide being complementary to the mid-sequence of the first. The overhang that is generated when the two oligonucleotides combine to form an adaptor is complementary to one end of the restriction site. The second restriction site end is complementary to the second adaptor. When the DNA is cut into fragments, the adaptors recognise the sticky ends generated by the restriction enzyme, and ligate to these fragments. In doing so they eliminate the restriction sites and then provide a template for amplification in the PCR.

1.12.3 Infrequent-restriction-site polymerase chain reaction (IRS-PCR)

The infrequent restriction site PCR (IRS-PCR) is based on the amplified fragment length polymorphism (AFLP) method. In the IRS-PCR method the enzymes that are used cut the DNA infrequently, whereas in the AFLP method the enzymes cut the DNA a greater number of times, and hence generate more restriction fragments. The AFLP method uses a one-step restriction and ligation, whereas in the IRS-PCR method restriction and ligation is expanded into two separate steps.

In the IRS-PCR method chromosomal DNA is restricted with two enzymes that cut the DNA infrequently. A subset of the resulting restriction fragments is isolated by ligating adaptors that recognise only those fragments that posses the recognition site of the first enzyme at one end of the fragment, and the second enzyme at the other end. Fragments that contain the recognition site of the same enzyme at each end of the fragment are not isolated. Primers specific for the individual adaptors and hence enzyme restriction sites are amplified by PCR to produce strain-specific electrophoretic patterns. The technique has been used for fingerprinting a wide range of bacterial species, including *Mycobacteria* species, *Pseudomonas* species and *Staphylococcus* species (Mazurek *et al.*, 1996).

1.12.4 Rapid amplification of polymorphic DNA (RAPD)

Bacterial strain differentiation also can be achieved by detecting DNA polymorphisms using single primers of arbitrary sequence. Primers may differ in length from 20-30 nucleotides (Welsh and McClelland, 1990) to 10 nucleotides (Williams *et al.*, 1990). Polymorphisms can be detected in the absence of specific nucleotide information. The method uses PCR cycles of low stringency annealing at 36°C to enable the non-specific primers to bind to the DNA of the organism under investigation. This technique has been termed Arbitrarily Primed PCR (AP-PCR) for primers of 20-30 nucleotides, and Random Amplification of Polymorphic DNA (RAPD) for primers of 10 nucleotides in length. They can be applied to a wide variety of organisms without requiring specific sequence information. Different complexity of banding patterns can be achieved depending on the primer sequence used. Primers of 10 nucleotides in length are available commercially from Operon Technologies, USA, and can be screened against the organism being tested to determine the level of complexity required.

This method has the advantage of being rapid and easy to perform compared to RFLP, which is more labour intensive because of the use of Southern blotting to detect the polymorphisms. A study that performed RAPD analysis on *Campylobacter jejuni* isolates found a correlation between serotype and the genotype generated by RAPD and allowed further differentiation of strains within a serotype (Mazurier *et al.*, 1992).

1.13 Measuring genetic variation

There are a number of methods that can be employed to measure genetic variation in populations of microorganisms. These include phenotypic methods such as protein electrophoresis where whole cell proteins or groups of proteins such as cell wall proteins or extracellular proteins can be examined using one dimensional or two-dimensional gel electrophoresis. Further differentiation can be achieved by examining the isoenzymes of a single protein (as used in the multilocus enzyme electrophoresis method). Genetic variation can also be determined more directly using molecular methods, such as pulsed field gel electrophoresis, amplified fragment length polymorphism and other methods such as those discussed in 1.14. These types of methods generally measure the genetic variation caused by rapid changes that may occur when an organism is under selective pressure, for example, from the host immune responses or environmental stress factors.

Other methods also detect genetic changes in a population of bacterial isolates, but these methods detect the changes that occur at a slower mutation rate, such as the changes that occur in the metabolic genes, or house-keeping genes. Multilocus enzyme electrophoresis (MLEE) detects genetic change by identifying allelic variations at the chromosomal loci that encode the enzymes by detecting the differences in the electrophoretic mobilities of the metabolic enzymes (Selander *et al.*, 1986). However, the rate of such genetic change is slow because the rate of recombination among the metabolic enzymes is low. Therefore, although these techniques are suitable for population studies they are not able to discriminate precisely between isolates that have recently evolved from a single source. PFGE and other molecular methods are able to identify isolates that recently have evolved from a single evolutionary line such as those found in an infection outbreak (Arbeit *et al.*, 1990).

Multilocus sequence typing (MLST) was first described by Maiden *et al* (1998). It provided a molecular version of MLEE that was easier to use and allowed the results to be more reliably compared between laboratories. The MLST method uses sequence information from a 470 base pair fragment from each of a number of house-keeping genes. Specific primers amplify each 470 bp fragment, which are then sequenced and compared to the sequence information in the database that is available on the world wide web at http://www.mlst.net. Identifying nucleotide variation or substitutions that reflect a change in the amino acid sequences of resulting proteins equates to the same variation detected at loci on alleles on the chromosome using the MLEE technique. The genetic change that is detected is the result of a slow rate of change, as the house-keeping genes constitute a stable part of the genome as opposed to an area that evolves rapidly because of positive selection pressure such as in the antibiotic-resistance genes. The results can be used for population studies or for epidemiological analysis to assist in the identification of localised disease outbreaks (Godoy *et al.*, 2003), or to monitor national and global disease trends (Meats *et al.*, 2003).

Analysis of an organism using a combination of molecular typing techniques to detect genetic changes that occur both rapidly and slowly can give a more accurate view of the population and how subgroups such as virulent clones emerge and evolve. This information can be used to monitor the population biology of an organism and can be applied to development of vaccines, monitoring antibiotic resistance or tracking virulent clones both locally and globally.

Estimating genetic similarity between isolates is important for estimating the spread of an infection. There is an assumption that genetic similarity amongst isolates is associated with close links in the transmission of the organism, so that genetic distance correlates to temporal and spatial relationships such that changes in the genome accumulate over time and distance. Estimating the genetic similarity depends upon the restriction enzymes used in techniques such as the PFGE method. Determining genetic distance can be improved by analysing the genome with more than one restriction enzyme, or by using more than one typing method. Because different restriction sites are assessed by either different enzymes or by different methods, then the likelihood of detecting changes in the genome caused by insertion or deletion at restriction sites is increased (Scherba *et al.*, 1999). Measuring genetic change in an organism in a disease outbreak situation requires methods that can detect rapid genetic change.

In the early stages of an outbreak the genetic diversity amongst isolates is assumed to be low, with no more than one genetic event difference. As the outbreak progresses over time, more random genetic changes will occur, which will be reflected in a greater diversity of restriction patterns (Tenover *et al.*, 1995). Genetic changes occur because of point mutations, recombination, insertion or deletion of DNA segments due to transposition of mobile genetic elements, plasmids, bacteriophage, other DNA re-arrangements and gene acquisition, and may be selected for as a result of, for example, pressures from the host's immune system. All these changes and pressures contribute to genome plasticity. It is these rapidly evolving genetic changes that are of interest to the epidemiologist, whereas the slowly evolving changes that occur in the area of the house-keeping genes or in metabolic function are of more importance to the evolutionary bacteriologist. Yet, these two areas together provide a picture of how the organism is evolving over time, and how these changes relate to each other in terms of virulence potential.

Even in the absence of a disease outbreak situation, genetic variation occurs in bacterial populations because of selective pressures, environmental conditions and mutagenesis. Variation can even be found in bacteria stored in stab cultures. Fresh subcultures show little or no genetic variation, whereas after more than ten years of storage pronounced polymorphism may be found (Arber *et al.*, 1994). Nevertheless, in a defined disease outbreak, closely related isolates will be identified more readily than isolates that have evolved over time from the initial source of infection.

Closely related strains of bacteria constitute a clonal group. These are strains that may be grouped as similar because they have identical phenotypic and genetic characteristics, even though they may be isolated from different sources (Ørskov and Ørskov, 1983). However, clonality is not absolute and, therefore, there is a degree of clonality in all bacteria relative to time because of normal genetic drift and selective pressure. Isolates may appear less clonal when tested by methods that are extremely sensitive to detecting genetic change. However, the assignment of clonal groupings can contribute to information about the organism. Clonal strains may share the same phenotypic factors that contribute to an organism's virulence. Thus, in a disease outbreak, isolates within a clonal group may possess the same virulence factors (Eisenstein, 1990).

Measuring the relatedness of isolates and the clusters of isolates is often subjective. Use of computational software that assigns genetic distance, relatedness and clusters of isolates by creating dendrograms may provide a more objective analysis, however there are still inaccuracies with this method. The mathematical algorithms in the software may not match identical isolates at 100% because it cannot fully recognise lane distortion or differences in band thickness due to different concentrations of DNA on the gel. Optimisation of the software settings is important for comparison of large databases of results and for comparing results between laboratories. Visual interpretation of results must also be done to provide accurate results (Duck *et al.*, 2003). In a defined outbreak, isolates can be assigned into clusters or clonal groups depending upon their relationship to the outbreak strain. Clonal groups may be identified as identical, similar or dissimilar to the outbreak strain depending upon, for example, the number of restriction bands in common in a PFGE profile (Tenover *et al.*, 1995; Goering and Tenover, 1997).

1.14 Current understanding of molecular epidemiology of footrot

The genetic diversity of *D. nodosus* has been examined in a number of studies. Techniques used include the application of primers designed to amplify the variable region of the fimbrial gene (*fim*A) (Zhou and Hickford, 2000b; Zhou *et al.*, 2001), restriction of the gene encoding the outer membrane protein (*omp*I) (Ghimire and Egerton, 1999), and by PFGE to investigate isolates from Malaysia (Zakaria *et al.*, 1998).

The variable region of the *fim*A gene is known to be diverse based on serological information and gene sequencing (Elleman, 1988; Mattick *et al.*, 1991). PCR primers were designed for use in a PCR/oligotyping method that used reverse dot-blot technology. Three primer sets were designed to amplify the regions that represented the genes encoding 10 different serogroups (A-I, M). The method was used to analyse material cultured from 14 hoof swabs taken from sheep on six farms in New Zealand. Eight serogroups were detected, of which serogroup B was predominant. This study identified 23 types, indicating the existence of great diversity within the *fim*A region. Multiple isolates and molecular types were found in a single hoof in 12 out of the 14 hoofs (86%) sampled. The authors noted that a disadvantage of this technique was that

the PCR/oligotyping approach would not be able to detect and identify new strains of *D. nodosus* (Zhou and Hickford, 2000b; Zhou *et al.*, 2001).

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was investigated to analyse *D. nodosus* isolates from Nepal (Ghimire and Egerton, 1999). The *omp*I gene that encodes the major outer membrane proteins was amplified and the product was restricted with *Hpa*II. A total of 66 representative isolates from serogroups B, C, E and M were typed, and produced 11 different fingerprints. Different fingerprint patterns were also found among isolates of different serogroups.

Pulsed field gel electrophoresis using restriction enzymes *Apa*I, *Sfi*I and *Sma*I was used to investigate the genetic diversity of 12 isolates of *D. nodosus* from three farms in Malaysia (Zakaria *et al.*, 1998). The isolates were differentiated into eight genomic types overall, which were broken down into three types identified by *Apa*I, four types by *Sfi*I and five types by *Sma*I.

All these studies were done on relatively small numbers of isolates, or in the case of the 66 isolates from Nepal, on isolates from a relatively confined geographical area. However, all the different techniques used in these studies demonstrated that *D. nodosus* is an extremely genetically diverse organism, whether in the variable regions of the fimbriae, the outer membrane proteins or the whole chromosomal DNA. None of these techniques was applied to a large number of isolates collected from diverse geographical areas, nor were they applied to a large group of epidemiologically-related isolates to determine their usefulness in trace-backs and trace-forwards in a disease outbreak. There was no investigation into the relationship between virulence and molecular type in the Malaysian study, nor was any epidemiological information known about the three farms involved in the study.

The PCR-RFLP method was investigated to determine whether there was a relationship between virulence and molecular type, and it was found that there were only two molecular types among the virulent isolates from Nepal (Ghimire and Egerton, 1999). Virulent footrot was introduced into Nepal thirty years earlier when a few infected rams were imported, and this may have been the reason why the genetic diversity was low. If this is the case, then the virulent isolates have shown little genetic

change since that time. However, the definition of virulence in this study was based on serogroup and clinical expression, rather than using protease thermostability as a definition of virulence.

1.15 Aims and objectives of this study

As discussed in the review of the literature, only small numbers of *D. nodosus* isolates have been examined using molecular techniques. An in-depth molecular epidemiological analysis of a large number of isolates, both epidemiologically-related and unrelated, has not been undertaken to date. The relationship between molecular genetic types also has not been examined to determine if there is a relationship between protease thermostability, isoenzyme types and molecular type. This epidemiological information is important to further our understanding of the *D. nodosus* organism, to examine other factors that may be involved in the spread of the disease, and to determine how the rate of genetic change impacts on the spread of the disease or the epidemiology of the disease. Finally, the rate of genetic change, and any possible effects on virulence need to be investigated.

Therefore, the aims or purpose of this thesis were:

1. To develop molecular methods suitable for the study of the molecular epidemiology of *Dichelobacter nodosus*.

2. To establish the genetic diversity of *D. nodosus* isolates within Australia, predominantly in WA.

3. To establish whether a relationship exists between a molecular type and virulence, as defined by protease thermostability.

4. To establish whether a relationship exists between molecular type and protease isoenzyme type, with particular emphasis on U5, U6 and T strains.

As a means to achieving these aims the objectives were:

1 To test and evaluate the methods of pulsed field gel electrophoresis, infrequent restriction-PCR, amplified fragment length polymorphisms, randomly amplified polymorphic DNA and multilocus enzyme electrophoresis for application to *D. nodosus*.

2 To obtain a large collection of *D. nodosus* isolates from WA, but also representative isolates from other Australian States where footrot occurs.

3 To use two of the molecular techniques to analyse this large group of isolates, and to examine epidemiological case studies on farms and between farms.

4 To analyse a large number of *D. nodosus* isolates characterised by molecular type, protease thermostability and isoenzyme profile.

5 To analyse the U5, U6 and T strains by molecular methods, and relate the findings to protease thermostability and isoenzyme profile

CHAPTER 2

General Materials and Methods

CHAPTER 2

2. General Materials and Methods

This chapter details the routine methods that were used throughout this study. It provides details of the number and source of *D. nodosus* isolates, the routine culture method that was used to isolate the bacterium from hoof lesion material, the methods used to characterise the isolates (zymogram and gelatin-gel method for protease thermostability), and the final optimised methods used for the pulsed field gel electrophoresis (PFGE) and infrequent restriction site (IRS-PCR) methods. The definitions used for the description of isolates, epidemiological definitions, and definitions used in the interpretation of fingerprints and the fingerprint analysis methods are also described in this chapter. Details of the optimisation of the PFGE and IRS-PCR methods for application to typing *D. nodosus* are described in chapter 3. A list of reagents, equipment used, and the manufacturer is provided in Appendix A**.**

2.1 Bacterial strains

A total of 796 isolates of *D. nodosus* were used for molecular typing. The majority (92%) of isolates came from WA: 735 isolates from 247 farms. The isolates were detected between 1976 and 2002, inclusive. Selection of isolates for molecular typing was not randomised. The database of isolates was composed of isolates that were detected between 1976 and 1994, inclusive, and were typed retrospectively from the culture collection. These were selected for investigation of their molecular type because of their zymogram type (eg U6 or U5), or because of on-farm investigation of molecular type. Isolates from the years 1995 to 1999 came from diagnostic samples submitted from the field and were composed of S and U zymogram profiles. Isolates from 2000 to 2002 were from diagnostic samples submitted from farms in quarantine and therefore were composed of mainly protease thermostable (S) types. The numbers of isolates tested from individual years and the corresponding number of farms is detailed in Table 2-1. The Australian Reference Laboratory for Ovine Footrot at the Department of Agriculture WA, Albany Regional Laboratory was responsible for reconstitution of isolates from the freeze-dried collection, and for the isolation of *D. nodosus* from diagnostic submissions of hoof swabs.

Table 2-1. Distribution of WA isolates according to year of isolation and protease type

Year Isolated	No. of Isolates	No. of Farms	No. of S	No. of U
1976	1	1		1
1978	1	1	1	
1985	4	3	3	1
1988	1	1	1	
1989	1	1	1	
1990	20	20	20	
1991	13	7	6	7
1992	17	6	15	2
1993	13	10	9	4
1994	35	24	17	18
1995	42	21	25	17
1996	79	21	20	59
1997	125	47	69	56
1998	19	8	9	10
2000	176	58	151	25
2001	131	58	116	15
2002	57	23	53	4
Totals	735		516	219

No. = number; S = protease thermostable strains; U = protease heat unstable strains. Total number of farms = 247. Farms may have had footrot detected in various years.

The protease thermostability and zymogram tests on these organisms were undertaken at the Albany Laboratory. Isolates from other Australian States (n = 61) included 54 from the National Culture Collection (NCC) maintained by Dr D. Stewart of the CSIRO Animal Health Laboratories, Geelong, which were composed of isolates from NSW (12 isolates from 12 farms), Vic (24 isolates from 22 farms) and SA (18 isolates from 18 farms). In addition, four isolates from one farm in NSW, and three isolates from three farms in SA had been obtained from colleagues in the State Department Veterinary Diagnostic Laboratories. The division of isolates into protease thermostable (S) and thermolabile (U) were: WA, 516 (70.2%) S strains and 219 (29.8%) U strains; NSW five (31.2%) S strains and 11 (68.7%) U strains; SA, 14 (66.6%) S strains and seven (33.3%) U strains; VIC, 20 (83.3%) S strains and four (16.7%) U strains.

Isolates from the NCC had been thoroughly characterised by gelatin-gel testing, zymogram type, and assessment of virulence by both clinical signs and pen testing. This collection of isolates resulted from a national project (CHP4) to investigate new molecular tests based on gene probes, and to reach agreement on definitions of virulence and the best tests to detect this virulence. The project was funded by

Australian Wool Innovation from 1994 to 1999. The strains are listed in the result tables in Appendix B.

2.2 Routine culture method

In WA, hoof lesions were scraped and the material collected into a modified Stuart's transport medium containing an increased agar concentration to 0.6% (Appendix A 3.1) and sent to the Australian Reference Laboratory for Ovine Footrot in Albany within 24 hours. The hoof material was used to inoculate a Trypticase Arginine Serine Hoof Isolation Medium (TASH IM) agar plate that contained powdered hoof horn (3%) and an agar concentration of 4% (Pitman *et al.*, 1994) (Appendix A 3.1). The plates were incubated at 37°C in an anaerobic atmosphere that was achieved using a MGC Gas Pak to generate an atmosphere of less than 1% oxygen and between 9-13% carbon dioxide. The plates were examined daily over four days for the appearance of colonies typical of *D. nodosus*, which are opaque and transparent and have a thin spreading growth (Figure 2-1). Individual colonies were subcultured for purity onto a Maintenance Medium plate (TASH MM) that contained 1.5% each of powdered horn and agar (Pitman *et al.*, 1994). Plates were packaged in an anaerobic atmosphere with a MGC Gas Pak and sent to the South Perth laboratory for DNA fingerprinting.

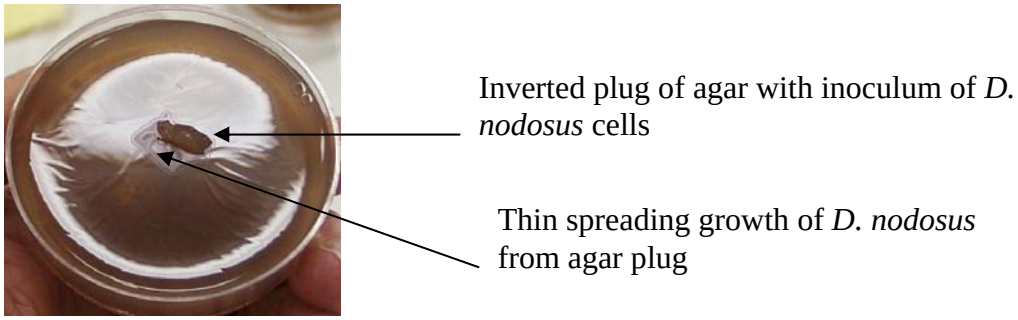


Figure 2-1. Growth of *D. nodosus* on TASH MM agar

2.3 Gelatin-gel test

Thermostable protease activity was detected using the gelatin-gel test at the Australian Reference Laboratory for Ovine Footrot. A pure culture of *D. nodosus* was grown in modified TAS broth (Depiazzi *et al.*, 1991) for 2 days at 37°C and diluted 1:4 in HEPES containing 0.01M $CaCl_2$, before testing. Aliquots of unheated broth, broth heated to 68°C for 8 minutes, and broth heated to 68°C for 16 minutes were placed into

wells in an agarose gel containing gelatin. The gel was incubated at 37°C for 18 hours before hydrolysis of gelatin was detected by precipitation of unhydrolysed gelatin using saturated ammonium sulphate. Strains producing stable protease were defined as those that retained gelatin-hydrolysing activity after heating at 68°C for 8 minutes, whereas benign strains were defined as those isolates that lost gelatinase activity (Palmer, 1993) (Figure 2-2).

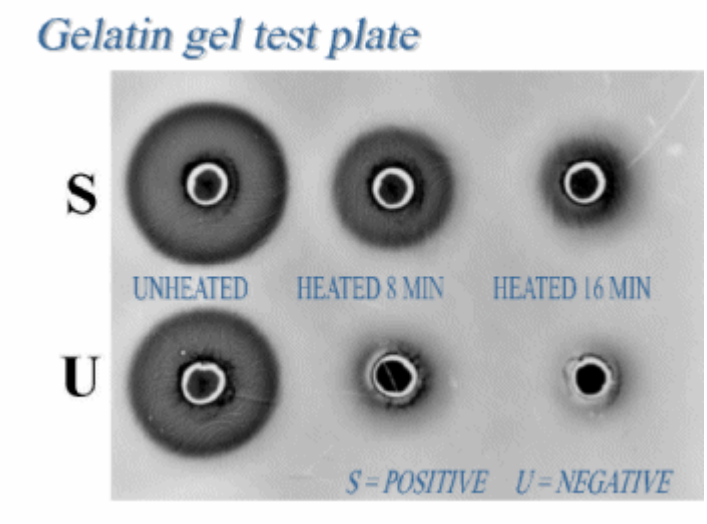


Figure 2-2. Gelatin-gel test for differentiation of thermostable and heat-labile protease

Photograph courtesy Mike Palmer, Australian Reference Laboratory for Ovine Footrot, Department of Agriculture, Albany, Western Australia.

D. nodosus is divided into either S strains (positive in the gelatin-gel test) or U strains (negative in the gelatin-gel test). Occasionally isolates produce an equivocal result in the gelatin-gel test and cannot be clearly assigned to either an S or a U. These isolates are termed E for equivocal.

2.4 Zymogram test

The zymogram test is used for the detection of protease isoenzymes, and was undertaken at the Australian Reference Laboratory for Ovine Footrot in Albany. An aliquot of a 2-day-old TAS broth culture was applied to a vertical polyacrylamide gel and subjected to electrophoresis (PAGE) for four hours. Gelatin-hydrolysing protease isoenzymes were detected by overlaying the gel with an agar-gelatin-gel. After incubation at 37°C for 1.5 hours the zymogram bands that hydrolysed gelatin were visualised using saturated ammonium sulphate (Depiazzi *et al.*, 1991; Palmer, 1993;

Pitman *et al.*, 1994). An example of these results is shown in Figure 2-3. Although a number of faint bands are seen on a gel, only those bands that are strongly staining, and therefore producing a large amount of isoenzyme, are used for classification.

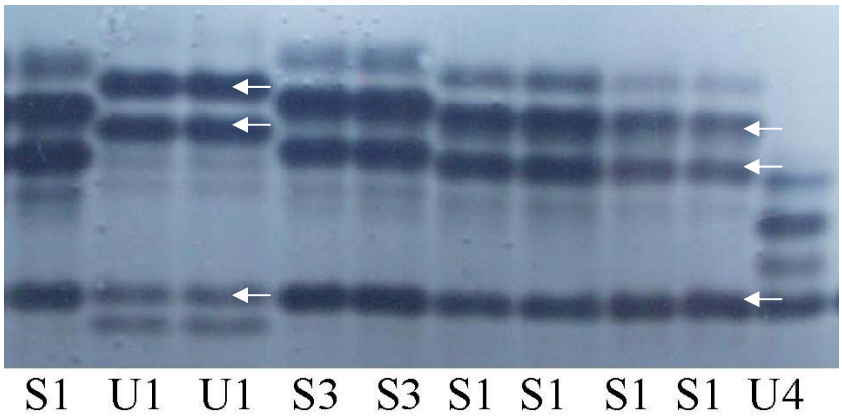


Figure 2-3. Zymogram gel showing isoenzymes of gelatin-degrading protease

S = Stable, U = Unstable. Arrows denote strongly staining isoenzyme bands that are used for classification.

Photograph courtesy Mike Palmer, Australian Reference Laboratory for Ovine Footrot, Department of Agriculture, Albany, Western Australia.

2.5 Current designation of strain type

Currently, 13 zymogram profiles are recognised. The zymogram profile is teamed with the result of the gelatin-gel test to give a strain type to the isolates. Thus in the S or protease thermostable strains, four zymogram types are recognised, S1, S2, S3, S4. In the U or protease labile group nine zymogram profiles are recognised, U1-U8 and T. One zymogram type, S3, is considered equivocal in the gelatin-gel test. Another zymogram type (T) has a profile that is the same as an S1, but the two upper bands are very weak. Some examples of patterns can be seen in Figure 2-3.

2.6 Pulsed field gel electrophoresis

The pulsed field gel electrophoresis (PFGE) method was adapted from previously reported methods for other bacteria (Cameron *et al.*, 1994; Wood, 1995; Matushek *et al.*, 1996) and was optimised for *D. nodosus*. Optimisation included determining the DNA concentration, suitable restriction enzymes and electrophoresis conditions. Details of this optimisation are reported in chapter 3. The methods described in this chapter are

the final optimised methods that were used for the work described in the rest of the thesis.

2.6.1 Growth and collection of bacterial cells

Each bacterial strain was lawn-inoculated onto a 50 mm plate of TAS MM medium (Pitman *et al*. 1994) and grown under anaerobic conditions for 3-4 days at 37°C (section 2.2). Bacterial growth was collected using a sterile cotton-tipped swab to scrape the cells into a 1.5 ml microfuge tube containing ice-cold 0.6 ml of wash buffer (1 M NaCl, 10 mM Tris Base, pH 8.0, 10 mM EDTA) (Appendix A 3.2). The cell suspension was adjusted visually to the opacity of a McFarland Turbidity Standard of 5 or 6. Cells were stored at –20°C if plugs were not prepared the same day. The purity of the cell suspension was checked by Gram staining and microscopic examination.

2.6.2 Preparation of agarose plugs containing bacterial cells

A 2% chromosomal grade agarose solution was prepared in wash buffer (Appendix A 3.2) and placed into a waterbath set at 55°C. 300 µl of bacterial cell suspension (section 2.6.1) was added to 300 µl of wash buffer in a 1.5 ml microfuge tube and placed into the 55°C water bath for 3 minutes. 300 µl of the agarose solution was added to the cell suspension in wash buffer and then quickly aspirated into plug moulds and allowed to set on ice. The final opacity was equivalent to a McFarland Turbidity Standard 3, which equated to a final cell suspension of 10^9 cells per ml. Solidified plugs were removed from the moulds and placed into 3 ml wash buffer in 5 ml tubes and stored at 4^oC until required. Five plugs were prepared for each isolate.

2.6.3 Extraction of DNA from agarose plugs

For DNA extraction the wash buffer was removed and replaced with 3 ml of lysis buffer (1M NaCl, 10 mM Tris pH 8.0, 100 mM EDTA Di-sodium salt, 0.5 % sarkosyl, 0.2 % sodium deoxycholate, 1 mg/ml lysozyme, and 2 µg/ml RNase). The plugs were incubated at 37^oC with gentle shaking for 1 to 2 hours. The lysis buffer was removed and replaced with 3 ml of ESP Buffer (0.5 M EDTA di-sodium salt, 1% sarkosyl, 100 µg/ml proteinase K) before incubating in a waterbath at 55^oC for 1–2 hours or overnight. The plugs were washed twice with TE buffer containing 30 µl of PMSF per 3

ml of TE for an hour each time. Two final washes with TE buffer only were performed, and then the plugs were stored at 4^{o}C in TE. Preparation of all reagents and buffers is detailed in Appendix 3.2.

2.6.4 Restriction and separation of DNA fragments

One-third (3mm) of an agarose plug containing DNA was placed into 0.6 ml of TE buffer and washed twice. DNA was restricted with 40 units of *Apa*I in a 100 µl volume (0.67 µl of *Apa*I at 40U/µl, 10 µl of 10x restriction buffer, 89.33 µl of milliQ water). For ease of use, a master mix (x29) of this restriction enzyme solution was prepared and 100 µl added to each agarose plug in a 1.5 ml microfuge tube. Restriction of the DNA was carried out for 2 hours at 25^{o}C. The restriction reaction was stopped by adding 0.6 ml of 0.5M TBE buffer to all tubes. The tubes, including slices of agarose containing the molecular weight markers, were placed in a waterbath at 50°C for 3 minutes. The tubes were then plunged into ice for 3 minutes to harden the plugs for ease of handling. The agarose plugs were placed into the wells of a cooled 1% agarose gel prepared with chromosomal grade agarose and 0.5 M TBE buffer (10x TBE is 89 mm Tris base, 89 mM boric acid and 2.5 mM disodium EDTA) (Appendix A 3.2). Filling the wells with 0.5M TBE buffer prior to loading the plugs prevented the formation of bubbles. A few drops of agarose (1% in 0.5M TBE) were used to ensure the plugs remained in the wells. An isolate (AC 7742), which was nominated as a restriction control, received the same treatment as the unknowns, and was placed in lane number 14 of each gel. A Lambda ladder molecular weight marker was placed in the first and last lanes of each gel. Two litres of 0.5M TBE buffer was placed into the electrophoresis tank of the CHEF DR III apparatus and allowed to cool to 12.9°C before loading the gel. The DNA fragments were separated at 6 volts/cm with pulse time of 0.1 to 16 seconds for 22 hours.

2.6.5 Staining and photographing gels

The gels were stained for 1 hour by immersion in a one-litre solution of ethidium bromide in distilled water at a concentration of 50 µl/l, and photographed over UV light. The gels were documented using Polaroid High Speed T57 film with a large format land camera, and digital imaging was done using an Alpha Imager.

In some cases isolates were further tested using the restriction enzyme *Xba*I. Restriction conditions and concentration were the same as those used for *Apa*I, however the pulse times were set to 0.5 to 10 seconds for 20 hours.

Details of the optimisation of restriction enzymes and electrophoresis conditions is presented in chapter 3.

2.7 Infrequent restriction site polymerase chain reaction (IRS-PCR)

The second molecular typing method used in this study was the infrequent restriction site PCR (IRS-PCR), and was based on the method as described by Mazurek *et al*. (1996). The theory behind the method is presented in the literature review, section 1.12.3. Other methods were attempted for application to *D. nodosus*, and these are presented in chapter 3 together with details on optimisation of the IRS-PCR.

A schedule for the IRS-PCR procedure is presented in Appendix A 3.4.

2.7.1 Extraction of DNA from agarose plugs

To ensure that the same DNA sample was tested by both typing methods, DNA was extracted from the agarose plug prepared for the PFGE method and used in the IRS-PCR method.

A 19 gauge needle was used to punch a hole in the base of a 0.6 ml microfuge tube. Aquarium filter wool was packed into the tube to a depth of 1 cm. This 0.6 ml tube was fitted inside a 1.5 ml microfuge tube labelled with the isolate identification number. An agarose plug containing the DNA from the isolate was selected and handled using a flame-sterilised spatula, and the plug placed into the 0.6 ml tube. The entire microfuge tube apparatus was frozen for 3 minutes in liquid nitrogen. The tubes were then centrifuged at 5000g (6,000 rpm) in a bench top centrifuge for 3 minutes. The tubes were inspected to make sure that the liquid containing the DNA had passed through into the larger tube. The 0.6 ml tube with the wool and unwanted agarose was discarded. DNA was stored at 4°C until required for the IRS-PCR method.

2.7.2 Restriction of DNA for IRS-PCR

A restriction mixture in a 10 μl volume containing 1 μl of *Xba*I enzyme (80U/μl), 1 μl of *Cfo*I enzyme (10U/μl), 1.25 μl of multi-core buffer (x10 as provided by the manufacturer), and 6.75 μl of ultra pure water was prepared as a master mix for 30 samples. A 12.5 μl aliquot of restriction mixture was added to 2.5 μl DNA and incubated at 37°C for one hour.

2.7.3 Preparation of adaptors

Adaptors were prepared from the appropriate oligonucleotides (as shown in Table 2-2). The AH adaptor was prepared from oligonucleotides AH1 and AH2, and recognises the restriction site generated by *Cfo*I (*Hha*I). The AX adaptor was prepared from AX1 and AX2 oligonucleotides (Table 2-2) and recognises the restriction fragment generated by *Xba*I. The adaptors (AH and AX) were prepared from the relevant oligonucleotides using 20 μl of each oligonucleotide (100 pmol), 10 μl of PCR buffer (x10 as supplied by the manufacturer), and 50 μl water. The adaptors were annealed by cooling from 80°C to 4°C over one hour. This procedure was carried out on a thermocylcer using a 5 degree drop in temperature every 4 minutes.

2.7.4 Ligation of adaptors

The AH and AX adaptors were ligated to the restriction fragments. A ligation mixture was prepared that contained 1 μl of each adaptor (20 pmol concentration), 1 μl of T4 ligase (1 U/μl), 2 μl ligase buffer and 2.5 μl of ultra pure water in a total volume of 7.5 μl. This mixture was prepared as a master mix and 7.5 μl was added to the 12.5 μl of restriction fragments in each tube. The ligation solution was incubated for 1 hour at 16°C. T4 ligase was then denatured at 65°C for 20 minutes. The restriction fragments were re-cut with 1 unit of each enzyme for 15 min at 37°C to ensure that no restriction fragments had reformed during ligation. The incubations were carried out using a thermocycler.

Table 2-2. Sequence information on oligonucleotides and primers used for the IRS-PCR

Designation	Sequence 5' – 3'
AH1	AGA ACT GAC CTC GAC TCG CAC G
AH2	TGC GAG T
AX1	PO_4-CTA GTA CTG GCA GAC TCT
AX2	GCC AGT A
PXT	AGA GTC TGC CAG TAC TAG AT
PXG	AGA GTC TGC CAG TAC TAG AG

AH1 + AH2 = adaptor AH; AX1 + AX2 = adaptor AX; PX-T and AH1 are the primers used in the PCR reaction; PX-G is an alternative primer used in the IRS-PCR. See chapter 3 for optimisation of primers.

2.7.5 Amplification of restriction fragments by PCR

A subset of fragments that contained a *Xba*I site at one end and a *Cfo*I recognition site at the other end were preferentially amplified using the AHI and PXT primers (Table 2-2). A 25 μl PCR reaction mix was used that contained 5 μl of DNA, 20 pmol of each primer, 2 μl of 10x PCR buffer, 0.2 μl of AmpliTaq at a final concentration of 1 unit, 1.25 μl of 2 mM dNTP mix (final concentration 100 μM), 2 μl of 25 mM $MgCl_2$ and 13.55 μl of ultra pure water. Each time new primers were purchased and adaptors prepared the concentration of dinucleotides was re-optimised. Accordingly, a final concentration of either 100 μM or 200 μM was optimal depending on the batch of primers. Details on preparation of stock and working solutions for IRS-PCR are detailed in Appendix A 3.4.

2.7.6 PCR amplification cycles

Amplification of the restriction fragment subset was performed on a Corbett FTS 320 thermocycler. The following cycling parameters were used.

Cycles 1-30	Cycle 31
95°C – 2 min	95°C - 1 min
55°C – 1 min	55°C - 1 min
72°C - 2 min	72°C – 10 min
x 30	4°C – infinity

2.7.7 Gel electrophoresis

Two isolates (AC 6 and AC 6034), previously nominated as restriction and amplification controls, were included in each batch of isolates being tested and placed in rows 14 and 15, respectively of each gel. Likewise, a DNA-free control was included and underwent the same restriction, ligation and amplification as the test and control isolates. A 100 base pair molecular weight marker was included on each gel and placed in the first and last rows of the gel.

The amplified fragments (5 μl) were separated by electrophoresis through a 4% high resolution agarose gel for 2.5 hour at 150 v with the electrophoresis tank packed in ice to prevent overheating of the gel. The preparation of this gel has certain requirements and is described in detail in Appendix A 3.4.

The gel was photographed over UV light following ethidium bromide staining as previously described in section 2.6.5.

2.8 Interpretation of fingerprints

The visual interpretation of the restriction patterns produced by the PFGE and IRS-PCR method for bacterial strain typing was based primarily on the guidelines proposed by Tenover *et al*. (1995).

2.8.1 Definitions used when referring to bacteria

Isolate: Refers to a pure subculture of a bacterium that was derived from a single colony for which the only information known is its genus and species (Tenover *et al.*, 1995).

Strain: Describes a group of isolates that form a subset within a genus and a species based on distinguishing phenotypic and genotypic traits, which are different from other groups or strains of isolates within the same genus and species (Tenover *et al.*, 1995).

Outbreak strain: The strain identified as the original strain in the outbreak of the infection.

Epidemiologically related isolates: Isolates that are possibly related to the disease outbreak being investigated and which have been collected within a defined time frame during the epidemiological investigation (Tenover *et al.*, 1995).

Genetically related isolates or clones: A collection of isolates that have been obtained from different geographical locations, from different hosts, and from different times. They have identical phenotypic and genotypic traits and are likely to have originated from the same source. These are termed clones (Eisenstein, 1990).

Virulence: "The relative capacity of a microbe to cause damage to the host" (Casadevall and Porofski, 1999).

Virulence factor: "A component of a pathogen that damages the host; can include components essential for viability including modulins" (Casadevall and Porofski, 1999).

2.8.2 Definitions used for the epidemiological categories of isolates

1 **Identical**: these isolates have the same restriction patterns as the outbreak strain when tested by molecular typing methods.

2 **Closely related**: An isolate is closely related to the outbreak strain if it has a DNA restriction banding pattern that differs from the outbreak strain by two to three bands (Tenover *et al.*, 1995). In this study closely related isolates were identified as such using this definition if they came from the same property or were identified as being epidemiologically related by trace-back or trace-forward information.

3 **Possibly related**: An isolate is possibly related if it differs from the outbreak strain by four to six restriction bands (Tenover *et al.*, 1995). In this study isolates were considered possibly related if the restriction band pattern was visually identifiable as originating from the outbreak strain – having a majority of bands in common between the two isolates, but with up to a four band difference.

4 **Unrelated**: An isolate is considered different to the outbreak strain if there are more than seven restriction bands different (Tenover *et al.*, 1995). There is some contention in regard to this absolute defisnition (Thal *et al*., 1997).

5 **Clonal group**: An isolate is said to belong to a particular clonal group of strains if the molecular type is closely related to a predominant molecular type.

6 **Molecular type**: Identified as the molecular type involved in a disease outbreak. For example, PFA 7 is a molecular type.

7 **Molecular subtype**: Identified as being closely related to an outbreak strain. For example PFA 7a is a subtype of PFA 7.

Tenover *et al*. (1995) based their guidelines for use on a small set of isolates, usually less than 30, which were derived from a known outbreak of a disease. In contrast, this study has used these recommendations, but applied them to a large number of isolates collected over a number of years.

2.8.3 Definitions used for computer analysis of DNA fingerprints

Cluster Analysis: Also known as numerical taxonomy, cluster analysis incorporates many different methods that search for groupings in data. The data are grouped in such a way that the degree of association between two groups of data (e.g. DNA fingerprints) is maximal if they belong to the same group, and minimal if they are from different groups (Everitt, 1974). There are many different algorithms and methods used for grouping data into categories. In this study, the dendrograms were constructed using the Dice unweighted pair group method with arithmetic averages (UPGMA) (section 2.8.5).

Dendrogram: A dendrogram is a two-dimensional, tree-like graphical representation of the clustering of the components within a data set (e.g. DNA fingerprints). It is a hierarchical classification system that gives a measure of the similarity of components within a cluster, and the dissimilarity between clusters. The calculation of similarity between DNA fingerprints is given as follows. If isolate B has seven of eight DNA bands in common with isolate A, then isolates A and B will be 88% similar (Table 2-3). If isolate C has six of seven bands in common with isolate B, then isolates B and C will be 86% similar. In this way a similarity matrix is developed for all isolates being examined.

Table 2-3. Example of similarity matrix showing percentage of similar bands

	A	B	C	D	E
A	.				
B	88	.			
C	75	86	.		
D	63	71	85	.	
E	50	57	67	80	.

Adapted from http://www.phplate.se/infofiler/php-exempel.doc

In the formation of the dendrogram, A and B will form a group, and B and C will form another group. Because both groups contain B, then isolates A, B and C will form a group at 86% similarity.

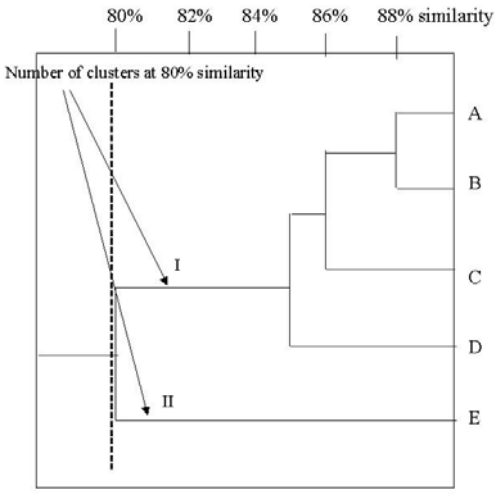


Figure 2-4. Example of construction of a dendrogram based on the similarity matrix

Adapted from http://www.phplate.se/infofiler/php-exempel.doc

A-E represents the original DNA fingerprints or data used in the calculation of the dendrogram (Figure 2-4). The solid vertical lines represent the links between the DNA fingerprints. The horizontal lines represent the "genetic distance" or genetic similarity between the DNA fingerprints. The greater the distance between isolates the less similarity between them.

Clusters: Clusters are identified in a dendrogram based on the similarity of the data within the clusters. The data within a cluster are more similar to each other than the data found in other clusters (Everitt, 1974). The numbers of clusters are formed at a level of "Y", and then isolates within individual clusters, cluster at a similarity level of "X". Clustering is hierarchical in that the joining method that is used groups the data into small groups that are contained in increasingly larger groups. This results in a small

number of groups at a high level of dissimilarity and a large number of groups at a low level of dissimilarity.

Deciding on the number of groups is subjective and depends upon the level of similarity that is chosen. A simple method is to draw a vertical line down the dendrogram at a similarity level (Y) chosen by the user, and then count the number of branches of the dendrogram that are cut by this line to arrive at the number of clusters (Urban, 2004). For example, if a similarity level of 80% is chosen, then two clusters will be identified in the dendrogram in Figure 2-4. Cluster I contains samples A-D, which are similar to each other at a level of 85% (X). Cluster II contains isolate E, which is similar to cluster I at a level of 80%.

A second method is to select the number of clusters based on the clusters that are ecologically interpretable, that is, by examining the species or the variables that characterise a cluster. In a large data set only a few high-level clusters are of interest (Urban, 2004).

Dice: The similarity coefficient used in the calculation to determine the similarity of the DNA fingerprints is based on band position, but places a weighting on matching bands (Dice, 1945).

Unweighted pair group method with arithmetic averages (UPGMA): This is a hierarchical cluster analysis method that uses the average linkage algorithm to calculate the distance between two clusters, which is identified as being the average distance between all pairs of DNA fingerprints in the two different clusters (Sneath and Snokal, 1973).

Diversity Index: Simpson's index of diversity describes the diversity of a species within an ecological habitat (Simpson, 1949). It estimates the probability that a single strain will belong to the j^{th} type. If the diversity index of a set of isolates is 0.8, this indicates that there is an 80% likelihood that any two strains in that population will belong to two different molecular types.

Diversity (D) is calculated as:

$$D = 1-1/N(N-1) \sum_{j=1}^{s} n_j(n_j-1)$$

N = total number of strains in the population, s = total number of types, n*j* = the number of strains belonging to the j^{th} type (Hunter and Gaston, 1988).

2.8.4 Assigning molecular type for PFGE

In this study the parental strain was given a fingerprint number and isolates that were genetically similar, with only one genetic event difference (3 or less restriction fragment difference), were called a subtype. A fingerprint number was assigned to each DNA fingerprint, which indicated the method used, the restriction enzyme used and then the identification of the molecular type and subtype. For example, the designation PFA 48a, as shown in Figure 2-5 is derived thus: PF refers to the method PFGE and A refers to the restriction enzyme used, *Apa*I; the outbreak or predominant strain is identified as molecular type 48, and the 'a' indicates the subtype. A clonal group may comprise a difference of up to 7 restriction bands between the subtype and the outbreak strain. In the example of PFA 48a, there is a two-band difference between PFA 48a and the outbreak strain PFA 48. The other 13 bands on both fingerprints are the same. Also in Figure 2-5 there are three isolates (80, 81 and 82) from Farm 4, and two isolates (12 and 18) from Farm 1. The isolates from Farm 4 and Farm 1 are clearly different because out of the 18 possible restriction bands, only nine are possibly the same, i.e. 50%, which indicates that they are different molecular types and therefore unrelated. The isolates on Farm 1 are designated PFA 24 and PFA 24a. There is a three-band difference between the two isolates, indicating that they are closely related and that one is a subtype of the other. In some cases, the restriction fragments smaller than 48.5 kb were unreliable in producing clear bands, which seemed to be related to the amount of DNA loaded onto the gel. However, very little genetic change in restriction fragments was seen below this molecular weight.

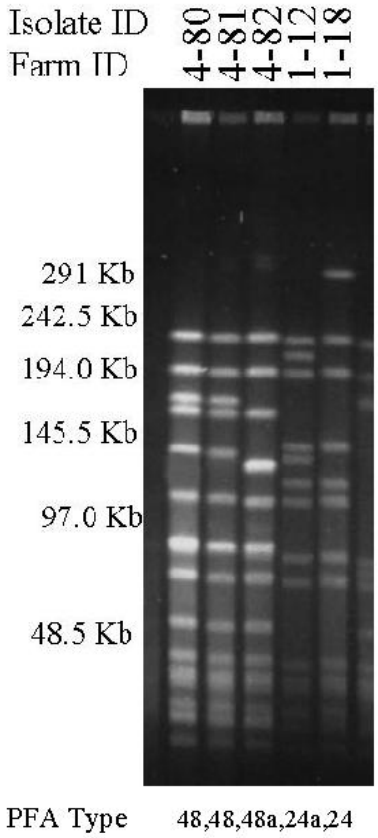


Figure 2-5. Example of interpretation of fingerprints for PFGE

Lane 1 = isolate 80 from farm 4 (PFA 48); Lane 2 = isolate 81 from Farm 4 (PFA 48); Lane 3 = isolate 82 from Farm 4 (PFA 48a); Lane 4 = isolate 12 from Farm 1 (PFA 24a); Lane 5 = isolate 18 from Farm 1 (PFA 24).

2.8.5 Assigning molecular type for IRS-PCR

For results with the IRS-PCR method, isolates were given the prefix IrsT to indicate the method (Irs), whilst the T refers to the extra nucleotide that is added to the primer for optimal results (see chapter 3 for explanation of optimisation of method).

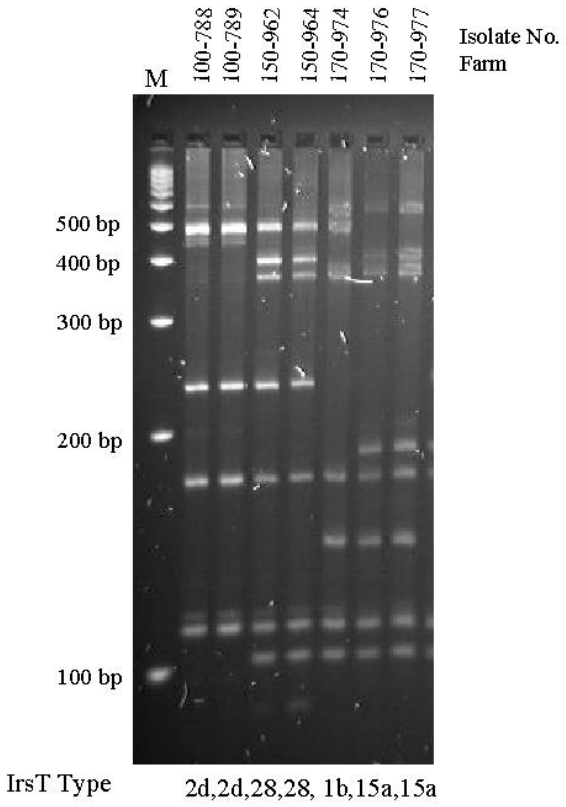


Figure 2-6. Example of interpretation of fingerprints for IRS-PCR

Lane 1 = isolate 788 from farm 100 (IrsT 2d); Lane 2 = isolate 789 from farm 100 (IrsT 2d); Lane 3 = isolate 962 from farm 150 (IrsT 28); Lane 4 = isolate 964 from farm 150 (IrsT 28); Lane 5 = isolate 974 from farm 170 (IrsT 1b); Lane 6 = isolate 976 from farm 170 (IrsT 15a); Lane 7 = isolate 977 from farm 170 (IrsT 15a).

Numbers were allocated at random to the isolates as they were tested, and therefore numerical relationship does not indicate genetic relationship except in the case of subtypes where an alphabetical letter is assigned to the subtype, for example 15a (Figure 2-6).

Some isolates had both an identical PFA type and an identical IrsT type, and therefore were considered to be genetically identical. Some isolates had the same PFA type but a different IrsT type, and therefore were considered to be genetically similar.

2.8.6 Calculation of genetic diversity

2.8.6.1 Simple diversity ratio

A simple ratio of genetic diversity was calculated by dividing the number of isolates tested by the number of molecular types obtained. The ratio of genetic diversity according to clonal group was obtained by dividing the number of isolates by the number of clonal groups. This was done for all isolates from Australia, for isolates from WA only, and for a ratio of genetic diversity on farms where more than one isolate was typed on that farm. The ratio of genetic diversity seen between virulent and benign isolates (as defined by protease thermostability) was also calculated.

2.8.6.2 Analysis of diversity based on Simpson's index of diversity.

Simpson's index of diversity (Simpson, 1949) was calculated to provide an estimation of the genetic diversity of isolates of *D. nodosus* within Australia, and within WA. The calculation was performed for the genetic diversity within molecular type and within clonal type.

2.8.7 Analysis of results by dendrogram

A dendrogram was prepared from all the standard types identified by PFA using the GelCompar II program version 3.1, Applied Maths, Kortrijk, Belgium. The data clustering method used was the Dice unweighted pair group method with arithmetic averages (UPGMA) (Dice, 1945; Sneath and Snokal, 1973). A band comparison position tolerance was set at 5% (Duck *et al*., 2003).

CHAPTER 3

OPTIMISATION OF PFGE AND IRS-PCR AND COMPARISON TO OTHER METHODS ATTEMPTED

CHAPTER 3

3. Optimisation of PFGE and IRS-PCR and comparison to other methods attempted

3.1 INTRODUCTION

A number of molecular typing methods are available for analysis of variation amongst microorganisms. These methods need to be trialled to determine which ones will produce the desired results for differentiation within the population of bacteria under analysis. In this thesis a number of methods were tested to determine what method would be suitable for a genetic analysis of a population of *D. nodosus* isolates, and produce the most discriminatory results suitable for an epidemiological study. These methods investigated were pulsed field gel electrophoresis (PFGE), infrequent restriction site polymerase chain reaction (IRS-PCR), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and multilocus enzyme electrophoresis (MLEE).

PFGE has been applied to many different bacteria, both Gram-positive and Gram-negative. The basic PFGE protocol needs to be optimised so as to obtain the best results for each bacterium. There is a relationship between DNA fragment length and mobility, and the separation of restriction fragments is proportional to the number of pulses (switch time) or alternating electric current used (Southern and Elder, 1995).

The IRS-PCR is basically an amplified fragment length polymorphism (AFLP) method. It has been described for a small number of bacteria and most of the protocol can be applied to these bacteria, however the primers used in the PCR reaction need to be optimised for results best suited to each organism (Mazurek *et al.*, 1996). Another AFLP method was tested and compared to the results obtained for the IRS-PCR to determine which method was best suited to strain differentiation of *D. nodosus*. The MLEE technique was also trialled to determine its usefulness in establishing genetic diversity and structure in a subset of isolates.

The optimisation of these techniques for application to strain typing of *D. nodosus* is presented in this chapter. The objectives were to develop the methods and their protocols so that an even spread of restriction bands was obtained, with an optimal

number of bands that enabled fine differentiation between strains, yet did not produce so many bands that interpretation of the gel was difficult. To achieve this outcome a number of restriction enzymes were tested, and different electrophoresis switch times and run times were applied for the PFGE method. For the IRS-PCR method the primers and the separation conditions were optimised, and the method compared to AFLP and RAPD methods. The methods were also optimised for ease of use and to reduce labour and time involved.

All media and buffers, molecular biology protocols, reagents, and equipment are detailed in Appendix A.

3.2 MATERIALS and METHODS

3.2.1 Bacterial isolates

Table 3-1. Details of the 27 isolates used for the assessment of the PFGE method

Isolate identification	Zym profile	Property identification no.
AC 6	U1	70
AC 915	U2	10
AC 2127	S1	105
AC 2407	S1	71
AC 2839	S1	33
AC 3264	U5	5
AC 4307	U5	95
AC 4834	U5	300
AC 5136	U6	129
AC 5404	U4	108
AC 5545	U4	26
AC 5615	S3	16
AC 5637	U6	301
AC 5659	U3	133
AC 5679	U5	68
AC 5709	S	115
AC 5944	U5	11
AC 6065	U1	20
AC 6161	U5	64
AC 6324	U1	21
AC 6361	U5	11
AC 6622	U1	20
AC 6623	U1	20
AC 6624	U1	20
97-55-79	U1	114

Table 3-1, continued

Isolate identification	Zym profile	Property identification no.
97-106-579	U5	115
97-118-849	S2/U4	98

no. = number; Zym = zymogram type.

Table 3-2. Details of the 30 isolates used for the assessment of the IRS-PCR method

Isolate identification	Zym profile	Property identification no.
AC 915	U2	10
AC 2407	S1	71
AC 2839	S1	33
AC 3264	U5	5
AC 4123	S3	31
AC 4307	U5	95
AC 4834	U5	300
AC 5136	U6	129
AC 5404	U4	108
AC 5545	U4	26
AC 5615	S3	16
AC 5637	U6	301
AC 5659	U3	133
AC 5679	U5	68
AC 5944	U5	11
AC 6065	U1	20
AC 6161	U5	64
AC 6167	S1	20
AC 63	U5	11
AC 6622	U1	20
AC 6623	U1	20
AC 6624	U1	20
AC 6324	U1	21
97-55-79	U1	114
97-106-579	U5	115
97-118-849	S2/U4	98
97-304-286	S1	34
97-327-411-1	U1	74
97-328-419	U1	49
97-328-424	S1	49
97-341-488	T	67
97-362-557	U1	313
97-369-626-2	U6	101
97-386-680	U1	24
97-405-765	U1	48
98-9-122	S1	226
98-9-124	S4	226
00-505-788	T	100

Table 3-2, continued

Isolate identification	Zym profile	Property identification no.
00-514-824	S1	227
00-514-825	S1	227
01-9-974	S1	170

no. = number; Zym = zymogram type.

Table 3-3. Details of the 36 isolates used for the assessment of the RAPD method

Isolate identification	Zym profile	Property identification no.
1213-127	U1	na
AC 5251	U1	na
1440-880	U1	na
1445-903	U1	na
AC 915	U2	10
AC 1087	U3	na
AC 2407	S1	71
AC 2839	S1	33
AC 4123	S3	31
AC 4292	S2	100
AC 4307	U5	95
AC 4346	U6	na
AC 5136	U6	129
AC 5404	U4	108
AC 5471	U1	na
AC 5503	U2	na
AC 5545	U4	26
AC 5593	S2	na
AC 5615	S3	16
AC 5637	U6	301
AC 5659	U3	133
AC 5679	U5	68
AC 6065	U1	20
AC 6167	S1	20
AC 6622	U1	20
AC 6623	U1	20
AC 6624	U1	20
97-106-579	U5	115
AC 3264	U5	5
AC 4307	U5	95
AC 4834	U5	300
AC 5679	U5	68
AC 5944	U5	11
AC 6161	U5	64
AC 6361	U5	11

na = not available; no. = number; Zym = zymogram type.

Table 3-4. Details of the 22 isolates used for the assessment of the AFLP method

Isolate identification	Zym profile	Property identification no.
96-168-906	U1	na
96-168-907	U1	na
96-182-27	U1	na
AC 7473	S1	na
AC 7475	U6/S1	na
96-184-41	na	na
AC 7470	S1	na
96-277-564	S1	na
AC 2127	S1	105
AC 2443	S1	88
AC 2452	S1	65
AC 2503	S1	110
AC 2839	S1	33
AC 6065	U1	20
AC 6623	U1	20
AC 6624	U1	20
97-55-79	U1	114
AC 3264	U5	5
AC 4834	U5	300
AC 5679	U5	68
AC 5944	U5	11
AC 6361	U5	11

na = not available; no. = number; Zym = zymogram type.

Table 3-5. Details of the ten isolates used for the assessment of the MLEE method

Isolate identification	Zym profile	Property identification no.
AC 5679	U5	68
AC 6487	S3	na
AC 7322	S1	na
AC 7327	U1	na
AC 2477	S	na
AC 6326	U3	129
97-418-228	S1	na
97-429-274	S	na
97-458-362	U	na
97-458-364	U	na

na = not available; no. = number; Zym = zymogram type.

Isolate AC 2407 was consecutively subcultured nine times, with each subculture tested by PFGE.

3.2.2 Optimisation of PFGE

3.2.2.1 Optimisation of the method for collecting bacterial cells for PFGE

Method 1

Method 1 was used for most of the strains tested, and was used prior to March 2001. It had the disadvantage of being time-consuming, however the amount of DNA present in each plug was very consistent.

Bacterial isolates were lawn inoculated onto basic TAS medium (Appendix A 3.1) prepared in 90 mm Petri dishes. Two plates were used per isolate. Plates were incubated for 3-4 days in an anaerobic box, with the anaerobic atmosphere produced using a Anaerogen gas pack (Appendix A 2) that creates an atmosphere of less than 1% O_2 and between 9-13% CO_2.

Ice-cold wash buffer (0.9 ml) (Appendix A 3.2) was added to each plate and the bacterial cells scraped from the media surface using a flame-sterilised wire loop. The bacterial cells were aspirated from the plate and placed into an appropriately labelled tube and kept on ice. Two 90 mm plates with a lawn inoculum of growth produced approximately 30 μl of packed cells. The bacterial cells were washed twice in ice-cold wash buffer by centrifuging at 16,060 g (13,000 rpm) for 10 minutes at room temperature. The supernatant was removed and replaced with 200 μl of cold wash buffer. The cells were stored at –20˚C when the plugs were not being prepared the same day.

Method 2

Method 2 was used from March 2001 onwards. It produced the same results for the PFGE and IRS-PCR as method 1, but had the advantage of being less time consuming. It is described in chapter 2, section 2.6.1.

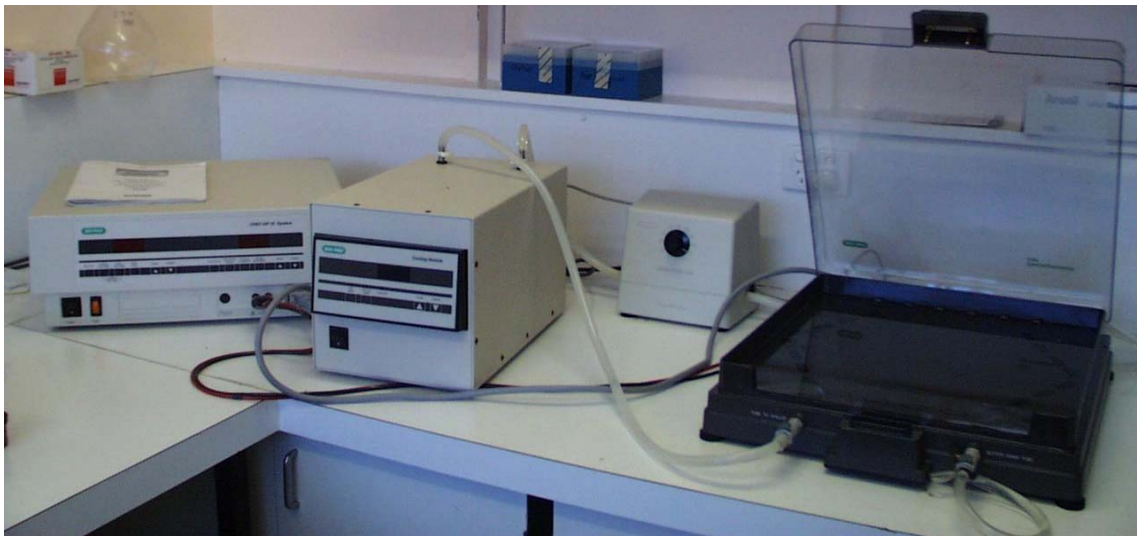


Figure 3-1. Photograph showing PFGE equipment

From left to right; power pack, cooling unit, pump, electrophoresis tank.

3.2.2.2 Optimisation of concentration of DNA for PFGE

Method 1. Counting cells in a counting chamber

When the PFGE method was first used, all bacterial suspensions were counted using a Thoma slide counting chamber viewed with the 20x objective under dark ground illumination. As the method became more familiar it was not necessary to ensure this accuracy of cell concentration, and therefore the McFarland opacity tubes were used to estimate bacterial cell concentration and hence DNA concentration. However, the method is included here as a reference, as more than half the isolates used in this thesis were tested using this method.

A 1:100 dilution was prepared by placing 10 µl of the cell suspension into 990 µl of wash buffer. The suspension was mixed by vortexing for 20 seconds. An aliquot of the cell suspension was aspirated into the well of a Thoma counting chamber and the 16 small squares were counted (Cruickshank *et al.*, 1975).

Calculation: N = No of cells counted/16 x 20, 000, 000 x dilution cells/ml.

The concentration of cells was adjusted to 1 x 10^{10} cells/ml. These cells were subsequently diluted 1:2 (section 3.2.2.3) when preparing agarose plugs, so the cell concentration for optimal concentration of DNA was 5 x 10^{9} cells/ml.

3.2.2.3 Preparation of agarose plugs containing bacterial cells

Method 1

Method 1 was used before March 2001. 50 µl of the suspension of cells in wash buffer (30 µl of packed cells in 200 µl of wash buffer) was added to 250 µl wash buffer, to give a cell count of 5 x 10^9 cells/ml. The cell suspension was warmed in a waterbath at 56ºC for 3 minutes. 300 µl of 2% agarose was added to the cell suspension and mixed thoroughly before being dispensed into plug moulds, which were set on ice. When set, the agarose plugs were placed into 3 mls of wash buffer in 5 ml tubes. The plugs were stored at 4ºC while waiting for DNA extraction to be done.

Method 2

Method 2 was used from March 2001 onwards and is described in section 2.6.2.

3.2.2.4 DNA extraction from bacterial cells embedded in agarose plugs

This method is described in chapter 2, section 2.6.3.

3.2.3 Optimisation of restriction enzymes and electrophoresis conditions for PFGE

Eight restriction enzymes were tested to determine optimal restriction and separation of DNA fragments of *D. nodosus*. These were *Apa*I, *Bgl*II, *Eco*RV, *Mlu*I, *Not*I, *Sfi*I, *Sma*I and *Xba*I. Two different unit concentrations (20 units/µl and 40 units/µl) were tested to determine optimal concentrations for restriction of chromosomal DNA.

A number of different electrophoresis times and switch times were tested for optimisation. The switch times tested were 0.1-16 seconds, 1-20 seconds, 5-50 seconds, 25-70 seconds, with combinations of electrophoresis run times of 20, 22 and 24 hours. A photograph of the PFGE equipment is shown in Figure 3-1.

3.3 Optimisation of IRS-PCR

The IRS-PCR method used two infrequently cutting restriction enzymes, *Xba*I and *Cfo*I to produce DNA fragments. A subset of restriction fragments was amplified by ligating adaptors to the ends of the restricted DNA. The adaptors were designed so that they recognised those fragments that have a *Xba*I recognition site on one end and a *Cfo*I recognition site on the other. One PCR primer, PX, was constructed to complement the AX1 sequence. The IRS-PCR was optimised to produce the best spread and number of restriction bands for analysis and differentiation of *D. nodosus* isolates. This was achieved by adding an extra nucleotide to the second PCR primer. Primers were tested with either an A, G, C, or T added to the 3 prime end (Table 3-6).

The IRS-PCR was optimised for PCR reagent concentrations. Dinucleotides were tested at a final concentration of 100 μM and 200 μM in the PCR master mix. Final primer concentrations were tested at 75 ng, 150 ng, 300 ng and 450 ng. Magnesium chloride concentrations were tested at 1.5 mM and 2 mM.

Table 3-6. IRS-PCR primers and their sequences

Primer	Sequence
PX	5’ – AGA GTC TGC CAG TAC TAG A – 3’
PXA	5’ – AGA GTC TGC CAG TAC TAG AA – 3’
PXC	5’ – AGA GTC TGC CAG TAC TAG AC – 3’
PXG	5’ – AGA GTC TGC CAG TAC TAG AG – 3’
PXT	5’ – AGA GTC TGC CAG TAC TAG AT – 3’

Figure 3-2. Photograph of horizontal gel bed packed in ice and used for the IRS-PCR method

3.3.1 Optimisation using PAGE compared to Metaphor agarose

The original IRS-PCR method used polyacrylamide gel electrophoresis (PAGE) to separate the restriction fragments (Mazurek *et al.*, 1996). PAGE was initially used in this study, but was later compared to Metaphor fine sieving agarose because this material was less hazardous and easier to prepare. A comparison of the methods is presented here.

3.3.1.1 PAGE method

A 6.5% large format polyacrylamide gel was prepared from 30% acrylamide-bisacrylamide (29:1) in 1x TBE buffer (0.045 M Tris-borate, 0.001 M EDTA). 18 μl of amplified product was loaded into each well with 2 μl of loading buffer, and the DNA fragments separated by electrophoresis at 200 volts for 2.5 hour. The gel was stained with ethidium bromide and photographed as described in section 2.6.5.

3.3.1.2 Agarose method

An alternative method to PAGE was tested using fine sieving agarose that the manufacturers claimed to have the same resolving power as PAGE. A 4% gel was prepared from fine sieving agarose. The gel was prepared with ice-cold 0.5 M TBE, as previously described in section 2.7.7 and Appendix A 3.2. To prevent overheating of the

gel during electrophoresis, the electrophoresis equipment was packed in ice (Figure 3-2).

3.4 Comparison of IRS-PCR to AFLP and RAPD methods

At the beginning of this project AFLP and RAPD were assessed for application to molecular typing of *D. nodosus*.

3.4.1 Amplified fragment length polymorphisms (AFLP)

The restriction and ligation of adaptor-oligonucleotides was carried out in one step. The primer and adaptor-oligonucleotide sequences are listed in Table 3-7. In a 20 µl volume, 3 µl of DNA (2 µg) was added to 1 µl of each adaptor (LG1 and LG2 at 0.2 µg each), 1 µl of *Pst*I (40U/µl), 1 µl of T4 ligase (1Unit), 2 µl of ligase buffer and 11 µl of ultra pure water. The reaction was carried out at 37°C for 3 hours. The tagged fragments (20 µl) were precipitated by adding 80 µl of ammonium acetate (10M) and 100 µl of chilled absolute ethanol for no longer than 5 min at room temperature. The mixture was centrifuged at 10,000 g (10,000 rpm) for 10 min at 4°C. The DNA was washed once in 70% ethanol and the pellet re-suspended in TE buffer (20 µl) and stored at 4°C.

The restriction fragments were amplified in a 20 µl reaction volume consisting of 1 µl of DNA (1 ng), 1 µl of each primer (PST1 and PST2 at 150 ng), 0.2 µl of AmpliTaq (5 U/µl), 2 µl of 10x buffer, 1.25 µl of a dinucleotide mix (100 µM final concentration), 4 µl of $MgCl_2$ (5 mM), and 9.44 µl of ultra pure water. The PCR reaction was performed on a Corbett FTS 320 thermocycler for 33 cycles consisting of 94°C for 1 minute, 60°C for 1 minute and extension at 72°C for 2.5 minutes. The amplified fragments were separated in a horizontal gel electrophoresis tank (wide mini cell) for 1.5 hour at 80 volts in a 1.5% agarose gel.

To test the ability of the *Pst*I restriction enzyme to cut the DNA of *D. nodosus*, 5 µl of a 150 ng/µl DNA stock was restricted with 1 µl *Pst*I (40U/µl), 2 µl of 10x restriction buffer and 12 µl of ultra pure water, at 37°C overnight. Fragments were separated on a 2% agarose gel using a horizontal electrophoresis bed at 80 volts for 2 hours. The gel

was strained in ethidium bromide and photographed as described previously in section 2.6.5.

Table 3-7. Primer and adaptor-oligonucleotide sequences used for AFLP

Primer	Sequence
PST1	5'–GAC TGC GTA CAT GCA G–3'
PST2	5'–CTG CAT GTA CGC AGT C–3'
LG1	5'–CTC GTA GAC TGC GTA CAT GCA–3'
LG2	5'–TGT ACG CAG TCT AC–3'

3.4.2 Random amplification of polymorphic DNA (RAPD)

RAPD analysis was applied to isolates of *D. nodosus* using two 10-mers that had been used for an assessment of *Campylobacter jejuni* and *Listeria* species (Mazurier *et al.*, 1992). These were HLWL74 (5'–ACG TAT CTGC–3') and HLWL85 (5'–ACA ACT GCTC–3'). A master mix was prepared consisting of: 2.5 µl of 10x PCR buffer, 1.25 µl of a 10 mM dNTP mix, 1 µl of primer (74 or 85 at 20 pmol), 0.2 µl of *Taq*, 2.5 µl of $MgCl_2$, 12.55 µl of water, and 5 µl of DNA (25 ng–5 ng/µl) to give a total volume of 25 µl. Amplification was performed with one cycle of 94°C for 10 minutes, annealing for 1 min at 34°C and extension at 72°C for 4 min. This was followed by 43 cycles of 94°C for 1 min, 34°C for 1 min and extension at 72°C for 4 min. A final cycle was done at the previous times and temperatures but with a final step at 4°C.

This technique was trialed before the PFGE method was investigated, and therefore the same source of DNA was not used when testing the RAPD method. DNA was extracted from bacterial cells grown on TASH MM medium, using the CTAB method (hexadecyltrimthyl ammonium bromide) (Ausubel *et al.*, 1990) (Appendix A 3.4). Bacterial cells were washed from a TASH MM plate using sterile normal saline and collected into a 1.5 ml microfuge tube before being centrifuged for 5 minutes at 16,060 g (13,000 rpm). The cell deposit (rice grain size) was resuspended in 567 µl TE, 30 µl 10% SDS and 3 µl 20 mg/ml Proteinase K, and the mixture was incubated at 37ºC for 1 hour. Next, 100 µl of 5 M NaCl and 80 µl CTAB reagent were added and incubated at 65ºC for 10 min. The solution was cooled and 600 µl of a chloroform/isoamyl alcohol (24:1) mixture was added and centrifuged for 5 min at 10,000 g (10,000 rpm). This step

was repeated once. 400 μl of ice-cold isopropanol was added to the upper aqueous layer and centrifuged for 5 min at 5,000 g (6,000 rpm). The precipitated DNA was washed twice with 400 μl of 70% alcohol. A final wash was performed using redistilled 95% alcohol. The pellet of DNA was dried in an incubator at 37°C for 1-2 hours. The DNA was reconstituted with 20 μl sterile milliQ water. The concentration of DNA was measured in a spectrophotometer at an optical density of 260 nm. The calculation was based on the concentration of double-stranded DNA at 50 μg/ml having an optical density (OD) of 1 at 260 nm. Thus, the concentration of DNA (μg/ml) = absorbance x dilution x weight per OD. The integrity of the DNA was checked on a 0.8% agarose gel, electrophoresed at 80 volts for 1.5 hours, stained in ethidium bromide and photographed as previously described.

3.5 Multilocus enzyme electrophoresis (MLEE)

The multilocus enzyme electrophoresis (MLEE) method was applied to *D. nodosus* isolates to determine if differences between isolates could be established that would differentiate between virulent and benign isolates, or lead to a greater understanding of the relationship of the zymogram groups to each other.

Bacterial cells were harvested from 15 large (9 cm) TAS plates after 4 days anaerobic growth at 37°C, as described previously under routine culture (section 2.2). Cells were harvested by washing with sterile phosphate buffered saline (pH 7.2) and collected into 5 ml bijou bottles. About 30 μl of packed cells was obtained from the plates. Extraction buffer (10% sucrose, 0.1% mercaptoethanol, 0.1% bromophenol blue) was added to the pelleted cells at less than 50% of total cell volume. Glass beads of 106 μm diameter were added to the cells, which were subjected to four x 1 minute cycles of sonication on ice using a Labsonic sonicator. Extracts were examined by Gram stain to ensure adequate cell lysis. Cell extracts were centrifuged at 15,000 g for 20 minutes at 4°C, and the supernatant collected. If cell extracts were not being used immediately they were kept frozen at –20°C overnight before testing.

Cell lysates were absorbed onto wicks (0.5 cm x 0.8 cm) prepared from Whatman's filter paper number 3. The wicks were applied to a 11.4% starch gel (Appendix A 3.3) prepared with different buffers according to the enzymes being tested. Electrophoresis

was carried out at 150–220 volts according to the buffer used, for 20 hours. Thirty-six enzymes were tested using up to six buffers. The enzymes were: acid phosphatase (ACP), aconitase (ACO), adenosine deaminase (ADA), adenylate kinase (AK), alanine dehydrogenase (ALA), alcohol dehydrogenase (ADH), alkaline phosphatase (ALP), arginine phosphokinase (APK NADP and NAD), aldolase (ALD), catalase, esterase, fructose-1, 6-diphosphate (fruc), fumarase, alpha-glycerophosphate dehydrogenase (α-GPD), glucose-6-phosphate dehydrogenase (G6PD), glutamate dehydrogenase (GDH), glutamate oxoacetate transaminase (GOT), glutamic pyruvic transaminase (GPT), guanine deaminase (GDA), glyceraldehyde-3-phosphate (3GP), hexokinase (HEX), iso-citrate dehydrogenase (ICH), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malonate dehydrogenase (MDH), mannose-6-phosphate isomerase (MPI), nucleoside phosphorylase (NP), L-leucyl-glycylglycine peptidase (LGG PEP), 6-phosphoglucose dehydrogenase (GPGD), phosphoglucose isomerase (PGI), phosphoglucose mutase (PGM), peptidase L-leucyl-proline (PEP LP), peptidase L-leucyl-L-tyrosine (PEP LT), peptidase L-leucyl-glycylglycine (PEP LGG), phosphokinase (NAPDP), and superoxide dismutase (SOD). The buffers were lithium hydroxide electrode buffer (LiOH); Poulik, sodium phosphate buffer (SP7); tris citrate buffer (TC8) pH 8.0; tris borate EDTA buffer (TEB); acid tris maleate ™ pH 8.2, based on previous studies (Selander *et al.*, 1986; Lymberry *et al.*, 1990; Trott *et al.*, 1995), (Appendix A 3.3). Enzymes were prepared fresh and placed over the gel in a 1% agarose overlay, or the gel was soaked in the enzyme solution according to the enzyme being assayed (Selander *et al.*, 1986). Gels were incubated at 37°C for up to four hours until colour had fully developed.

The presence of enzyme bands was recorded and each band was given a number according to its position in terms of the distance it had travelled from the cathode.

3.6 RESULTS

3.6.1 Results for optimisation of PFGE

3.6.1.1 Collecting bacterial cells and estimation of DNA concentration

Method 1 for collecting cells and achieving a suitable DNA concentration was successful and produced a reliable and consistent concentration of DNA in the plugs. This method had the disadvantage of being time consuming because of the number of steps taken and time required to prepare the cell dilutions, to perform the counts under the microscope, and to subsequently prepare the dilutions to make the agarose plugs. Method 2 was less time consuming, as it only involved one step to estimate the cell dilution for the preparation of the agarose plugs, and produced results as clear as those obtained when cells were counted in method 1. The optimal cell concentration was 1 x 10^{10} cells/ml, which was then diluted 1:2 with agarose when making the agarose plugs.

3.6.1.2 Results of evaluation and optimisation of restriction enzymes and electrophoresis conditions for PFGE

Of the seven restriction enzymes (*Bgl*II, *Eco*RV, *Mlu*I, *Not*I, *Sfi*I and *Sma*I) tested and evaluated, *Apa*I produced the best results. No restriction of *D. nodosus* DNA was seen with *Not*I or *Sfi*I when tested at 20 units of enzyme. *Bgl*II, *Eco*RV and *Mlu*I produced too many restriction fragments for ease of analysis, whereas *Apa*I and *Sma*I produced about the same number of fragments, but *Apa*I gave a more even spread of restriction fragments throughout the gel and was much cheaper than *Sma*I. Switch times were tested for an optimal spread of restriction fragments, which was provided using a switch time of 0.1-16 seconds as opposed to other times of 1-20 seconds, 5-50 seconds, and 25-70 seconds. Figure 3-1 shows an example of the results obtained with the different enzymes.

In gel 1, Figure 3-1, three isolates (AC 2127, AC 6 and AC 2839) were restricted with 20 units of enzymes *Apa*I, *Bgl*II, and *Mlu*I. *Sma*I was also tested against isolate AC 2839. A switch time of 5-50 secs for 20 hours was used. Under these conditions *Apa*I and *Sma*I produced the best separation of restriction fragments.

Gel 2, Figure 3-3, shows the results of testing isolates AC 6 and AC 6488 at two concentrations (20 units and 40 units, respectively) of the enzymes *Sma*I, and *Eco*RV. The two isolates were also tested with 40 units of *Apa*I. A switch time of 5-50 secs for 19 hours was used. *Apa*I and *Sma*I produced the best separation of restriction fragments under these conditions. Some restriction fragments resulting from *Eco*RV had run off the end of the gel. There was not as much differentiation between the isolates using this enzyme compared to the differentiation see with *Apa*I and *Sma*I.

Gel 3, Figure 3-3, shows duplicate results for isolates AC 6 and AC 6488 restricted with *Sma*I and *Apa*I in the same tube.

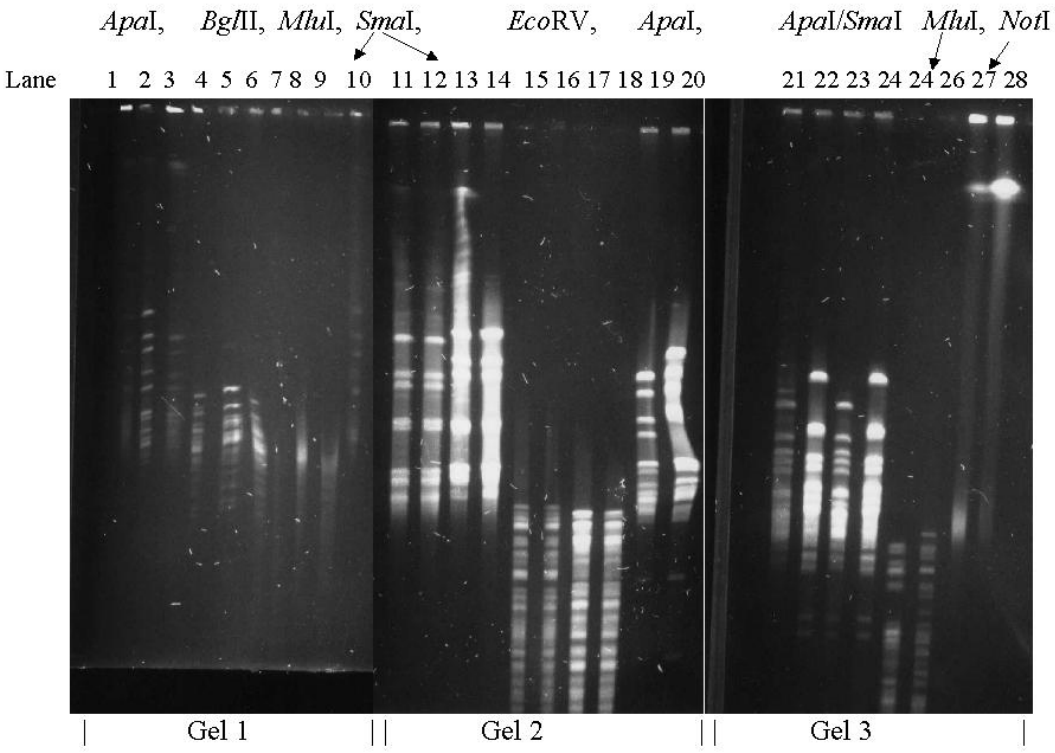


Figure 3-3. Composite gel picture showing the results of PFGE with different restriction enzymes

Lane 1 = AC 2127 (20 units/µl *Apa*I); Lane 2 = AC 6 (20 units/µl *Apa*I); Lane 3 = AC 2839 (20 units/µl *Apa*I); Lane 4 = AC 2127 (20 units/µl *Bgl*II); Lane 5 = AC 6 (20 units/µl *Bgl*II); Lane 6 = AC 2839 (20 units/µl *Bgl*II); Lane 7 = AC 2127 (20 units/µl *Mlu*I); Lane 8 = AC 6 (20 units/µl *Mlu*I); Lane 9 = AC 2839 (20 units/µl *Mlu*I); Lane 10 = AC 2839 (20 units/µl *Sma*I); Lane 11 = AC 6 (20 units/µl *Sma*I); Lane 12 = AC 6 (40 units/µl *Sma*I); Lane 13 = AC 6488 (20 units/µl *Sma*I); Lane 14 = AC 6488 (40 units/µl *Sma*I); Lane 15 = AC 6 (20 units/µl *Eco*RV); Lane 16 = AC 6 (40 units/µl *Eco*RV); Lane 17 = AC 6488 (20 units/µl *Eco*RV); Lane 18 = AC 6488 (40 units/µl *Eco*RV); Lane 19 = AC 6 (40 units/µl *Apa*I); Lane 20 = AC 6488 (40 units/µl *Apa*I); Lane 21 = AC 6 (20 units/µl *Apa*I/*Sma*I); Lane 22 = AC 2839 (20 units/µl *Apa*I/*Sma*I); Lane 23 = AC 6 (40 units/µl *Apa*I/*Sma*I); Lane 24 = AC 2839 (40 units/µl *Apa*I/*Sma*I); Lane 25 = AC 6482 (36 units/µl *Mlu*I); Lane 26 = AC 5709 (36 units/µl *Mlu*I); Lane 27 = AC 6 (40 units/µl *Not*I); Lane 28 = AC 6488 (40 units/µl *Not*I).

Isolates AC 6482 and AC 5709 were tested with 36 units of *Mlu*I, and isolates AC 6 and AC 6488 were tested using 40 units of *Not*I. The conditions tested were a run time of 20 hours and a switch time of 1-20 sec.

Results obtained using a combination of the two enzymes *Apa*I and *Sma*I gave no additional data when compared to the results obtained using each enzyme alone. More restriction fragments were produced from the enzyme *Mlu*I, but some fragments were lost from the end of the gel using a switch time of 1-20 sec.

3.6.1.3 Reproducibility of the PFGE method

The PFGE method was reproducible, as the same restriction fragments were obtained after isolate AC 2407 was consecutively subcultured nine times. Cells collected at each subculture and tested by PFGE produced identical DNA fingerprints identified as PFA 6 (Figure 3-4). On the gel shown in Figure 3-4, DNA was denatured in lanes 2, 3 and 4, but this was a function of either too low a cell concentration or the presence of nucleases.

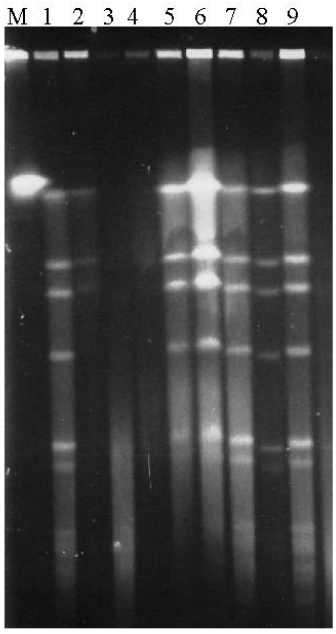


Figure 3-4. Results of PFGE typing of consecutive cultures of isolate AC 2407

Lanes 1-9 represent isolates from 1-9 consecutive subcultures. M = molecular weight marker. In this gel, the marker failed to separate into individual fragments.

3.6.2 Results for optimisation of IRS-PCR

Unlike the AFLP method, the IRS-PCR produced results for *D. nodosus* the first time it was attempted using the published reagent concentrations and electrophoresis conditions that had been used for other microorganisms (Mazurek *et al.*, 1996). All primers were tested at a range of concentrations for all PCR master mix reagents. A $MgCl_2$ concentration of 2 mM produced the best definition between bands, although there was no difference in the number or position of the bands that were amplified using different concentrations of $MgCl_2$. Primers were tested at concentrations of 75 ng, 150 ng, 300 ng and 450 ng. No difference in banding patterns were seen at 75 or 150 ng of each primer, but at higher concentrations amplification of all bands was reduced. Either 100 μM or 200 μM final concentration of dinucleotides could be used, but this needed to be optimised each time a new primer was ordered. For an initial batch of primers, 100 μM dNTP concentration of primers was optimal, whereas when a further batch was ordered, a final concentration of 200 μM primers was optimal and no amplification occurred when 100 μM was used.

Primers gave the same restriction banding results at 50°C and 55°C annealing temperatures.

3.6.2.1 Comparison of different PCR primers

Primers PX, PXA, PXC, PXG and PXT (Table 3-6) were tested for application to *D. nodosus* isolates. The addition of an extra "T" to the primer (i.e. PXT) produced the best differentiation between isolates and the most intense bands, as shown in Figure 3-5.

The composite gel, as shown in Figure 3-5, is an example of the results for these primers. A number of other isolates were tested and these results are presented in Table B-1, Appendix B. The restriction fragments that were amplified with primer PXG (Figure 3-5) and primer PXA were not amplified to the same intensity as other primers, although the amplification of the bands may have been improved with further optimisation.

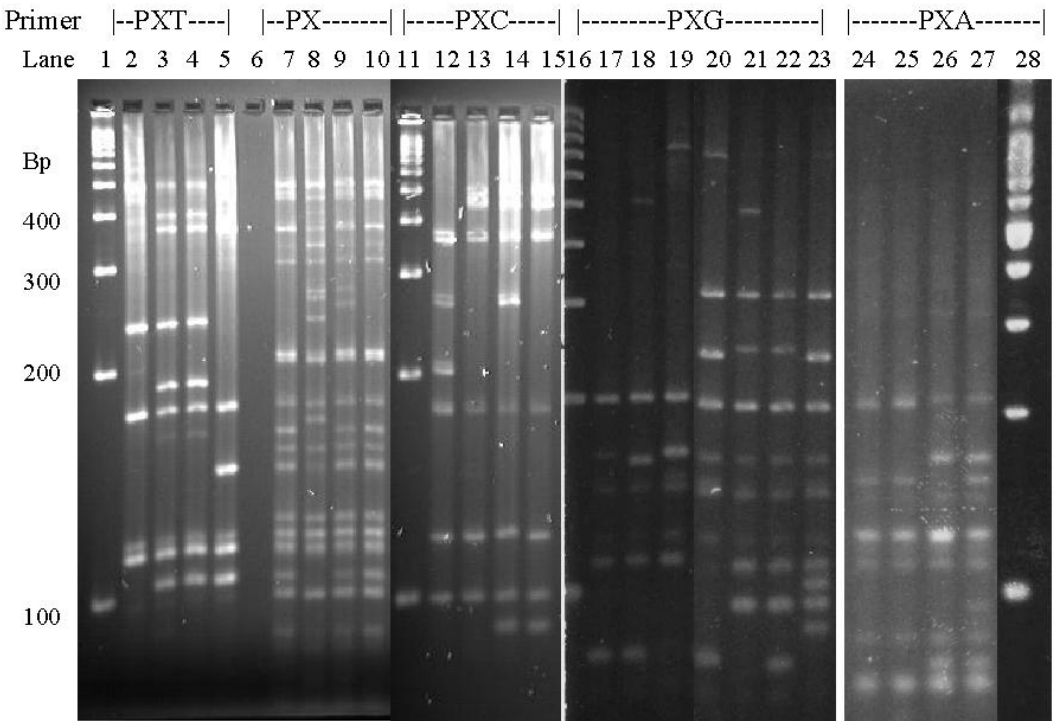


Figure 3-5. Composite gel showing amplification of restriction fragments using different primers for the IRS-PCR method

Lane 1 = 100 base pair molecular weight marker; Lane 2 = 00-505-788 (primer PXT); Lane 3 = 00-514-824 (primer PXT); Lane 4 = 00-514-825 (primer PXT); Lane 5 = 01-9-974 (primer PXT); Lane 6 = DNA-negative control; Lane 7 = AC 6065 (primer PX); Lane 8 = AC 6167 (primer PX); Lane 9 = AC 6622 (primer PX); Lane 10 = AC 6623 (primer PX); Lane 11 = 100 base pair molecular weight marker; Lane 12 = AC 3264 (primer PXC); Lane 13 = AC 4307 (primer PXC); Lane 14 = AC 4834 (primer PXC); Lane 15 = AC 5679 (primer PXC); Lane 16 = 100 base pair molecular weight marker; Lane 17 = 97-405-765 (primer PXG); Lane 18 = 97-386-680 (primer PXG); Lane 19 = 97-362-557 (primer PXG); Lane 20 = 97-369-626-2 (primer PXG); Lane 21 = 97-341-488 (primer PXG); Lane 22 = 98-9-122 (primer PXG); Lane 23 = 98-9-124 (primer PXG); Lane 24 = 97-304-286 (primer PXA); Lane 25 = 97-327-411-1 (primer PXA); Lane 26 = 97-328-419 (primer PXA); Lane 27 = 97-328-424 (primer PXA); Lane 28 = 100 base pair molecular weight marker.

A greater number of faintly staining bands were amplified with primers PXG and PXA than primer PXT. As predicted, primer PX amplified the most restriction fragments (Mazurek *et al.*, 1996).

All primers were able to differentiate between similar strains of *D. nodosus*. For example, PFGE and the primers used in IRS-PCR were all able to provide discrimination between zymogram strains such as the U5 strains (Table 3-8). These results in relation to protease thermostability and genetic type are discussed further in chapter 6. Any of these primers could be used for analysis of *D. nodosus*, however primer PXT produced the best intensity of restriction bands, particularly at molecular weights of 400 to 600 base pairs. At these molecular weights some of the bands

generated from primers PX and PXC were difficult to interpret on some gels (results not shown).

Table 3-8. Comparison of primers tested in the IRS-PCR with results from PFGE

Isolate ID	Farm	Zym	PFGE	IRS-PCR primers			
			PFA type	IrsT type	IrsPX type	IrsG type	IrsC type
6065	20	U1	9a	1	4	3	NT
6167	20	S1	NT	2	5	NT	NT
6622	20	U1	26b	1d	6	NT	NT
6623	20	U1	9a	1	4	3	NT
6624	20	U1	9a	1	4	3	NT
6324	21	U1	19	1d	NT	NT	1
97-55-79	114	U1	19	1	NT	4a	1
106-579	115	U5	9e	1d	7	NT	NT
3264	5	U5	7k	13g	8	1	4
4307	95	U5	9r	30	9	NT	1
4834	300	U5	23	29d	10	2	2
5679	68	U5	18	1d	11	5	3
5944	11	U5	12	5	12	5	1
6161	64	U5	8	6	13		5
6361	11	U5	12	5	12	5	1
97-118-849	98	S2/U4	19b	18	NT	NT	9

ID = identification number; NT = not tested; U = protease unstable; S = protease stable; Zym pattern = zymogram electrophoresis pattern; PFA type = molecular type using enzyme *Apa*I and PFGE; IrsT type = molecular type using PXT primer and IRS-PCR; IrsPX type = molecular type using PX primer and IRS-PCR; IrsG = molecular type using primer PXG and IRS-PCR; IrsC type = molecular type using primer PXC and IRS-PCR.

3.6.2.2 Comparison of electrophoretic media (PAGE and agarose)

Metaphor fine sieving agarose gave an equivalent resolving power to polyacrylamide. The restriction bands were evenly distributed and the same bands appeared on each gel medium. The agarose was easier to handle because the gel was smaller and thicker than the large format PAGE, yet it still had the same resolving power. It also was less hazardous because it did not contain acrylamide, which is poisonous until it is set. Hence the use of fine sieving agarose was easier and safer.

3.6.3 Results for RAPD

3.6.3.1 Optimisation of the RAPD method

The reagent concentrations and reaction conditions were optimised for the two 10-mer primers, HLWL74 and HLWL85. Although the primers were optimised individually, the same reagent concentrations gave optimised results for each primer. An optimum concentration of dinucleotide mix was a final concentration of 100 μM for both primers. At concentrations of 50 μM and 200 μM the intensity of the amplified bands was reduced compared to a concentration of 100 μM. Similarly, the optimal volume of $MgCl_2$ was 2.5 μl, as at 3 μl the intensity of most bands was reduced. Some amplified bands of DNA were not affected at any concentration, but other bands were reduced in intensity when 2 μl or 3 μl was used.

Different volumes of *Taq* enzyme were used: 0.1, 0.2 and 0.4 μl. There was very little effect on the intensity of the bands, although at 0.4 μl the background staining increased.

Different concentrations of DNA template were tested at 1 ng, 25 ng, 50 ng and 100 ng. Slightly more background staining occurred in the electrophoresis lane when 100 ng of DNA was used, and one band was reduced in intensity when 1 ng was used. The optimal DNA concentration ranged from 25 to 50 ng.

Three different concentrations of primer 74 (22 ng, 66 ng, and 132 ng) were tested with isolates AC 2839, 1213-128 and 1443-897. All three primer concentrations produced different banding patterns for each isolate. A major band that was produced at 1500 kb for all isolates was reduced at a primer concentration of 132 ng, whereas other major bands at 180 bp, 450 bp, and 800 bp were reduced or absent at 22 ng. A number of minor bands were present or absent at the different primer concentrations to the extent that there was no consistency in the results (Figure 3-6). Of all the parameters that were tested, the primer concentration was the most critical for consistency of results and repeatability of the test.

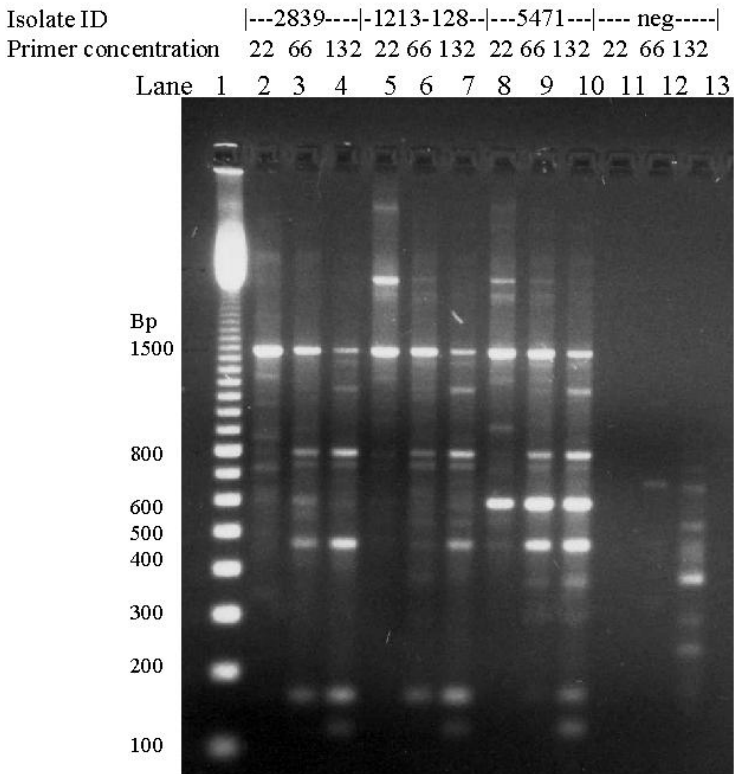


Figure 3-6. Variation in RAPD banding pattern found with different primer concentrations

Lane 1 = 100 base pair ladder; Lane 2 = Isolate AC 2839, primer concentration 22 ng; Lane 3 = Isolate AC 2839, primer concentration 66 ng; Lane 4 = Isolate AC 2839 primer, concentration 132 ng; Lane 5 = Isolate 1213-128, primer concentration 22 ng; Lane 6 = Isolate 1213-128, primer concentration 66 ng; Lane 7 = Isolate 1213-128, primer concentration 132 ng; Lane 8 = Isolate AC 5471, primer concentration 22 ng; Lane 9 = Isolate AC 5471, primer concentration 66 ng; Lane 10 = Isolate AC 5471, primer concentration 132 ng; Lane 11 = negative control, primer concentration 22 ng; Lane 12 = negative control, primer concentration 66 ng; Lane 13 = negative control, primer concentration 132 ng. These results were obtained with primer HLWL74.

Another major factor affecting reproducibility of the technique was the method of DNA extraction. When the RAPD banding patterns were compared for DNA from an isolate that had been extracted by both the boiled cells method and the CTAB method, differences in the bands were seen. Some bands were present in DNA extracted by the CTAB method, but absent from DNA extracted by the boiling method. To maintain consistency in the results the CTAB method was used, and the concentration of DNA was measured by spectrophotometer so that a known concentration of DNA (5 ng/μl) was used in the RAPD reaction.

Differences in the presence and intensity of DNA bands was noted with different batch number of *Taq* enzyme, even from the same supplier.

3.6.3.2 RAPD results using primer HLWL74

Primer HLWL74 was tested with a range of strains of *D. nodosus* that reflected the range in protease thermostability and zymogram types. All isolates produced a major band at 1500 base pairs. Only a few other bands, of greatly reduced staining intensity, were seen (Figure 3-7).

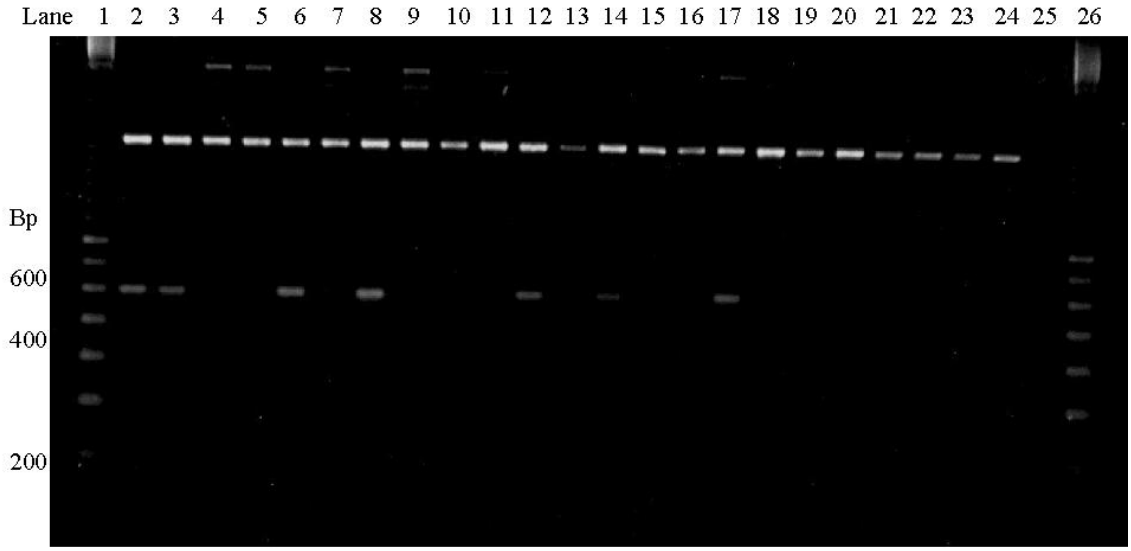


Figure 3-7. RAPD results for primer HLWL74

Lane 1 = 100 base pair molecular weight marker; Lane 2 = 1443-893; Lane 3 = 1440-880; Lane 4 = 1213-127; Lane 5 = 1213-128; Lane 6 = AC 5471; Lane 7 = AC 5503; Lane 8 = AC 915; Lane 9 = AC 5659; Lane 10 = AC 1087; Lane 11 = 1445-903; Lane 12 = AC 5404; Lane 13 = AC 5545; Lane 14 = AC 4307; Lane 15 = AC 5679; Lane 16 = AC 5637; Lane 17 = AC 4346; Lane 18 = AC 5136; Lane 19 = AC 2407; Lane 20 = AC 2839; Lane 21 = AC 4292; Lane 22 = AC 5593; Lane 23 = AC 4123; Lane 24 = AC 5615; lane 25 = negative DNA control; Lane 26 = 100 base pair molecular weight marker.

The presence of these bands was not consistent and at times duplicates from the same DNA gave inconsistent results. Therefore, this primer was considered to be unsuitable for use in a molecular typing method.

3.6.3.3 RAPD results using primer HLWL85

Primer HLWL85 was tested against the same set of isolates, and results are presented in Figure 3-8. The method differentiated between isolates within a strain, such as the U2 zymogram group. All zymogram groups could be further differentiated using this primer. Of the 23 isolates tested, 15 different types were identified, indicating that the primer was sensitive for identifying genetic differences. None of the banding patterns

appeared to provide a differentiation between strains with thermostable protease or heat labile protease.

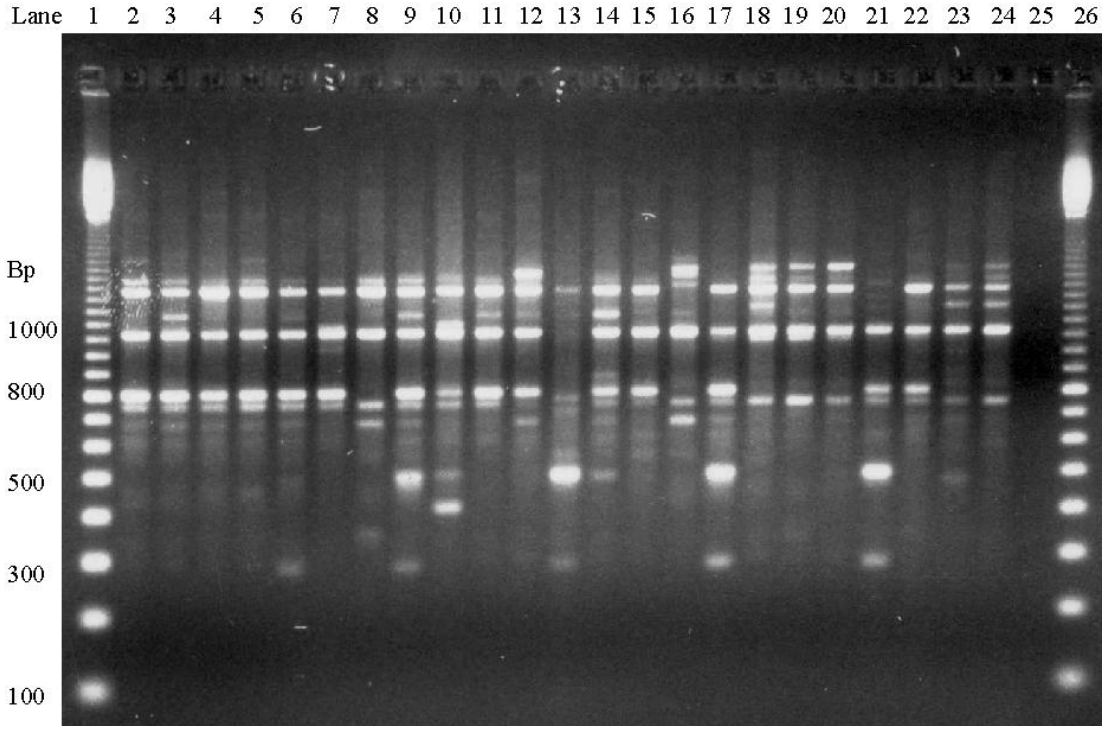


Figure 3-8. RAPD results for primer HLWL85

Lane 1 = 100 base pair molecular weight marker; Lane 2 = 1443-893; Lane 3 = 1440-880; Lane 4 = 1213-127; Lane 5 = 1213-128; Lane 6 = AC 5471; Lane 7 = AC 5503; Lane 8 = AC 915; Lane 9 = AC 5659; Lane 10 = AC 1087; Lane 11 = 1445-903; Lane 12 = AC 5404; Lane 13 = AC 5545; Lane 14 = AC 4307; Lane 15 = AC 5679; Lane 16 = AC 5637; Lane 17 = AC 4346; Lane 18 = AC 5136; Lane 19 = AC 2407; Lane 20 = AC 2839; Lane 21 = AC 4292; Lane 22 = AC 5593; Lane 23 = AC 4123; Lane 24 = AC 5615; lane 25 = negative DNA control; Lane 26 = 100 base pair molecular weight marker.

The RAPD method was prone to problems with reproducibility mainly due to the type of DNA extraction method, primer concentrations, and variation in band intensity obtained with different brands of *Taq* polymerase.

Results with primer HLWL85 are contrasted with those for primer HLWL74 in Table 3-9. There were no bands or banding patterns generated from either primer that could differentiate between the different zymogram patterns or indicate a difference between thermostable protease strains and protease heat labile strains.

Table 3-9. Comparison of results for RAPD primers HLWL74 and HLWL85

Isolate	Zym	RAPD 74	RAPD 85
AC 2407	S1	1	2b
AC 2839	S1	1	2b
AC 4292	S2	1	10
AC 5593	S2	1	1a
AC 5615	S3	2 or 4a	11a
AC 4123	S3	4 or 1	11
AC 5136	U6	5 or 1	9
AC 5659	U3	1	4
AC 5404	U4	1	2a
AC 4307	U5	?3 or 1	3
AC 5679	U5	1	1a
AC 5637	U6	1	7
1213-127	U1	2	1a
1213-128	U1	2	1
AC 1087	U3	2	5
AC 5251	U1	2	
AC 5503	U2	2a	1a
1441-893	U1	3	1
1440-880	U1	3	2
1443-897	U1	3	1a
1445-903	U3	3	2
AC 915	U2	3	3
AC 4346	U6	3 or 6	8

AC = Albany Laboratory culture collection number; Zym = Zymogram pattern; RAPD 74 = molecular type generated from primer HLWL 74; RAPD 85 = molecular type generated from primer HLWL 85. Prefix a or b indicates a subtype as identified by a one-band difference (For an explanation of assignment of molecular type, refer to section 2.8). Results for some strains were not clear due to faintly staining bands, hence the designation of two possible molecular types.

3.6.4 Results for comparison of typing methods

The results obtained with RAPD, IRS-PCR and PFGE were compared to determine which method indicated the most genetic diversity between isolates. Although only a limited number of isolates were compared for all four methods, it can be seen from Table 3-10, that the PFGE and IRS-PCR methods gave further differentiation between isolates that was not provided with the RAPD method with primer HLWL85. The fingerprint pattern 2b obtained with primer 85 was further differentiated using both PFGE and IRS-PCR. This finding also occurs with fingerprint pattern 3 from the primer HLWL85.

Due to the problems encountered with reproducibility of the RAPD method that were not found with either the PFGE or the IRS-PCR methods, the RAPD method was not investigated further.

Table 3-10. Comparison of results from PFGE, IRS-PCR, and RAPD

Isolate	Zym	PFA	IrsT	RAPD 74	RAPD 85
AC 2407	S1	6	2	1	2b
AC 2127	S1	7a	2	1	
AC 2839	S1	7	13	1	2b
AC 4123	S3		15a	4 or 1	11
AC 5615	S3	46a	29c	2 or 4a	11a
AC 5637	U6	31	60	1	7
AC 5136	U6	7	2	5 or 1	9
AC 5659	U3	9e	13g	1	4
AC 5404	U4	9i	30	1	2a
AC 5545	U4	69	1	3	6
AC 4307	U5	9r	30	?3 or 1	3
AC 915	U2	9a	1g	3	3
AC 5679	U5	18	1g	1	1a

AC = Albany Laboratory culture collection number; Zym = Zymogram pattern; PFA = molecular types generated by the PFGE method using *Apa*I restriction enzyme; IrsT = molecular type generated by the IRS-PCR method using primer PXT; RAPD 74 = molecular type generated from primer HLWL 74; RAPD 85 = molecular type generated from primer HLWL 85. Prefix a or b indicates a subtype as identified by a one-band difference (For an explanation of assignment of molecular type, refer to section 2.8).

3.6.5 Results for AFLP

The AFLP was followed using restriction enzyme *Pst*I (Valsangiacomo *et al.*, 1995). However, results were poor when applied to isolates of *D. nodosus*. After amplification of the restriction fragments that had been ligated with the adaptors for the PCR primers, only a smear of DNA was seen on the electrophoresis gel. Some individual bands were seen in a few lanes (Figure 3-9), but these results were not those expected according to the method. A simple restriction digest using *Pst*I and chromosomal DNA was performed (results not shown) to establish that the enzyme was capable of cutting the DNA of *D. nodosus*. Numerous restriction fragments were seen as expected, however these were not amplified in the subsequent PCR. This method may have been successful if further optimisation was undertaken, however because the IRS-PCR method produced results with little further work, this latter method was then used for all subsequent typing.

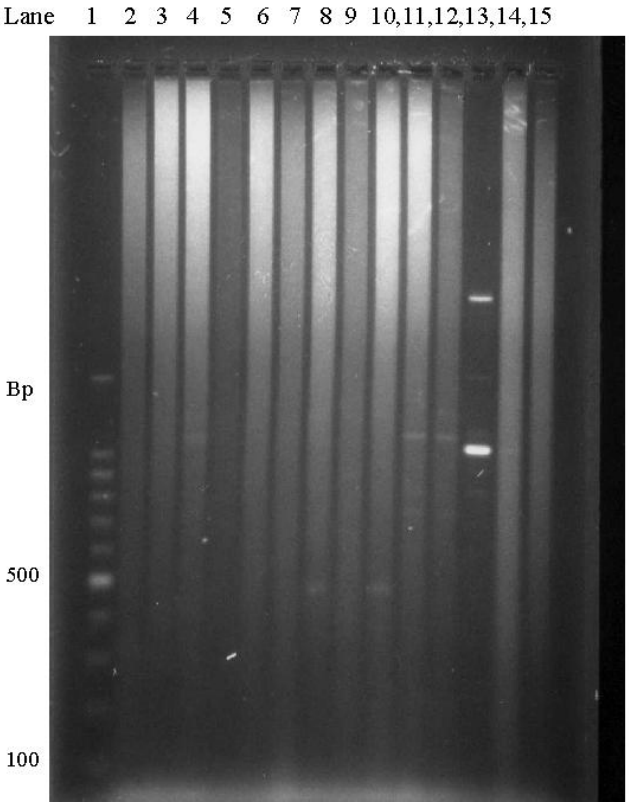


Figure 3-9. Results of AFLP using *Pst*I

Lane 1 = Molecular weight marker 100 base pair ladder; Lane 2 = AC 2127; Lane 3 = AC 2839; Lane 4 = AC 2839; Lane 5 = AC 2443; Lane 6 = AC 2452; Lane 7 = AC 2503; Lane 8 = 168-906; Lane 9 = 168-907; Lane 10 = 182-27; Lane 11 = 230-332; Lane 12 = 230-324; Lane 13 = 184-41; Lane 14 = 230-328; Lane 15 = 277-564.

3.6.6 Results for multilocus enzyme electrophoresis (MLEE)

Thirty-six enzymes were screened using all buffer systems. No activity was detected for 15 of these enzymes; peptidase L-leucyl proline, 6-phosphoglucose dehydrogenase, guanine deaminase, phosphoglucose mutase, lactate dehydrogenase, alcohol dehydrogenase, alkaline phosphatase, catalase, aconitase, glutamate dehydrogenase, glutamate oxaloacetate transaminase, iso-citrate dehydrogenase, fumarase, superoxide dimutase, and glucose-6-phosphate dehydrogenase. Only six enzymes produced results that had strongly staining enzymes; nucleoside phosphorylase, phosphoglucose isomerase, peptidase L-leucyl tyrosine, peptidase L-leucyl glycyl glycine, hexokinase, esterase and leucine aminopeptidase. The remaining enzymes produced variable results. Some enzymes were not detected in some isolates, and in others detection was at a very low level. In addition, the cell lysate extract from some isolates did not contain a high enough concentration of enzymes for adequate

detection. Some results were difficult to interpret as bands were not seen as discrete banding patterns, but were seen as a smear or smudge on the gel.

The results were variable between isolates using different buffer systems. For example, nucleoside phosphorylase (NP) was detected at varying intensity of staining in isolates AC 5679 (U5), AC 6487 (S3), AC 7322 (S1) and AC 7327 (U1) when TC8 buffer was used. The enzyme was only detected in AC 7327 when lithium hydroxide electrode buffer was used. Gel 3 in Figure 3-10 shows the results for NP in isolates AC 2477 (S), AC 6326 (U3) and 97-418-228 (S1) when Tris EDTA boric acid (TEB) electrode buffer was used.

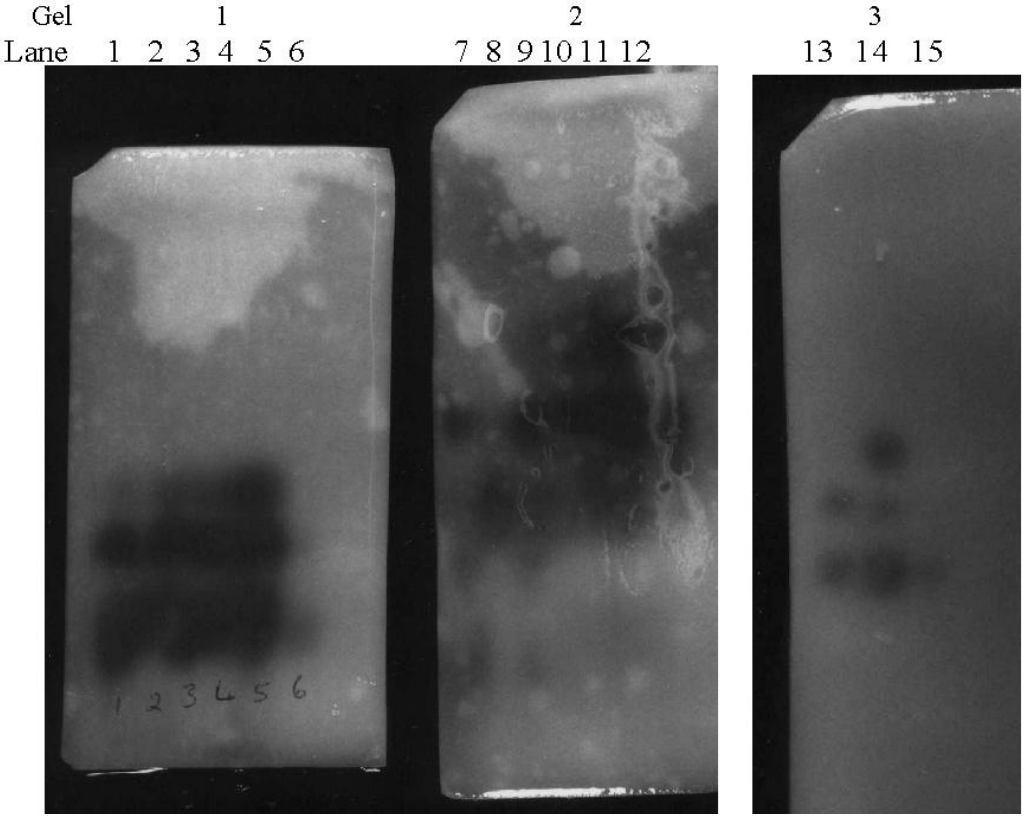


Figure 3-10. Example of starch gel for MLEE

Lane 1 = AC 2477 (S); Lane 2 = AC 6326 (U3); Lane 3 = 97-418-228 (S1); Lane 4 = 97-429-274 (S); Lane 5 = 97-458-362 (U); Lane 6 = 97-458-364 (U); Lane 7 = AC 2477 (S); Lane 8 = AC 6326 (U3); Lane 9 = 97-418-228 (S1); Lane 10 = 97-429-274 (S); Lane 11 = 97-458-362 (U); Lane 12 = 97-458-364 (U);Lane 13 = AC 2477 (S); Lane 14 = AC 6326 (U3); Lane 5 = 97-418-228 (S1). Gel 1 shows the results for peptidase L-leucyl glycyl glycine and lithium hydroxide electrode buffer; Gel 2 shows the results for peptidase L-leucyl glycyl glycine using poulik electrode buffer; Gel 3 shows the results for nucleoside phosphorylase using Tris EDTA boric acid (TEB) electrode buffer.

Gels 1 and 3 in Figure 3-10 show the variation in results for the enzyme peptidase L-leucyl glycyl glycine when lithium hydroxide electrode buffer was used (Gel 1), compared to the results when poulik electrode buffer was used (Gel 2).

Because of the variability of the results, no assessment could be made of any differences seen between virulent and benign strains.

3.7 DISCUSSION

Molecular typing methods offer improved discrimination of strains compared to conventional phenotypic methods such as biochemical characterisation and serotyping. Two such molecular methods, namely PFGE and IRS-PCR, were optimised for *D. nodosus* and proved to be discriminatory for strain differentiation compared to the other methods trialed in this study (ie. AFLP, RAPD and MLEE).

Although the RAPD method produced diverse banding patterns for *D. nodosus* and could be used for differentiating strains, the test was not entirely reproducible, especially when a different batch number of *Taq* enzyme was used from the same supplier. This finding has also been reported with other techniques such as ERIC (enterobacterial repetitive intergenic consensus), which use low stringency annealing (Tyler *et al.*, 1997). Other reports indicated reproducibility problems with RAPD methods, as concentration of primer and quality of the template DNA were found to affect reproducibility of the result, with new targets being amplified or other targets disappearing (Muralidharan and Wakeland, 1993). The variation in the current study could not be overcome even by using a reliable DNA extraction method as opposed to obtaining DNA by boiling the cells, which is used in some methods. With the random amplification of DNA using the short 10-mer primers, the bands of DNA that are amplified can be a combination of artifactual variation and true polymorphism. It had been thought that arbitrary primers would be consistent in their amplification or detection of polymorphism, however the method is prone to variations. The method is affected by the same factors that affect conventional PCR such as $MgCl_2$ concentration, primer:DNA ratio, thermocycler model, primer synthesis etc.

The AFLP method did not produce meaningful results. The major difference between this method and the IRS-PCR was the restriction site target of the enzymes, and the restriction-ligation steps. The AFLP method uses an enzyme that cuts frequently, whereas the IRS-PCR uses two enzymes, one that cuts frequently and one that cuts infrequently. Through the use of different enzymes and adjusting the nucleotide on the 5' end of the PCR primers, the number of restriction fragments to be amplified can be controlled to obtain a meaningful number of bands for analysis. The AFLP method performs restriction and ligation in a single step, whereas the IRS-PCR performs this as a two-stage process. It is possible that the AFLP method could be further modified to make it more useful for typing *D. nodosus* isolates, but this was not pursued here because both IRS-PCR and PFGE gave good results.

Unfortunately no meaningful results could be obtained from the MLEE method. This was largely because of the difficulty in identifying enough conserved "housekeeping" genes that were expressed to a detectable level in all isolates. As a result of time limitations, this approach was abandoned – although it would be worth pursuing in the future. One of the main problems encountered with the MLEE method was the difficulty in producing sufficient cell growth so as to obtain a high enough concentration of enzyme for adequate examination. The absence of a band in one isolate compared to another was difficult to interpret as a true absence because it could not be determined if the band absence was a true reflection of locus diversity, or absent due to loading of insufficient sample onto the gel.

The cells of *D. nodosus* were difficult to disrupt by sonication. Some enzyme may have been destroyed during prolonged sonication, although samples were held on ice between rounds of sonication to reduce the heat generated. Subsequently, use of polyacrylamide gels rather than starch gels might have produced sharper bands and required less enzyme for analysis. Because of the limitations of the technique when applied to *D. nodosus*, a large number of isolates could not be tested for a number of enzymes. This meant that insufficient genetic loci were analysed and therefore no results or interpretations could be made in regard to the population genetics of *D. nodosus*. Results from assessing the allelic variation in the proteins would have provided an estimation of genetic distance between isolates and this information would have complemented the results obtained from the PFGE and IRS-PCR methods. It had been hoped that data from MLEE analysis would provided information on how the

clonal groups detected in the PFGE method related to each other. Likewise, no assessment of any differences between virulent and benign strains could be made.

In the future, when a number of house-keeping genes have been sequenced for *D. nodosus*, multilocus sequencing typing (MLST) could be used to overcome the technical limitations experienced in this project for MLEE analysis of *D. nodosus*.

Primer synthesis affected the results from the IRS-PCR on one occasion, after ordering a new batch of primers and adaptors. To correct for this, the concentration of dinucleotides had to be re-optimised. However, this was the only noted variation with the IRS-PCR method and this variation was quite obvious, because no amplification was seen at the sub-optimal concentration of dinucleotides. No variation of DNA profiles was seen in isolates that were repeat tested, or were tested months apart. Two restriction and amplification controls were included with each gel, and no variation was ever seen with their results, even when the isolates were reconstituted from storage and re-tested. The IRS-PCR method was relatively easy to perform, taking two days, and only required equipment that was already part of a molecular biology laboratory (i.e a gel electrophoresis tank and a thermocycler).

The PFGE method was optimised for enzyme, enzyme concentration, switch time and run time. The enzyme concentration did not have a great effect on the restriction of the DNA, and no great difference was seen between 20 units or 40 units of enzyme. The most significant effect was the ability of the enzyme to cut the DNA. The enzyme that was selected (*Apa*I) cut the DNA to produce banding patterns that were easy to interpret, produced a sufficient number of bands to enable differentiation between isolates, and was relatively cheap. The concentration of the bacterial cells and hence the DNA was another major factor for producing good results. Too little DNA produced weakly staining bands that could not be analysed clearly, and too much DNA produced smearing of the DNA on the gel, or two bands that were close together may have appeared as one band because of overstaining. This problem was overcome once the optimal concentration of DNA was assessed and a simple method for estimating cell concentration was used (i.e. use of the McFarland standards). The major disadvantages with the PFGE technique were the cost of the specialised equipment, the time taken to extract the DNA (1-2 days), and the electrophoresis run time (overnight).

Both PFGE and IRS-PCR gave reproducible results, producing a repeatable pattern of fingerprints from serial subcultures of the same organism. This not only showed the reproducibility of the method, but also indicated that the isolate did not undergo detectable genetic change after nine consecutive subcultures.

In conclusion, it was found that PFGE and IRS-PCR were robust, reliable techniques that provided fine discrimination between isolates in a population, and could therefore be used for epidemiological analysis of *D. nodosus* in this study.

CHAPTER 4

MOLECULAR TYPING OF *DICHELOBACTER NODOSUS* AND INVESTIGATION OF GENETIC RELATEDNESS

CHAPTER 4

4. Molecular typing of *Dichelobacter nodosus* and investigation of genetic relatedness

4.1 INTRODUCTION

Molecular typing techniques are powerful tools for analysing microorganisms to provide information for molecular epidemiological studies, including strain identification, estimating interstrain genetic relatedness and identifying clonal groups (Branger *et al.*, 2003).

In Chapter 3 the molecular typing methods of pulsed field gel electrophoresis (PFGE), infrequent restriction site polymerase chain reaction (IRS-PCR), randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLP) were assessed for application for a study of isolates of *D. nodosus*. PFGE and IRS-PCR proved to be the best methods that showed discrimination between strains and produced reliable and repeatable results.

Pulsed field gel electrophoresis had been used to analyse a small number of isolates of *D. nodosus* from Malaysia (Zakaria *et al.*, 1998). To date, there have been no reports of large-scale studies on molecular typing of isolates of *D. nodosus* in Australia or elsewhere. No data are available for an assessment of the genetic diversity of Australian isolates from cases of footrot, nor is it known how isolates from different States are related. Anecdotal evidence suggests that because of the eradication program, infected sheep in WA no longer carry the highly virulent strains that cause foot lesions of score 4 and 5 that are still seen in infections in VIC and NSW.

In this chapter, PFGE and IRS-PCR were applied to *D. nodosus* strains mainly from WA, but also, for comparison, from NSW, SA and VIC. The aim was to determine the genetic diversity of isolates in WA, to compare the molecular types found in WA to those found in NSW, SA and VIC, and to determine the distribution of isolates between States. The extent of genetic diversity revealed by each typing method was analysed and the results compared. The genetic diversity of the protease thermostable isolates was investigated, and compared to the diversity seen in the heat-labile isolates. The genetic similarity of molecular types identified by PFGE was investigated using computer-

assisted analysis to generate dendrograms, and the results were compared to those obtained from visual examination of the fingerprints.

The application of molecular typing to epidemiological investigation is presented in chapter 5. The investigation of the molecular type in relation to virulence as defined by protease thermostability forms the basis of chapter 6.

4.2 MATERIALS and METHODS

4.2.1 Bacterial isolates

A total of 735 *D. nodosus* isolates from 247 farms in WA isolated in the years 1976 to 2002 were collected and tested by PFGE (Table 2-1). A subset of 616 isolates was tested by IRS-PCR. A policy decision by the footrot program at the Department of Agriculture WA determined that up to two S strains from each property in quarantine should be typed by molecular typing. This created a bias towards S strains between the years 2000 to 2002. Of the 735 isolates tested in WA, 364 (49.5%) were isolated from 2000 onwards. Of these, 320 (87.9%) were S strains and 44 (12.1%) were U strains (Table 2-1). The remaining 371 (50.5%) isolates were isolated prior to 2000, and were composed of 195 (52.6%) S strains and 174 (46.9%) U strains.

In addition, 24 isolates from 22 properties in VIC, 21 isolates from 21 properties in SA and 16 isolates from 13 properties in NSW were tested. The details of these isolates including their zymogram profiles are presented in Appendix B, Tables B-1, B-2, B-3, and B-4. All isolates tested from VIC, SA and NSW were tested by both PFGE and IRS-PCR. The isolates represented a range of zymogram patterns, and included multiple isolates from some farms, and multiple isolates from some animals.

4.2.2 Typing Methods

The isolates were tested by PFGE using the restriction enzyme *Apa*I, and IRS-PCR using primer PXT, as described in chapter 2.

4.2.3 Analysis of molecular fingerprints

Gels were analysed visually, and nomenclature for molecular type assigned as described in chapter 2. One isolate from each representative PFA type was selected and used to prepare a dendrogram of "standard" molecular PFA types using the GelCompar II software program. Analysis of molecular types was performed using the Dice unweighted pair group method with arithmetic averages (UPGMA), with a band comparison position tolerance of 5%. Only those DNA bands occurring between 48.5 and 291 kilobase pairs were selected for analysis. Dendrograms were prepared for "standard" PFA molecular types using 168 isolates. Individual dendrograms for protease thermostable (S) strains and protease heat-labile (U) strains from WA were also prepared.

A total of 26 PFGE gels that contained representative PFA types (standard PFA types) were used in the analysis for construction of a dendrogram showing relatedness of strains. A molecular weight marker (Lambda DNA) was run in lanes 1 and 30 of each gel. The molecular weights from these gels were aligned using the GelCompar II software to check the accuracy of the alignments. Dendrograms of IrsT types were not constructed.

4.2.4 Calculation of Simpson's index of diversity

Simpson's index of diversity (D) was calculated as described in section 2.8.1. The calculations were performed using Microsoft Excel. Calculations of Simpson's index of diversity were done for all molecular types, all clonal types, all S strains, and all U strains.

4.2.5 Calculation of diversity ratio of isolates and molecular types

A simple method for determining the ratio of genetic diversity was calculated by dividing the number of isolates by the number of molecular types (section 2.8.4.1). The calculation was also applied to obtaining the diversity ratio for clonal groups. Calculations were done for all molecular types, all clonal types, all S strains, and all U strains.

4.2.6 Interpretation of dendrogram

Dendrograms were interpreted as described in section 2.8.3.

4.2.7 Persistence of clonal groups in WA

The 735 isolates tested from WA were selected from isolates detected between 1976 and 2002, inclusive (Table 2-1). One representative PFA clonal type was selected from each farm and used to estimate the persistence of clonal groups in WA throughout the years from which the isolates were selected. Equal numbers of isolates from each year were not tested. A simple diversity ratio was calculated based on the number of isolates detected in a year divided by the number of clonal groups identified in that year.

4.2.8 Comparison of PFGE results to IRS-PCR results

The relationship between the molecular type obtained by PFGE (PFA type) and the molecular type obtained by IRS-PCR (IrsT type) was analysed using the results obtained for the PFA 7 clonal group, using 42 isolates from WA (see Table 4-5, results).

4.3 RESULTS

The raw data for the PFGE and IRS-PCR methods is presented in Tables B-1, B-2, B-3, and B-4, in Appendix B. A summary of the results is presented in Table 4-1.

4.3.1 Molecular types and genetic relatedness identified by PFGE

For the PFGE analysis, a total of 214 PFA types were obtained from the 796 isolates tested throughout Australia, which indicated a diversity ratio of 1:3.7. The isolates were grouped into clonal groups based on the definitions in chapter 2. For the 796 isolates tested from all States in Australia, the 214 PFA types were classified into 82 clonal groups, and gave a diversity ratio of 1:9.7 (Table 4-1).

Table 4-1. Summary of results for PFGE and IRS-PCR molecular typing of Australian isolates of *D. nodosus*

Data	WA	VIC	SA	NSW	Total
PFGE					
No. of properties	247	22	21	13	303
No. of isolates	735	24	21	16	796
No. of PFA types	181	21	19	14	214
No. of clonal groups	67	18	15	10	82
Overall diversity ratio of PFA types	1:4	nc	nc	nc	1:3.7
Overall diversity ratio of clonal groups	1:11	nc	nc	nc	1:9.7
Simpson's diversity for molecular type	0.98	nc	nc	nc	0.98
Simpson's diversity for clonal type	0.90	nc	nc	nc	0.91
IRS-PCR					
No. of properties	247	22	21	13	303
No. of isolates	616	24	21	16	677
No. of IrsT Types	77	17	17	13	94
No. of clonal groups	36	13	12	11	48
Overall diversity ratio of IrsT types	1:8	nc	nc	nc	1:7.2
Overall diversity ratio of clonal types	1:17	nc	nc	nc	1:14
Simpson's diversity for molecular type	0.95	nc	nc	nc	0.96
Simpson's diversity for clonal type	0.87	nc	nc	nc	0.89

IrsT = Molecular type using primer PXT and IRS-PCR method; nc = not calculated; No. = number; NSW = New South Wales; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; SA = South Australia; VIC = Victoria; WA = Western Australia.

The numbers of isolates tested from SA, NSW and VIC were considered too small to provide an accurate estimate of genetic diversity in those States.

In WA where 735 isolates were tested from 247 properties, 181 PFA types were obtained (Table B-1, Appendix B), thus giving a PFA diversity ratio of 1:4 (Table 4-1). These could be grouped into 67 clonal groups. The diversity ratio for clonal groups was 1:11.

In VIC, 24 isolates were tested from 22 farms and resulted in 21 PFA types. Three isolates were subgroups, thus identifying 18 clonal groups (Table B-4, Appendix B). In SA, the 21 isolates tested from the 21 farms were divided into 19 PFA types, which

could be classified into 15 clonal groups (Table B-2, Appendix B). In NSW, 16 isolates from 13 properties were divided into 14 PFA types, and these could be classified into 10 clonal groups (Table B-3, Appendix B).

The numerical index of the discriminatory ability of the PFGE using *Apa*I and testing isolates from WA produced a Simpson's index of diversity of 0.982. This was based on N = 735 so that N(N-1) = 539490, s (number of types) = 181, and the sum of the strains in a molecular type where $nj(nj\text{-}1) = 9552$.

D = 1-(9552 ÷ 539490) = 0.98 for PFA types in WA.

D = 1-(10226 ÷ 632820) = 0.98 for PFA types in Australia.

The diversity for the clonal groupings was calculated in the same way. For clonal PFA groups from WA, N(N-1) = 539490, and $\Sigma nj(nj\text{-}1) = 53506$, which produced a clonal genetic diversity of 0.90. Calculations for all clonal groups from Australia, where $\Sigma nj(nj\text{-}1) = 57614$, gave a diversity index of 0.91. These results are listed in Table 4-1.

4.3.1.1 Genetic relatedness identified by dendrogram analysis of PFA types

A dendrogram alignment of all the molecular weight markers from the PFA gels that were used for analysis by the GelCompar II software showed an alignment of 100% (data not shown).

A dendrogram was constructed from 168 selected standard PFA types, and indicated that the genetic relatedness varied from 100% to 63% (Figure 4-1). Six clusters (I-VI) were distinguished at a level of 75% similarity.

Cluster I contained 78 isolates that were related at a level of 77% similarity. These comprised 60.3% (n = 47) S strains and 39.7% (n = 31) U strains. Within cluster I, seven groups (A-G) were identified (Figure 4-1). A range of protease type, zymogram type, PFA type and IrsT type were found in all groups, with the exception of group E, which only contained S strains. These represented 46.8% of all S strains found in cluster I. The 22 S strains in group E were composed of 36.4% (n = 8) that belonged to clonal group PFA 7.

A total of 15 isolates from clonal group PFA 9 were represented in the dendrogram, with ten (66.7%) of these occurring in cluster I. The majority of these (80%, n = 8) belonged to group C.

Isolates that were identified from a farm and were closely related PFA subtypes were located in the same group or cluster on the dendrogram. For example, three isolates (00-444-580, 000-444-581#1, 00-444-583) from Farm 195 were all closely related by PFA type (PFA 21, 21b, 21c, respectively) and were located in cluster I, group A at a level of 85.5% similarity (Figure 4-1). Two other closely related isolates (01-323-40, 01-466-524) from Farm 100 were identified as PFA 21aand PFA 21e, respectively, and these isolates were also located in cluster I, group A.

Cluster II contained 36 isolates related at 77% similarity and comprised 52.8% U strains and 47.2% S strains. No predominant molecular type occurred in cluster II.

Cluster III comprised 36 isolates with 86.1% (n = 31) S strains and 13.9% (n = 5) U strains, all of which were U5 zymogram type. These isolates were related to each other at a level of 75% similarity.

Clusters IV, V, and VI harboured 13, four and one strain, respectively.

Further investigation into the predominant clonal groups PFA 7, 9 and 11 and the relationship to protease thermostability is described in chapter 6.

The diversity of molecular types seen at the farm level and hoof level is discussed in chapter 5 in relation to epidemiology of *D. nodosus*.

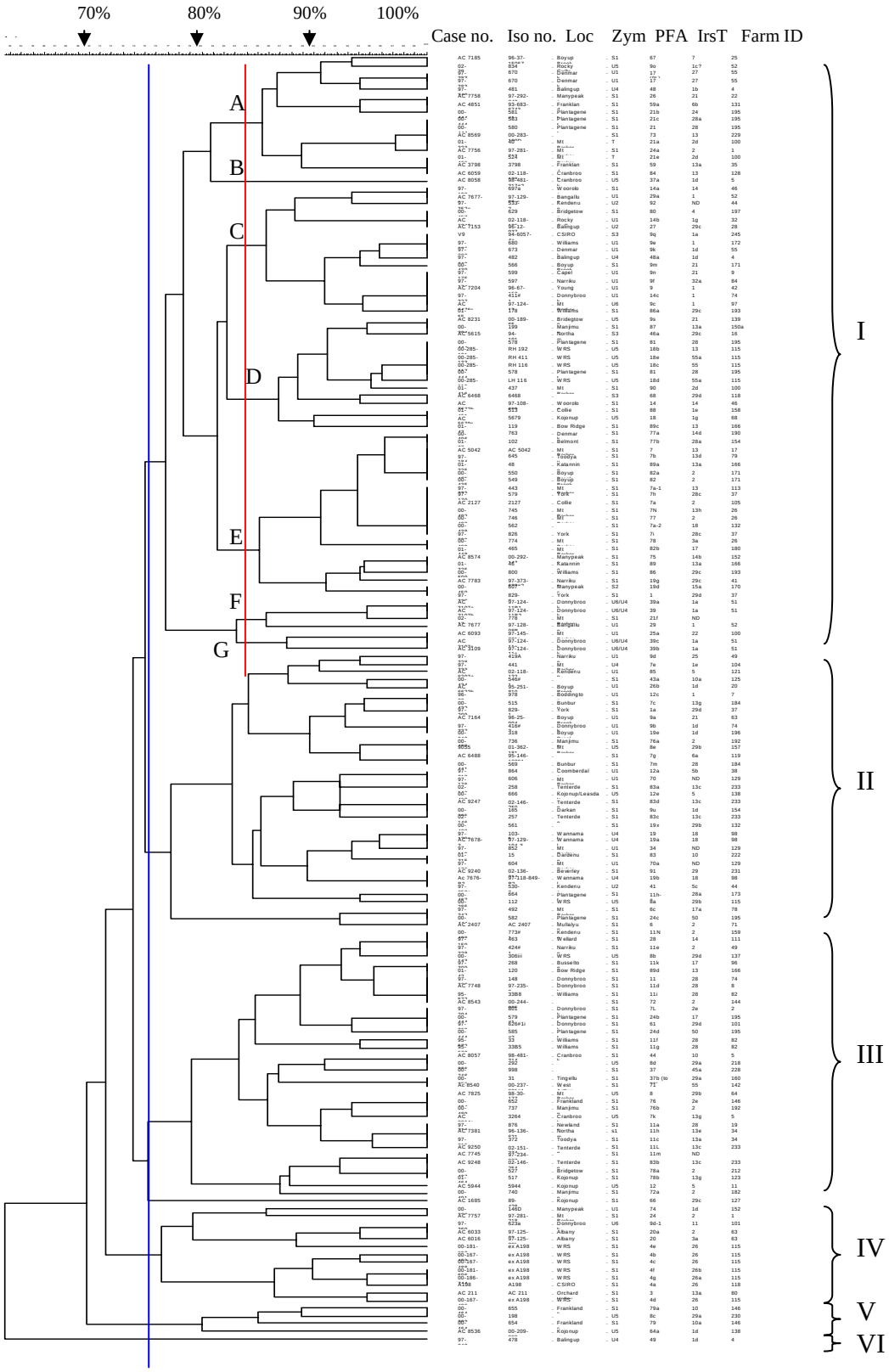


Figure 4-1. Dendrogram showing genetic relatedness of standard PFA types

The case number, isolate identification code, zymogram profile, protease thermostability (S or U), and farm identification number are indicated. Clusters I-VI are indicated at a level of 75% genetic relatedness.

4.3.1.2 Genetic relatedness of protease thermostable and unstable isolates in WA

Amongst the S strains (n = 516), 124 PFA types were identified, and these were grouped into 48 PFA clonal groups. The genetic relatedness of S strains was 0.97 as calculated by Simpson's index of genetic diversity (S = 516; N(N-1) = 39402; Σ*nj*(*nj*-1) = 1176). A simple diversity ratio based on the number of isolates and the number of molecular types was 1:4.2. For the PFA clonal groups, Simpson's index of diversity was 0.85, and the simple diversity ratio of clonal groups compared to the number of S strains was 1:4.2 (Table 4-2).

Table 4-2. Genetic relatedness of protease thermostable and unstable *D. nodosus* isolates in WA

Data	S	U
PFGE		
No. of isolates	516	219
No. of PFA types	124	81
No. of clonal groups	48	33
Overall diversity ratio of PFA types	1:4.2	1:2.7
Overall diversity ratio of clonal groups	1:10.8	1:6.3
Simpson's index of diversity for molecular type	0.97	0.97
Simpson's index of diversity for clonal type	0.85	0.97
IRS-PCR		
No. of isolates	429	187
No. of IrsT Types	60	40
No. of clonal groups	28	22
Overall diversity ratio of IrsT types	1:7.1	1:4.7
Overall diversity ratio of clonal types	1:15.3	1:8.5
Simpson's index of diversity for molecular type	0.94	0.92
Simpson's index of diversity for clonal type	0.84	0.73

IrsT type = molecular type identified using primer PXT and the IRS-PCR method; No. = number; PFA type = the molecular type identified using the restriction enzyme *Apa*I and the PFGE method; S = protease thermostable isolate; U = protease thermolabile isolate.

For the U strains (n = 219), there were 81 PFA types identified and these grouped into 33 PFA clonal groups. The simple diversity ratio of PFA molecular types for U strains was 1:2.7, and 1:6.3 for clonal groups. Simpson's index of diversity for U strains was 0.97 for both PFA molecular types and PFA clonal groups (Table 4-2).

Amongst the S strains (n = 429) typed by IRS-PCR using primer PXT, there were 60 IrsT types that were grouped into 28 clonal groups. This produced a simple diversity ratio of 1:7.1 for all IrsT types, and a ratio of 1:15.3 for clonal groups of S strains. Simpson's index for diversity was 0.94 for IrsT types and 0.84 for clonal groups (Table 4-2).

There were 187 U strains that were typed by IRS-PCR and primer PXT, and 40 IrsT types were identified, with 22 clonal groups. The overall simple diversity ratio was 1:4.7 for IrsT types and 1:8.5 for clonal groupings. The Simpson's index of diversity for IrsT molecular types was 0.92 and the diversity for IrsT clonal groups was 0.73 (Table 4-2).

Dendrogram constructed from selected standard PFA types for S strains

A dendrogram of 99 standard PFA molecular types for the S strains was prepared (Figure 4-2), which indicated that the strains were similar at a level of 71% to 100%. At a level of 79% the molecular types grouped into seven clusters (I-VII) (Figure 4-2).

Cluster I harboured 29 PFA molecular types that represented 13 PFA clonal groups that were related to each other at 80% similarity. The predominant PFA clonal group was PFA 11, with 44.8% (n = 13) of the molecular types belonging to this clonal group. An association was noted between clonal group PFA 11 and clonal groups IrsT 13 and IrsT 28. Of the 13 molecular types located in clonal group PFA 11, there were three IrsT 13 molecular types (13a, 13c,13e) and six IrsT 28 molecular types.

Cluster II harboured two isolates, the type strain A198 and a WA strain isolated in 1989. The two strains were related to each other at 83%.

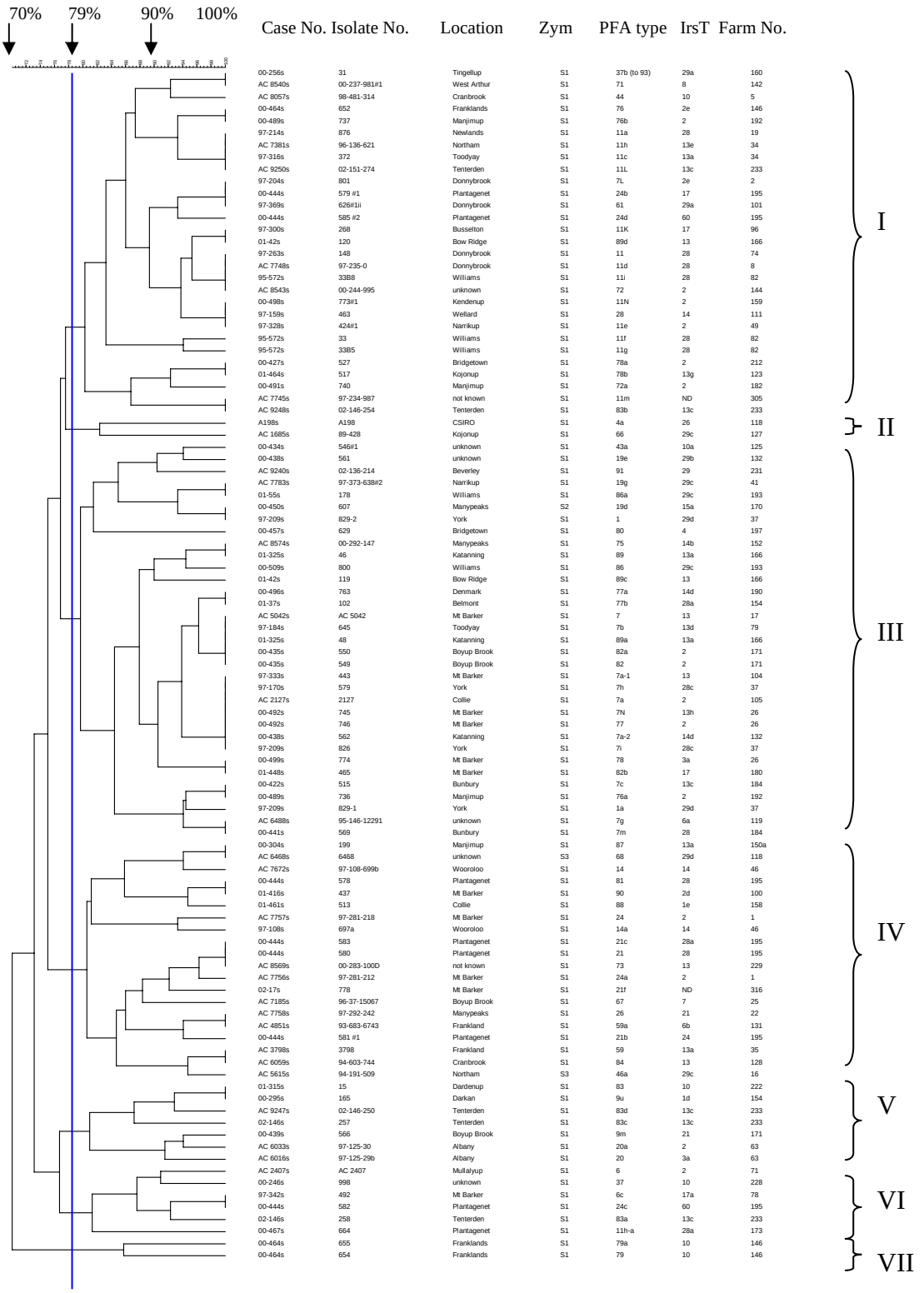
Cluster III harboured 33 PFA molecular types, and the predominant clonal group was PFA 7, with 33.3% (n = 11) of strains belonging to this group. These strains were similar at a level of 84% on the dendrogram. The 11 molecular types associated with PFA 7 clonal group were associated with five molecular types from IrsT 13 clonal group. Three molecular types from PFA clonal group 7 had a corresponding IrsT type of 28. The remaining three PFA 7 molecular types had corresponding IrsT types of IrsT 2, 6a and 14d (Figure 4-2).

Cluster IV harboured 20 strains representing 14 PFA clonal groups. Two strains from clonal group PFA 24 were found in this cluster, but clusters I and VI also harboured one strain each from this clonal group.

Cluster V harboured seven strains with a predominance (42.8%, n = 3) belonging to clonal group PFA 83. One strain from this clonal group was found in cluster VI.

Cluster VI harboured six strains from five clonal groups, and cluster VII harboured two strains both from clonal group PFA 79.

A dendrogram based on the IrsT types was not constructed.



Case No.	Isolate No.	Location	Zym	PFA type	IrsT	Farm No.	
00-256s	31	Tingellup	S1	37b (to 93)	29a	160	I
AC 8540s	00-237-981#1	West Arthur	S1	71	8	142	I
AC 8057s	98-481-314	Cranbrook	S1	44	10	5	I
00-464s	652	Franklands	S1	76	2e	146	I
00-489s	737	Manjimup	S1	76b	2	192	I
97-214s	876	Newlands	S1	11a	28	19	I
AC 7381s	96-136-621	Northam	S1	11h	13e	34	I
97-316s	372	Toodyay	S1	11c	13a	34	I
AC 9250s	02-151-274	Tenterden	S1	11L	13c	233	I
97-204s	801	Donnybrook	S1	7L	2e	2	I
00-444s	579 #1	Plantagenet	S1	24b	17	195	I
97-369s	626#1ii	Donnybrook	S1	61	29a	101	I
00-444s	585 #2	Plantagenet	S1	24d	60	195	I
97-300s	268	Busselton	S1	11K	17	96	I
01-42s	120	Bow Ridge	S1	89d	13	166	I
97-263s	148	Donnybrook	S1	11	28	74	I
AC 7748s	97-235-0	Donnybrook	S1	11d	28	8	I
95-572s	33B8	Williams	S1	11i	28	82	I
AC 8543s	00-244-995	unknown	S1	72	2	144	I
00-498s	773#1	Kendenup	S1	11N	2	159	I
97-159s	463	Wellard	S1	28	14	111	I
97-328s	424#1	Narrikup	S1	11e	2	49	I
95-572s	33	Williams	S1	11f	28	82	I
95-572s	33B5	Williams	S1	11g	28	82	I
00-427s	527	Bridgetown	S1	78a	2	212	I
01-464s	517	Kojonup	S1	78b	13g	123	I
00-491s	740	Manjimup	S1	72a	2	182	I
AC 7745s	97-234-987	not known	S1	11m	ND	305	I
AC 9248s	02-146-254	Tenterden	S1	83b	13c	233	I
A198s	A198	CSIRO	S1	4a	26	118	II
AC 1685s	89-428	Kojonup	S1	66	29c	127	II
00-434s	546#1	unknown	S1	43a	10a	125	III
00-438s	561	unknown	S1	19e	29b	132	III
AC 9240s	02-136-214	Beverley	S1	91	29	231	III
AC 7783s	97-373-638#2	Narrikup	S1	19g	29c	41	III
01-55s	178	Williams	S1	86a	29c	193	III
00-450s	607	Manypeaks	S2	19d	15a	170	III
97-209s	829-2	York	S1	1	29d	37	III
00-457s	629	Bridgetown	S1	80	4	197	III
AC 8574s	00-292-147	Manypeaks	S1	75	14b	152	III
01-325s	46	Katanning	S1	89	13a	166	III
00-509s	800	Williams	S1	86	29c	193	III
01-42s	119	Bow Ridge	S1	89c	13	166	III
00-496s	763	Denmark	S1	77a	14d	190	III
01-37s	102	Belmont	S1	77b	28a	154	III
AC 5042s	AC 5042	Mt Barker	S1	7	13	17	III
97-184s	645	Toodyay	S1	7b	13d	79	III
01-325s	48	Katanning	S1	89a	13a	166	III
00-435s	550	Boyup Brook	S1	82a	2	171	III
00-435s	549	Boyup Brook	S1	82	2	171	III
97-333s	443	Mt Barker	S1	7a-1	13	104	III
97-170s	579	York	S1	7h	28c	37	III
AC 2127s	2127	Collie	S1	7a	2	105	III
00-492s	745	Mt Barker	S1	7N	13h	26	III
00-492s	746	Mt Barker	S1	77	2	26	III
00-438s	562	Katanning	S1	7a-2	14d	132	III
97-209s	826	York	S1	7i	28c	37	III
00-499s	774	Mt Barker	S1	78	3a	26	III
01-448s	465	Mt Barker	S1	82b	17	180	III
00-422s	515	Bunbury	S1	7c	13c	184	III
00-489s	736	Manjimup	S1	76a	2	192	III
97-209s	829-1	York	S1	1a	29d	37	III
AC 6488s	95-146-12291	unknown	S1	7g	6a	119	III
00-441s	569	Bunbury	S1	7m	28	184	III
00-304s	199	Manjimup	S1	87	13a	150a	IV
AC 6468s	6468	unknown	S3	68	29d	118	IV
AC 7672s	97-108-699b	Wooroloo	S1	14	14	46	IV
00-444s	578	Plantagenet	S1	81	28	195	IV
01-416s	437	Mt Barker	S1	90	2d	100	IV
01-461s	513	Collie	S1	88	1e	158	IV
AC 7757s	97-281-218	Mt Barker	S1	24	2	1	IV
97-108s	697a	Wooroloo	S1	14a	14	46	IV
00-444s	583	Plantagenet	S1	21c	28a	195	IV
00-444s	580	Plantagenet	S1	21	28	195	IV
AC 8569s	00-283-100D	not known	S1	73	13	229	IV
AC 7756s	97-281-212	Mt Barker	S1	24a	2	1	IV
02-17s	778	Mt Barker	S1	21f	ND	316	IV
AC 7185s	96-37-15067	Boyup Brook	S1	67	7	25	IV
AC 7758s	97-292-242	Manypeaks	S1	26	21	22	IV
AC 4851s	93-683-6743	Frankland	S1	59a	6b	131	IV
00-444s	581 #1	Plantagenet	S1	21b	24	195	IV
AC 3798s	3798	Frankland	S1	59	13a	35	IV
AC 6059s	94-603-744	Cranbrook	S1	84	13	128	IV
AC 5615s	94-191-509	Northam	S3	46a	29c	16	IV
01-315s	15	Dardenup	S1	83	10	222	V
00-295s	165	Darkan	S1	9u	1d	154	V
AC 9247s	02-146-250	Tenterden	S1	83d	13c	233	V
02-146s	257	Tenterden	S1	83c	13c	233	V
00-439s	566	Boyup Brook	S1	9m	21	171	V
AC 6033s	97-125-30	Albany	S1	20a	2	63	V
AC 6016s	97-125-29b	Albany	S1	20	3a	63	V
AC 2407s	AC 2407	Mullalyup	S1	6	2	71	V
00-246s	998	unknown	S1	37	10	228	VI
97-342s	492	Mt Barker	S1	6c	17a	78	VI
00-444s	582	Plantagenet	S1	24c	60	195	VI
02-146s	258	Tenterden	S1	83a	13c	233	VI
00-467s	664	Plantagenet	S1	11h-a	28a	173	VI
00-464s	655	Franklands	S1	79a	10	146	VII
00-464s	654	Franklands	S1	79	10	146	VII

Figure 4-2. Dendrogram showing genetic relatedness of standard PFA types obtained from protease thermostable (S) strains found in WA

The case number, isolate identification code, zymogram profile, and farm identification number are indicated. Numbers I-IV indicate cluster number, and A, B indicate groups identified within cluster II.

Dendrogram constructed from selected standard PFA types for U strains

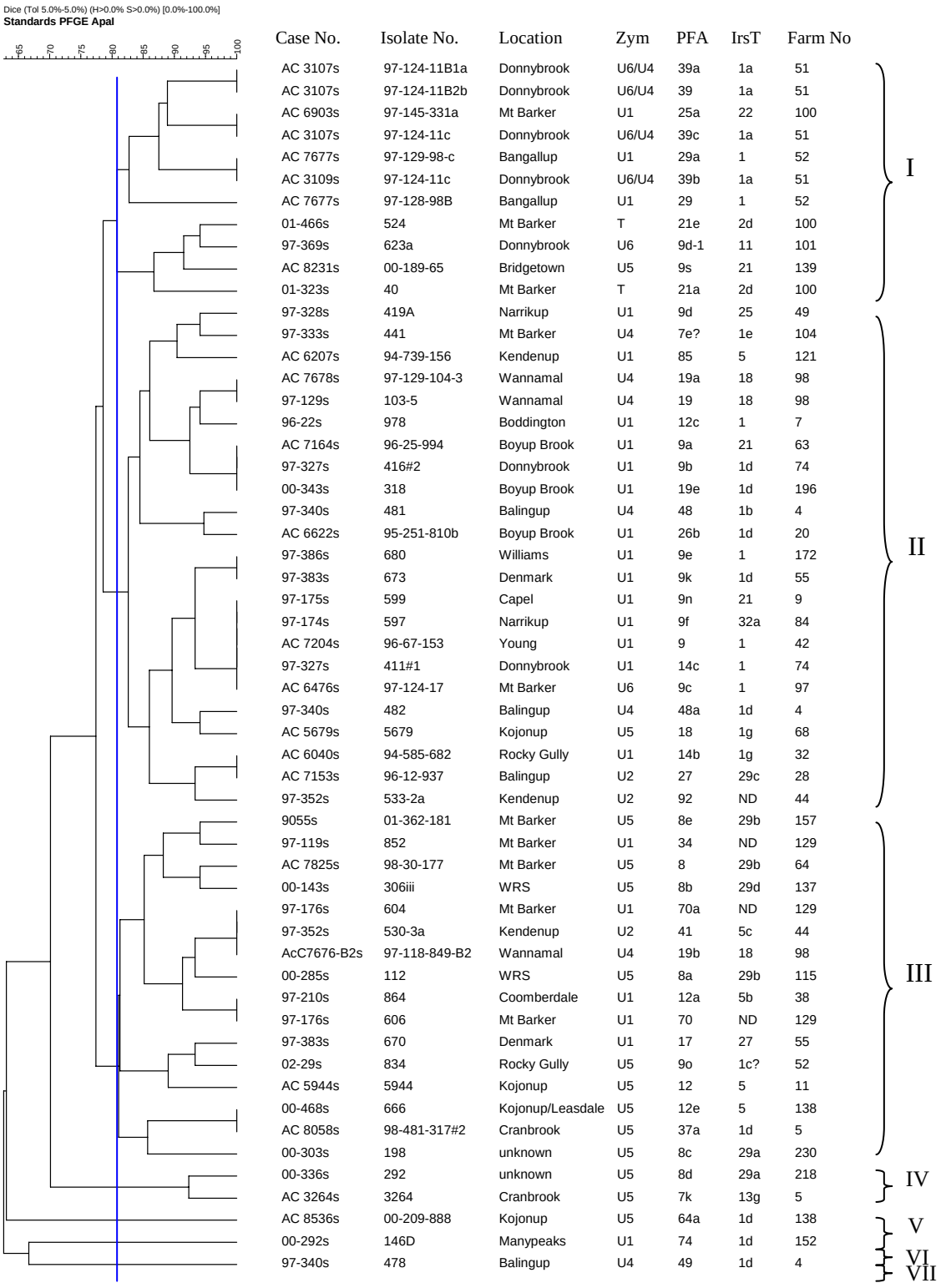
A dendrogram constructed using 55 standard PFA molecular types for the U strains indicated that these strains were 64% to 100% similar (Figure 4-3). Seven clusters (I-VII) were identified at a level of 80% similarity.

Cluster I harboured 11 strains that represented five PFA clonal groups, which were 80% related to each other. Strains from clonal group PFA 39 predominated with 36.4% (n = 4) of isolates belonging to this clonal group. Isolates from zymogram types U1, U6, U6/U4 and T strains occurred in this cluster. One U6 strain was identified in cluster II.

Cluster II harboured 23 strains from 11 PFA clonal groups that were 82% related. Strains from clonal group PFA 9 predominated with 39.1% (n = 9) of strains occurring in this group. All except one isolate in the clonal group PFA 9 in this cluster were identified as U1 strains.

Cluster III harboured 16 strains from nine PFA clonal groups that were related at a level of 85% similarity. The predominant clonal group was PFA 8 with 31.2% (n = 5) of strains belonging to this clonal group. Strains with U5 zymogram profile predominated in cluster III with 56.2% (n = 9) of strains having this zymogram profile. One isolate identified as U5 was found in each of clusters I and II while the sole isolate in cluster V was identified as U5. The only two strains in cluster IV were both identified as U5.

Clusters V, VI and VII each had only one isolate in the cluster.



Case No.	Isolate No.	Location	Zym	PFA	IrsT	Farm No	
AC 3107s	97-124-11B1a	Donnybrook	U6/U4	39a	1a	51	I
AC 3107s	97-124-11B2b	Donnybrook	U6/U4	39	1a	51	
AC 6903s	97-145-331a	Mt Barker	U1	25a	22	100	
AC 3107s	97-124-11c	Donnybrook	U6/U4	39c	1a	51	
AC 7677s	97-129-98-c	Bangallup	U1	29a	1	52	
AC 3109s	97-124-11c	Donnybrook	U6/U4	39b	1a	51	
AC 7677s	97-128-98B	Bangallup	U1	29	1	52	
01-466s	524	Mt Barker	T	21e	2d	100	
97-369s	623a	Donnybrook	U6	9d-1	11	101	
AC 8231s	00-189-65	Bridgetown	U5	9s	21	139	
01-323s	40	Mt Barker	T	21a	2d	100	
97-328s	419A	Narrikup	U1	9d	25	49	II
97-333s	441	Mt Barker	U4	7e?	1e	104	
AC 6207s	94-739-156	Kendenup	U1	85	5	121	
AC 7678s	97-129-104-3	Wannamal	U4	19a	18	98	
97-129s	103-5	Wannamal	U4	19	18	98	
96-22s	978	Boddington	U1	12c	1	7	
AC 7164s	96-25-994	Boyup Brook	U1	9a	21	63	
97-327s	416#2	Donnybrook	U1	9b	1d	74	
00-343s	318	Boyup Brook	U1	19e	1d	196	
97-340s	481	Balingup	U4	48	1b	4	
AC 6622s	95-251-810b	Boyup Brook	U1	26b	1d	20	
97-386s	680	Williams	U1	9e	1	172	
97-383s	673	Denmark	U1	9k	1d	55	
97-175s	599	Capel	U1	9n	21	9	
97-174s	597	Narrikup	U1	9f	32a	84	
AC 7204s	96-67-153	Young	U1	9	1	42	
97-327s	411#1	Donnybrook	U1	14c	1	74	
AC 6476s	97-124-17	Mt Barker	U6	9c	1	97	
97-340s	482	Balingup	U4	48a	1d	4	
AC 5679s	5679	Kojonup	U5	18	1g	68	
AC 6040s	94-585-682	Rocky Gully	U1	14b	1g	32	
AC 7153s	96-12-937	Balingup	U2	27	29c	28	
97-352s	533-2a	Kendenup	U2	92	ND	44	
9055s	01-362-181	Mt Barker	U5	8e	29b	157	III
97-119s	852	Mt Barker	U1	34	ND	129	
AC 7825s	98-30-177	Mt Barker	U5	8	29b	64	
00-143s	306iii	WRS	U5	8b	29d	137	
97-176s	604	Mt Barker	U1	70a	ND	129	
97-352s	530-3a	Kendenup	U2	41	5c	44	
AcC7676-B2s	97-118-849-B2	Wannamal	U4	19b	18	98	
00-285s	112	WRS	U5	8a	29b	115	
97-210s	864	Coomberdale	U1	12a	5b	38	
97-176s	606	Mt Barker	U1	70	ND	129	
97-383s	670	Denmark	U1	17	27	55	
02-29s	834	Rocky Gully	U5	9o	1c?	52	
AC 5944s	5944	Kojonup	U5	12	5	11	
00-468s	666	Kojonup/Leasdale	U5	12e	5	138	
AC 8058s	98-481-317#2	Cranbrook	U5	37a	1d	5	
00-303s	198	unknown	U5	8c	29a	230	
00-336s	292	unknown	U5	8d	29a	218	IV
AC 3264s	3264	Cranbrook	U5	7k	13g	5	
AC 8536s	00-209-888	Kojonup	U5	64a	1d	138	V
00-292s	146D	Manypeaks	U1	74	1d	152	VI
97-340s	478	Balingup	U4	49	1d	4	VII

Figure 4-3. Dendrogram showing genetic similarity of 55 standard PFA types obtained from protease thermolabile (U) strains found in WA

The case number, isolate identification code, zymogram profile, and farm identification numbers are indicated. Numbers I-VII indicate cluster number.

4.3.2 Molecular types and genetic relatedness identified by IRS-PCR

A subgroup of 677 isolates from the 303 properties throughout Australia was tested by IRS-PCR, and 94 IrsT types were obtained, indicating a diversity ratio of 1:7.2 (Table 4-1). The IrsT types could be further grouped into 48 clonal groups. The diversity ratio based on clonal groups was 1:14. The diversity ratio of IrsT types in WA was 1:8 where 616 isolates were tested, and produced 77 IrsT types. A diversity ratio of 1:17 was obtained for the 36 clonal groups identified in WA.

For the calculation of Simpson's level of diversity for the IrsT types from WA, N = 616, N(N-1) = 378840, s = 77, and $\Sigma nj(nj\text{-}1) = 17408$. This produced a level of genetic diversity of 0.95 (Table 4-1).

The Simpson genetic diversity based on IrsT clonal groups in WA was calculated using N(N-1) = 378840, and $\Sigma nj(nj\text{-}1) = 47284$, which produced a diversity of 0.87 (Table 4-1).

For IrsT types from Australia, N = 677, N(N-1) = 457652, s = 94, and $\Sigma nj(nj\text{-}1) = 18302$, which gave a Simpson's diversity of 0.96 (Table 4-1).

The genetic diversity of clonal IrsT groups throughout Australia was calculated using N(N-1) = 457652, and $\Sigma nj(nj\text{-}1) = 51898$, which gave a diversity of 0.89 (Table 4-1).

A dendrogram of IrsT types was not constructed.

4.3.3 Distribution of clonal groups throughout Australia

The distribution of clonal groups over the four States examined is presented in Table 4-3. Of the total 82 PFA clonal groups identified, three (PFA 7, 9 11) were common between all four Australian States.

In WA, PFA 46 clonal group was not detected in this collection of isolates, but this clonal group was found in NSW, SA and VIC.

Table 4-3. Clonal types common between States

	PFA	IrsT
WA	3, 6, 7, 9, 11	1, 2, 3, 5, 13, 14, 15, 23, 25, 29, 60
NSW	3, 6, 7, 9, 11, 46	1, 2, 13, 23, 29, 39
SA	6, 7, 9, 11, 46	1, 5, 13, 15, 29, 60
VIC	3, 7, 9, 11, 46	1, 2, 3, 5, 13, 14, 25, 29, 39
Total Types	214	94
Total No of Clonal groups	82	48

IrsT = molecular type using primer PXT and method IRS-PCR; NSW = New South Wales, PFA = molecular type using restriction enzyme *Apa*I and PFGE method; SA = South Australia; VIC = Victoria; No. = number; WA = Western Australia.

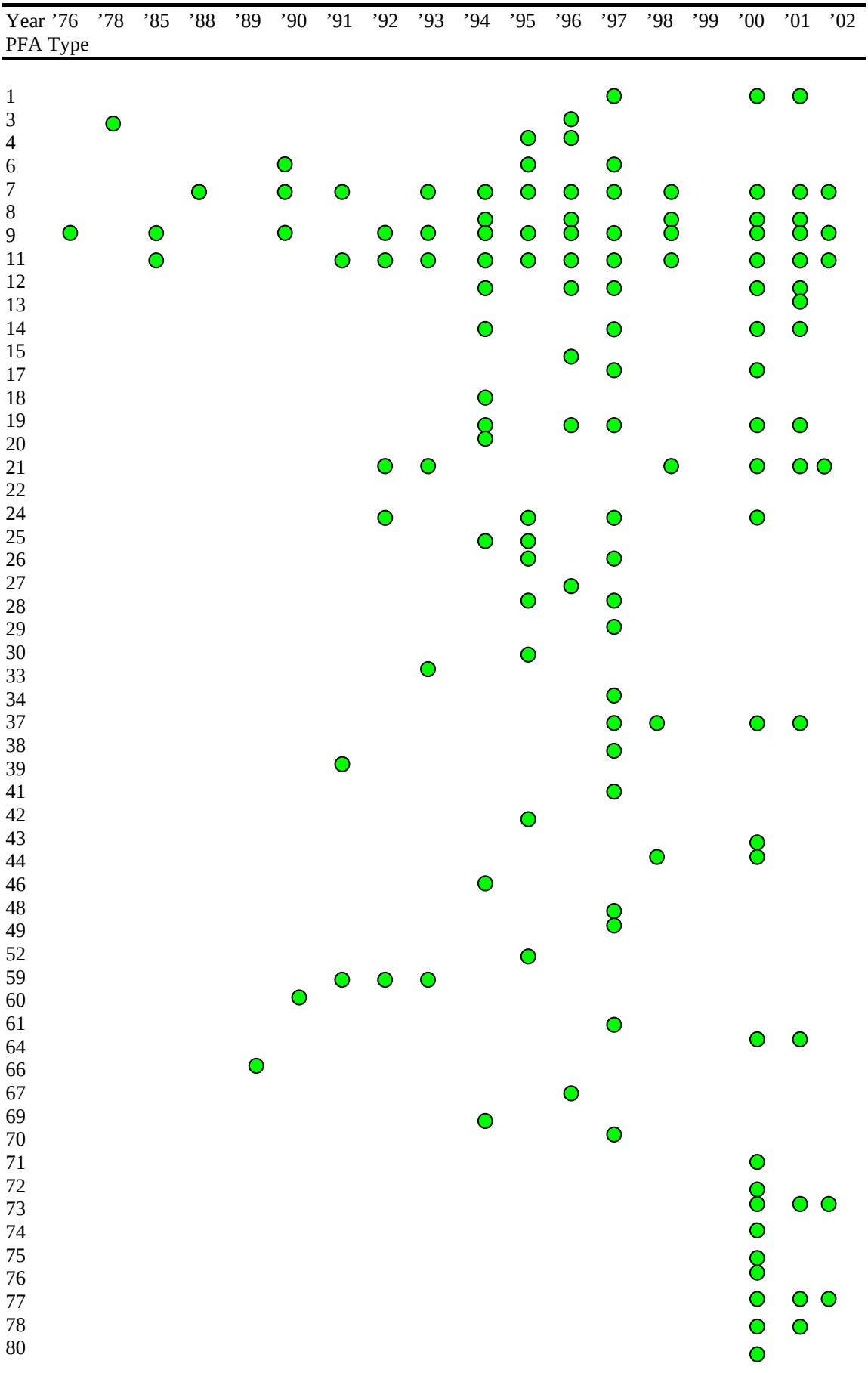
Clonal groups PFA 3 and PFA 6 were common amongst a majority of States (Table 4-3). In SA, 15 PFA types were identified from 21 isolates tested, and five of these belonged to clonal groups that were common between the other States. In NSW, 14 PFA types were identified from the 16 isolates tested, and six of these were from clonal groups common throughout Australia. Similarly, in VIC where 24 isolates were tested and 21 PFA types identified, five of these belonged to common clonal groups.

The results with the IRS-PCR method confirmed the findings of the PFGE typing, in that clonal groups common between the Australian States were identified (Table 4-3). The 94 IrsT types identified from the 677 isolates tested could be grouped within 48 clonal groups. As was found with the PFGE typing, and despite the small numbers of strains tested from NSW, SA and VIC, the IRS-PCR identified three IrsT clonal types common in all four States, and 11 clonal types that were common amongst two or more States.

4.3.3.1 Persistence of clonal groups in WA throughout a 26 year period

The distribution of PFA clonal groups from 91 farms in WA over the period 1976 to 2002 is shown in Table 4-4, where each spot in the table represents one isolate from a clonal PFA group.

Table 4-4. Clonal groups isolated on farms between 1976 and 2002



(Table 4-4 continued)

Year	'76	'78	'85	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02
PFA Type																		
81																●		
82																●	●	●
83										●							●	
84										●								
85										●								
86																●	●	
87									●							●	●	
88																●	●	●
89																	●	
90																	●	
91																		●
Total No. of isolates tested from each year																		
	1	1	4	1	1	20	13	17	13	35	42	79	125	19	0	176	131	57
Total No. of clonal groups identified																		
	1	1	2	1	1	4	4	5	7	15	12	11	21	7	0	29	22	9
Total No. of farms																		
	1	1	4	1	1	20	7	6	10	24	21	21	47	8	0	58	58	23
No. of S strains																		
	0	1	3	1	1	20	6	15	9	17	25	20	69	9	0	151	116	53
No. of U strains																		
	1	0	1	0	0	0	7	2	4	18	17	59	56	10	0	25	15	4
Genetic diversity ratio of clonal groups per year of X:1																		
X =	1	1	2	1	1	5	3.25	3.4	1.8	2.3	3.5	7.2	5.9	2.7	0	6.1	5.9	6.3

Some isolates from some clonal groups were present for the entire period, whereas other clonal groups were not detected in all years. Clonal group PFA 7 was detected in 12 of the years between 1985 and 2002, and clonal group PFA 11 was detected in 12 years from 1985 onwards. Isolates belonging to clonal group PFA 9 were detected in 13 of the years between 1976 and 2002. Other clonal groups such as PFA 3, 15, 60, 66, 84 and 85 were detected in the 1990s, but were not seen in recent years.

The simple genetic diversity ratio of number of isolates compared to the number of molecular types in a single year ranged from 1:1 to 7.2:1 (Table 4-4). In years 1990, '94, '95, '96, '97, 2000, 2001, 2002, where 20 isolates or more were examined from 20 or more farms, the diversity ratio ranged from 2.3:1 to 6.3:1. In 1996, a total of 79 isolates were typed, and were classified into 11 PFA clonal groups to give a diversity ratio of 7.2:1. In later years (2000 and 2001) where 176 and 131 isolates were typed, diversity ratios of 6.1:1 and 5.9:1, respectively, were obtained. For the isolates typed in 1997 and 2001, a genetic diversity ratio of 5.9:1 was calculated for each year. In 1997, 125 isolates were typed and these were divided into 69 S strains and 56 U strains,

whereas in 2001, 131 isolates were examined and were composed of 116 S strains and 15 U strains.

The relationship between virulent (S) strains and benign (U) strains and their molecular type is investigated further in chapter 6.

4.3.4 Relationship of PFGE type to IRS-PCR type

The genetic type obtained by PFGE was compared to the genetic type obtained from the IRS-PCR. When comparing the typing results between isolates, a number of isolates that had the same PFA type had the same IrsT type. Some isolates had the same PFA molecular type, but had different IrsT types. Table 4-5 presents the data showing the relationship between the results obtained with the PFGE method and the molecular type obtained with the IRS-PCR method, using PFA 7 clonal group as an example. In WA, 134 or 18.2% of isolates belonged to clonal group PFA 7, which was composed of 13 PFA molecular types (PFA 7, 7a, 7b, 7c, 7e, 7f, 7g, 7h, 7i, 7k, 7L, 7m, 7N). The predominant type was PFA 7 with 50 (37.3%) isolates. For the isolates with the PFA 7 molecular type there were seven corresponding IrsT types (IrsT 2, 9, 13, 13a, 13g, 13h and 14). Some of these IrsT types also occurred in other molecular types within the clonal group PFA 7. For example, IrsT 13 not only occurred in PFA 7 molecular type but also occurred in PFA 7a, and 7c molecular types.

A cluster of isolates that was identified as PFA 7 and IrsT 13 was detected on 19 properties from 1990 to 2001. Although 15 of these farms were involved in one outbreak originating from Farm 17 in 1990, this cluster also occurred in 2000 and 2001 on farms apparently uninvolved in the 1990 outbreak. This cluster persisted on the source farm (Farm 17) for 11 years. Another cluster type on the source farm 17, cluster PFA 7 and IrsT 14, originally identified in 1991, was also found on three other farms in 2001 and 2002. The results of molecular typing from these farms and the application to epidemiology are presented in chapter 5.

Another cluster of PFA 7 and IrsT 13h, which comprised three isolates or 6% of the clonal group PFA 7, was found on three farms (41, 175 and 214) in 1997, 2001 and 2002. Other clusters only contained one isolate from one farm.

Table 4-5. Relationship between clonal group PFA 7 and IRS-PCR type

PFA Type	No. of isolates	IrsT Type	No. of isolates (%)	No. of properties
7	50	2	2 (4)	2
		9	1 (2)	1
		13	27 (54)	19
		13a	4 (8)	3
		13g	1 (2)	1
		13h	3 (6)	3
		14	4 (8)	4
7a	22	2	3 (13.6)	3
		13	2 (9)	2
		13g	4 (18)	3
		13h	4 (18)	3
		14c	2 (9)	2
		14d	1 (4.5)	1
		28a	1	1
7b	5	13d	2 (40)	1
		17	1 (20)	1
		28	1 (20)	1
7c	20	5a	1 (5)	1
		13	6 (30)	1
		13c	2 (10)	2
		13g	6 (30)	2
		28	2 (10)	2
		28a	1	1
7e	2	13c	1	1
7f	19	2	3 (15.8)	2
		2e	3 (15.8)	1
		13c	2 (10.5)	1
		13g	2 (10.5)	2
		14	2 (10.5)	1
		28a	1 (5.2)	1
		50b	1 (5.2)	1
7g	1	6a	1 (100)	1
7h	4	28	1 (25)	1
		28c	3 (75)	3
7i	3	2	2 (66)	2
		28c		1
7k	1	13g	1 (100)	1
7L	4	2	1 (25)	1
		2e	2 (50)	2
7m	2	28	1 (50)	1
		29a	1 (50)	1
7N	1	13h	1 (100)	1

IrsT Type = molecular type obtained with IRS-PCR method using primer PXT; No. = number; PFA Type = molecular type obtained with restriction enzyme *Apa*I and PFGE method.

4.4 DISCUSSION

Typing of bacterial strains for epidemiological purposes can be performed by a number of different methods. These methods need to be efficient, reproducible and provide sensitive discrimination between isolates. The ability of a typing method to discriminate between strains is determined by the number of types identified by the typing method and the relative frequencies of the types. The discriminatory ability of a typing method can be measured using Simpson's index of diversity. Calculation of the index of diversity should only be done on a large database of strains, as the equation does not correct for a small population size. A typing method that has an index of greater than 0.90 offers good discrimination between strains (Hunter and Gaston, 1988).

In this study on isolates of *D. nodosus* the discriminatory ability of the PFGE (D = 0.98) and IRS-PCR (D = 0.95) methods were both very high, with the PFGE method being more discriminatory than the IRS-PCR. The diversity index for WA strains compared to the diversity index for all Australian strains was almost the same, and this was due to the large number of strains typed from WA, with only a small number typed from the other States. Typing by the PFGE method means that in WA the probability of two strains having different molecular types is 98%, whereas with the IRS-PCR method the probability is 95%. The numbers of strains that were tested from each of the other Australian States were too small to allow an accurate calculation for genetic diversity for each State.

The simple diversity ratio calculated for both methods also showed that isolates of *D. nodosus* were genetically diverse. PFGE was more sensitive at detecting genetic differences, as indicated by the diversity ratio of 1:4 for molecular types achieved with this technique compared to a ratio of 1:10 with the IRS-PCR method. Therefore, the PFGE method is 2.5 times more likely to detect a genetic difference between two isolates than the IRS-PCR method. This assessment agrees with numerous studies that also indicate that PFGE is the molecular typing method of choice for sensitive detection of genetic change (Saulnier *et al.*, 1993; Skov *et al.*, 1995a; Feizabadi *et al.*, 1996). However, it is recognised that the IRS-PCR technique offers almost as good a differentiation as PFGE, but has the advantage of being easier to use, does not require specialised equipment and is more rapid than PFGE if DNA is extracted direct from bacterial cells (Riffard *et al.*, 1998).

The molecular types were grouped into clonal groups based on their similarity of restriction bands, as defined by Tenover *et al*. 1995 and explained in chapter 2. Simpson's index of diversity for clonal groupings for the PFGE and IRS-PCR methods reflected the results obtained for individual molecular types. Clonal genetic diversity was 90% for PFGE and 87% for IRS-PCR, indicating that less diversity was seen at the level of clonal groupings. A lower level of diversity at the clonal level would be expected because, by definition, clonal groups encompass a number of minor molecular changes seen between genetically similar isolates.

The extent of the distribution of common molecular types throughout Australia can be determined from the results. Despite testing only a small number of isolates from NSW, VIC and SA it is clear that certain groups of the organism are distributed throughout the Australian States, as indicated by the clonal groups that are common between States. Three clonal groups (PFA 7, 9, 11) were common between States, despite only a small number of isolates being tested from NSW, VIC and SA. Reports had suggested that footrot came into Australia soon after British colonisation, with sheep imported by the early settlers (Mohler and Washburn, 1905). The fact that isolates are clonal throughout the Australian States would validate this report and suggest a common ancestor. A different scenario could suggest that the presence of major clonal groups common to the various States might be due to independent import of these clonal groups to the individual States followed by transmission of the clones between States.

Visual examination of the DNA fingerprints of the isolates from clonal groups PFA 7 and PFA 11 show that they are very similar in appearance because of the pattern of the distribution of the DNA bands on the gel. It is possible (but untested) that one clonal type originally derived from the other. The fingerprint of clonal group PFA 9 may also have originated from either PFA 7 or 11 clonal groups because of similarities in the banding pattern on the gel. Other fingerprint patterns from other clonal groups are visually quite different in pattern and spread of the DNA bands on the gel, which suggests that they were not derived from any of the three major clonal groups. These visual interpretations would suggest that clonal groups PFA 7, 9 and 11 had a common ancestor many years ago, and that geographical spread and genetic change has led to the rise of these three main clonal groups. This idea is purely conjecture from visual interpretation, but may be able to be tested in the future by methods such as multilocus sequence typing.

When trying to interpret the origin of the genetic diversity observed in Australian isolates, it would be instructive to examine *D. nodosus* strain diversity in other countries such as Spain, Portugal and France where the disease has been present for more than 200 years. Investigation of the genetic diversity in these isolates would determine whether isolates from these countries have major clonal groups and a high level of diversity similar to Australian isolates.

The three PFA clonal groups (PFA 7, 9, 11) that were common between WA and other States exist despite WA having had an eradication program for protease thermostable strains since 1974, and despite strict border quarantine regulations. This suggests that these molecular types were present in WA before the enforcement of the eradication program, and that these types have continued to persist since that time. Unfortunately, there is no data available to investigate the flow of the spread of isolates throughout Australia.

In Western Australia these same three common PFA clonal groups that were detected in all States had persisted on farms in WA for over a 20-year period despite the eradication program. This finding may result from the fact that isolates from these clonal groups are the most common in this State, or it may be that genetic types associated with these clonal groups have an ability to persist longer than other genetic types. Another factor that may have resulted in the high numbers of these genetic types in WA might be due to an outbreak farm selling infected sheep to a large number of farms. If these farmers had then continued to on-sell infected sheep before quarantine measures were put in place, especially before the introduction of the eradication program in 1974, then this would have led to the transmission of these strains throughout WA. Whatever the mechanism for the spread of strains, the fact remains that of all the isolates tested half of these belonged to one of these three clonal groups.

In the isolates from WA, equal numbers of isolates from each year were not tested. The isolates were selected from the culture collection, or from current diagnostic submissions, or were selected to investigate a footrot outbreak, and did not reflect a true random sample. Also, the isolates that were selected for molecular typing after the year 2000 were biased towards protease thermostable (S) strains. This was due to the Department of Agriculture’s policy to only type strains on quarantined farms. Despite the bias present in the collection of isolates, the results for the assessment of genetic

diversity by both the simple ratio and the Simpson index indicate that *D. nodosus* isolates are genetically diverse. A range in the genetic diversity ratio was seen in the isolates tested from different years in WA. This ratio did not appear to be affected by the total number of isolates typed in a single year in that the greatest number of isolates examined did not produce a greater diversity ratio. The number of protease thermostable (S) strains compared to the number of protease heat labile strains (U) examined also did not affect the diversity ratio.

The Simpson's diversity index showed very little difference between the genetic diversity of molecular types of protease thermostable and protease heat labile strains, as detected by both PFGE and IRS-PCR. This diversity index was almost the same value as that obtained for the overall genetic diversity index for all strains, regardless of protease thermostability. A difference in the genetic diversity index for clonal groups was detected by both methods. For the PFGE method the diversity index for protease thermostable strains was less that that for clonal groups detected in the protease heat labile strains. This finding was reversed for the IRS-PCR method, which found that protease thermostable strains had a greater genetic diversity index for clonal groups than the protease heat labile strains. Overall, these results suggest that isolates of *D. nodosus* are genetically diverse and that this diversity is high regardless of protease thermostability.

Assessment of the genetic relatedness of PFA molecular types of isolates in WA using dendrograms indicated that strains (regardless of protease thermostability) were related at a level of 70% to 100%. Clusters of isolates were differentiated at particular levels of relatedness, and these clusters contained isolates with a range of clonal groupings, and a range of protease thermostability types. The initial assignment of molecular type to an isolate was done visually, rather than constructing a dendrogram of unknowns and then allocating molecular type. In this manner there was no bias in the allocation of molecular type to an isolate based on dendrogram analysis alone. A subtype was allocated to an isolate that only had been isolated on the same farm as the parent molecular type identified on that farm. Visual allocation of molecular type may lead to mis-identification of molecular type. In some instances where there was doubt about two isolates being the same type, they were tested on the same electrophoresis gel, which also contained the "standard" type as a control. In a number of cases the dendrogram identified some molecular types as identical, yet visual examination of the

fingerprint gels where the isolates were co-located on the same gel clearly indicated a shift in one band position relative to the other isolate. Another instance occurred where one fingerprint pattern showed three bands close together and a second fingerprint pattern had two bands identical to the first pattern but the third band was absent. In this case the software was unable to differentiate the two fingerprint patterns. Some of this may be due to the band comparison position tolerance, which in this assessment of isolates was set at 5%, as recommended (Duck *et al*., 2003). Changing the band comparison tolerance can alter the similarity of one isolate to another as identified by the dendrogram. The band comparison tolerance level must be standardised for all gels so as not to introduce more inconsistencies into the analysis. The width of the DNA fragments generated by the restriction of the chromosomal DNA is determined by the amount of DNA loaded onto the gel, or in the case of the PFGE method, the number of bacterial cells set into the agarose plug from which the DNA is extracted. The greater the accuracy in determining the number of bacterial cells, and hence the concentration of DNA, the better will be the standardization of DNA fragment width on the gels. Selecting which method to use for an assessment of DNA concentration is a trade-off between ease of use and accuracy of the method. Method 1 (section 3.2.2.1) that was used in the beginning of the project was extremely accurate, but was labour intensive. Method 2 was very quick to perform, but was more dependent on operator skill. To accurately assign a molecular type, a combination of dendrogram assessment and visual assessment of molecular type is recommended. Once a large collection of molecular types is amassed, assignment of molecular type by initial visual inspection becomes time-consuming and tedious, as each fingerprint has to be compared to all previous fingerprints. This can be made easier by the generation of gels of “standard” molecular types, but as the number of these standard types increases this too becomes time-consuming. An initial assessment using the GelCompar II software followed by visual inspection of the fingerprints is recommended as the database of isolates increases.

The use of dendrograms is a guide for genetic relatedness and the differentiation of clusters. It was noted in this assessment, and by others (Skov *et al.*, 1995b), that the number and position of the clusters can change when new isolates are added to the dendrogram, or other isolates are left out of the analysis. Generally, there was good correlation between the visual assignment of molecular type and dendrogram analysis.

Analysis of the dendrogram revealed that there was no clearly defined distinction between protease thermostable (S) and protease heat labile strains (U), that is, the strains did not fall into two groups on the dendrogram. Despite this there was one group (group D, cluster I) that contained only S strains, and there were some clusters that had a predominance of S or U strains. Of interest was the similarity between five U5 zymogram types that occurred in a cluster (III) predominated by S strains. The investigation of zymogram profiles and molecular type forms the basis of chapter 6. The molecular type for the U5 strains in cluster III was variable, but amongst the S strains, the PFA 11 clonal group predominated. Only one S strain identified as belonging to clonal group PFA 11 occurred outside cluster III. This isolate may indicate a significant genetic change from the other isolates within the PFA 11 clonal group, and possibly represents a new molecular type outside the PFA 11 clonal group. This assessment is complicated by the fact that the IrsT type for this isolate was IrsT 28a, which is a common IrsT type for other PFA 11 types. It may be a reflection of the fact that the PFGE technique is more sensitive at detecting genetic change than the IRS-PCR method.

In group B in cluster I a predominance of U strains occurred compared to S strains, and these were predominantly from the PFA 9 clonal group. In other clusters, such as cluster II there was a mixture of protease thermostability types with no predominant clonal group.

Molecular typing can give an indication of the genetic similarity of one strain compared to another, and by using two methods the confidence in detecting strain relatedness is increased. Genetic change is random and occurs in different parts of the genome, and this change may be detected with one typing method but not another. As time progresses the random genetic change that occurs in two different isolates will be reflected by a difference in molecular type. Accurate detection of genetic change is important in the eradication program where DNA fingerprinting is being used to confirm stock records that indicate that an epidemiological link exists between isolates. Further research conducted in this project, and reported in chapter 7, indicates that genetic change in *D. nodosus* isolates occurs at a rapid rate, and therefore accurate and discriminatory methods are vital for assessment of strain relatedness.

In this study it was found that isolates that were genetically identical had both the same PFA and IrsT type. Other isolates that had the same PFA type had subtypes of the IrsT type. There was another group of isolates that had the same PFA type but did not correspond with their IrsT type. These results give an indication of the genetic change that is occurring in the genome and the genetic relationship of one isolate to another. Thus two isolates that have the same PFA types and the same IrsT type can be said to be genetically identical. Two isolates that have the same PFA type and a closely related IrsT type are still genetically closely related but some change has occurred in one of the genomes. This would indicate a slight genetic distance between the two isolates. Two isolates that have the same PFA type but completely different IrsT types would still be closely related based on the PFGE results but would be indicating further genetic distance than either of the other two examples. Within a PFA group there may be a number of IrsT types (and visa versa).

It can be expected that as the eradication program nears completion that there will be a reduction in the number of genetic types in WA. This will make it easier to continually monitor strains on infected properties, and to assist in the detection of entry of previously unknown types into the State.

The results presented here using the PFGE and IRS-PCR methods agree with another report that isolates of *D. nodosus* are genetically diverse, that clonal groupings occur, and that PFGE is suitable for strain differentiation (Zakaria *et al.*, 1998; Zhou and Hickford, 2000b).

An investigation of virulence in relationship to molecular type is investigated in chapter 6 and 7.

CHAPTER 5

APPLICATION OF MOLECULAR TYPING TO INVESTIGATE THE EPIDEMIOLOGY OF OVINE FOOTROT

CHAPTER 5

5. Application of molecular typing to investigate the epidemiology of ovine footrot

5.1 INTRODUCTION

A study of the epidemiology of footrot involves the identification of risk factors that contribute to this disease occurring in an individual sheep or a flock. Risk factors for the disease include transmission of the organism, susceptibility of the host, environmental factors, geographical distribution, phenotype of the organism, and the potential virulence capacity of strains. An understanding of these risk factors can assist in developing measures to improve disease control. Further knowledge about the epidemiology of the organism and the disease can be gained using molecular methods, which have proved to be very sensitive for differentiating between bacterial strains.

At the start of this work, strain typing of *D. nodosus* for epidemiology and trace-back purposes was confined to differentiation based on protease type and zymogram isoenzyme type. With isolates classified as either protease heat-stable or protease heat-labile, and further differentiation based on 13 isoenzymes, this classification did not provide the fine discrimination between strains that is required for proving definite epidemiological links when investigating a disease outbreak. Two molecular methods were shown to be suitable for strain differentiation of *D. nodosus*, as outlined in chapter 3. The molecular typing methods of PFGE and IRS-PCR were applied to a large collection of isolates of *D. nodosus* to determine the genetic diversity of the organism, its clonal nature, and the relatedness of strains (chapter 4). This information could be used as 'background' information on the organism, to gain a greater understanding of what is happening on a smaller scale such as on-farm, or amongst small groups of epidemiologically related strains.

In the current chapter, three case studies are presented to help demonstrate that PFGE and IRS-PCR are suitable for trace-back and epidemiological studies of *D. nodosus*, and to investigate on-farm epidemiology of the organism. The results from these investigations led to an examination of the genetic diversity of *D. nodosus* strains within a farm, and within an individual hoof, and these results are presented in this chapter.

5.2 MATERIALS and METHODS

5.2.1 Typing Methods

Methods for PFGE and IRS-PCR typing are described in Chapter 2, General Materials and Methods. For case study 1, isolates were typed by PFGE, using two restriction enzymes, *Apa*I and *Xba*I. They were also typed by IRS-PCR using two primers, PXT and PXG. Isolates for case study 2 were typed by both molecular methods, using enzymes *Apa*I and *Xba*I for PFGE, but only primer PXT was used in the IRS-PCR. Case study 3, the investigation of genetic diversity on farms, and the investigation of epidemiology in the infected hoof, was performed using the restriction enzyme *Apa*I in the PFGE method, and primer PXT in the IRS-PCR method.

5.2.2 Bacterial isolates used in epidemiological studies

The bacterial strains were those from WA, Table B-1, Appendix B. Details of the case studies, the isolates and specifics of each method are presented as follows.

5.2.2.1 Case Study 1. Trace-back from a defined disease outbreak

In 1990, Farm 17 sold stud rams to a number of purchasers. Rapid trace-back by the Department of Agriculture WA detected footrot in purchased rams on 16 properties. The isolates cultured from these infected rams were typed retrospectively, in that the outbreak occurred in 1990 and the isolates were typed in 1996. For the trace-back investigation one isolate from Farm 17, and one isolate each from Farms 3, 14, 18, 27, 29, 30, 32, 45, 56, 62, 65, 88, 89, 90, 110 and 134 isolated in 1990 were tested. The isolates were tested by PFGE using enzymes *Apa*I and *Xba*I, and IRS-PCR using primers PXT and PXG.

Footrot continued to be found on Farm 17 from 1990 until 1997. The typing from these isolates is presented in Case Study 3.

5.2.2.2 Case Study 2. Typing of isolates with epidemiological links

Six individual epidemiological events were investigated in a retrospective study to determine if science could support the anecdotal claim of a common causal relationship between the outbreaks.

Epidemiological Event 1 involved an outbreak of footrot in 1985 on three properties (Farms 77, 106 and 107) when the same truck was borrowed and used to transport sheep. Apart from the use of the truck there appeared to be no other link between the sheep on all three properties. One isolate from each farm was typed by PFGE using the restriction enzymes *Apa*I and *Xba*I, and also typed by IRS-PCR using primer PXT. Isolates AC 857, AC 968 and AC 830 came from Farms 77, 106, 107, respectively, and were from the Albany laboratory's footrot culture collection.

The next 5 epidemiological events (Events 2 to 6) to be investigated were discrete events in different geographical areas, but in each case the common link for the isolates in each event was that they came from sheep on neighbouring farms. Two isolates from each event were typed by PFGE using the enzymes *ApaI* and *Xba*I, and also by IRS-PCR using primer PXT. In Event 2, isolates AC 1572 and AC 1685 from Farm 69 and Farm 127, respectively, were tested. For Event 3, isolates AC 4223 and AC 4953 were tested from Farms 47 and 75, respectively. Event 4 examined isolates AC 3798 and AC 4851 from Farms 35 and 131, respectively. Event 5 examined isolates AC 5113 and AC 5260 from Farm 73 and Farm 83, respectively. Finally, Event 6 examined isolates AC 6095 and AC 6386 from Farms 13 and 92, respectively. These are detailed in the results, Table 5-2.

5.2.2.3 Case Study 3. Investigation of isolates from a farm over a prolonged period

The PFGE and IRS-PCR methods were used to investigate isolates that came from two individual farms that had sheep infected with footrot over a number of years.

Investigation of isolates from Farm 100

Sheep on Farm 100 were infected with footrot in various years from 1992 until 2002. Twenty-nine isolates were fingerprinted by PFGE using *Apa*I enzyme and fingerprinted by IRS-PCR using primer PXT. In 2000, 2001 and 2002 sheep on four

neighbours to Farm 100 became infected with footrot. One representative isolate from Farms 26, 176 and 195, and two isolates from Farm 195 were tested.

Investigation of isolates from Farm 17

Footrot was detected on Farm 17 in 1990 (Case study 1). Footrot then persisted in infected sheep on this farm for the next seven years until 1997. Eleven isolates obtained in the years 1990, 1991, 1993, 1994, 1995 and 1997 from Farm 17 were typed by PFGE using restriction enzyme *Apa*I and by IRS-PCR using primers PXT and PXG.

5.2.2.4 Case Study 4. Epidemiology within a hoof

During the course of this study there were a number of occasions where different isolates were found in a single hoof. These were usually detected because of differences in culture morphology at the time of initial isolation at the Australian Reference Laboratory for Ovine Footrot in the Albany Regional Laboratory. Sometimes disparate results in either the gelatin-gel test or the zymogram led to further investigation for confirmation of culture purity. These isolates were then sent to the South Perth laboratory for molecular typing.

There were 68 isolates from 31 animals on 15 farms in this category that were selected for molecular typing. These are detailed in Table 5-6.

5.2.2.5 Assessment of genetic diversity of isolates on infected farms

Data used to estimate the genetic diversity of the isolates seen in infected sheep on farms was obtained from the database of isolate information generated by molecular typing using PFGE and IRS-PCR (Table B-1, Appendix B). Only farms that had more than one isolate typed were used in the assessment. Therefore, results for an assessment of the genetic diversity seen on farms was based on two or more isolates being typed from that farm. For PFGE using *Apa*I restriction enzyme, the data was taken from 133 farms, and for the IRS-PCR using primer PXT the data was collected from 118 farms.

5.3 RESULTS

5.3.1 Case Study 1. Trace-back of a defined outbreak from Farm 17

Footrot was detected in rams on 16 farms that had purchased these animals from Farm 17. When the isolates were typed by PFGE using enzyme *Apa*I, all but one of the purchased rams was infected with the same PFA type (PFA 7) that was present on the source farm. An exception was Farm 90 that had an isolate (AC 2434) that was PFA 60, and this type was not detected on the source farm (Table 5-1, Figure 5-1).

Table 5-1. Molecular typing of trace-back of footrot outbreak from Farm 17 on 16 farms

Farm ID	Isolate ID	Year Isolated	PFA	PFX	IrsT	IrsG
17	2378	1990	7		13	8
17	3009	1991	7	1	13	8
134	2406	1990	7	1a	13	8
90	2434	1990	60	19	15	9
56	2441	1990	7	1	13	8
88	2443	1990	7	1	13	8
32	2444	1990	7	1	13	8
18	2447	1990	7	1a	13	8
3	2449	1990	7	1a	13	8
65	2452	1990	7	1a	13	8
30	2458	1990	7	1	13	8
27	2459	1990	7	1a	13	8
110	2503	1990	7	1a	13	8
14	2505	1990	7	1a	13	8
45	2508	1990	7	nr	13	8
29	2512	1990	7	nr	13	8
89	2588	1990	7	1	13	8
62	2469	1990	7	nr	13	8

Farm ID = farm identification number; IrsG = IrsG type obtained using primer PXG in the IRS-PCR method; IrsT = IrsT type obtained using primer PXT in the IRS-PCR method; nr = no result; PFA = molecular type obtained using enzyme *Apa*I in the PFGE method; PFX = molecular type obtained using enzyme *Xba*I in the PFGE method; Numbers refer to Albany laboratory culture number followed by farm identification number (AC number).

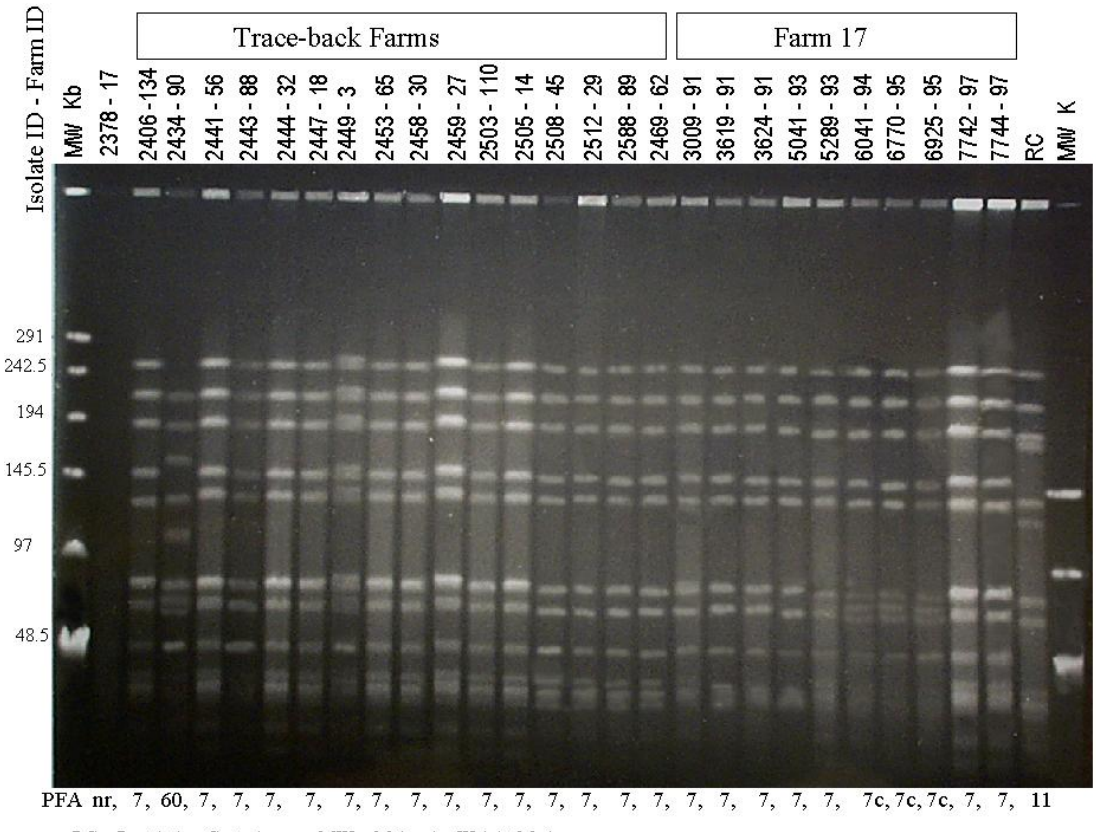


Figure 5-1. PFGE using *Apa*I for trace-back of footrot outbreak from Farm 17

Kb = kilobase pairs; MW = molecular weight marker; nr = no result; PFA = molecular type obtained using enzyme *Apa*I in the PFGE method; RC = restriction control. Numbers refer to Albany laboratory culture number followed by farm identification number (AC number). Figure 5-1 shows the results obtained for isolates from the trace-back farms, and results for isolates from Farm 17.

The results obtained from PFGE using enzyme *Xba*I confirmed the results found with enzyme *Apa*I. Isolates from the trace-back farms gave the result of either PFX 1 or PFX 1a with the exception of isolate AC 2434 from Farm 90. This farm had an isolate that typed as PFX 19. One isolate from the source farm (isolated in 1991) gave the type PFX 1.

Restriction using the enzyme *Xba*I was slightly more sensitive than using *Apa*I in this case study, as a slight genetic change was detected in seven isolates (Figure 5-2). These were subtype PFX 1a, whereas the other isolates and the one isolate from the source farm that was tested, typed as PFX 1. The difference between PFX 1 and PFX 1a was a restriction fragment at approximately 60 kb. These isolates were clonal and closely related because they were similar except for a difference of one restriction band. One isolate only from the source farm was typed using *Xba*I, therefore it cannot be determined whether PFX 1a types existed on this property. DNA from some isolates

(AC 2377, AC 2378, AC 2450, AC 2461, AC 2477, AC 2904, AC 2508, AC 2512) shown in Figure 5-2 was denatured due to unknown factors, and results could not be obtained for restriction with *Xba*I.

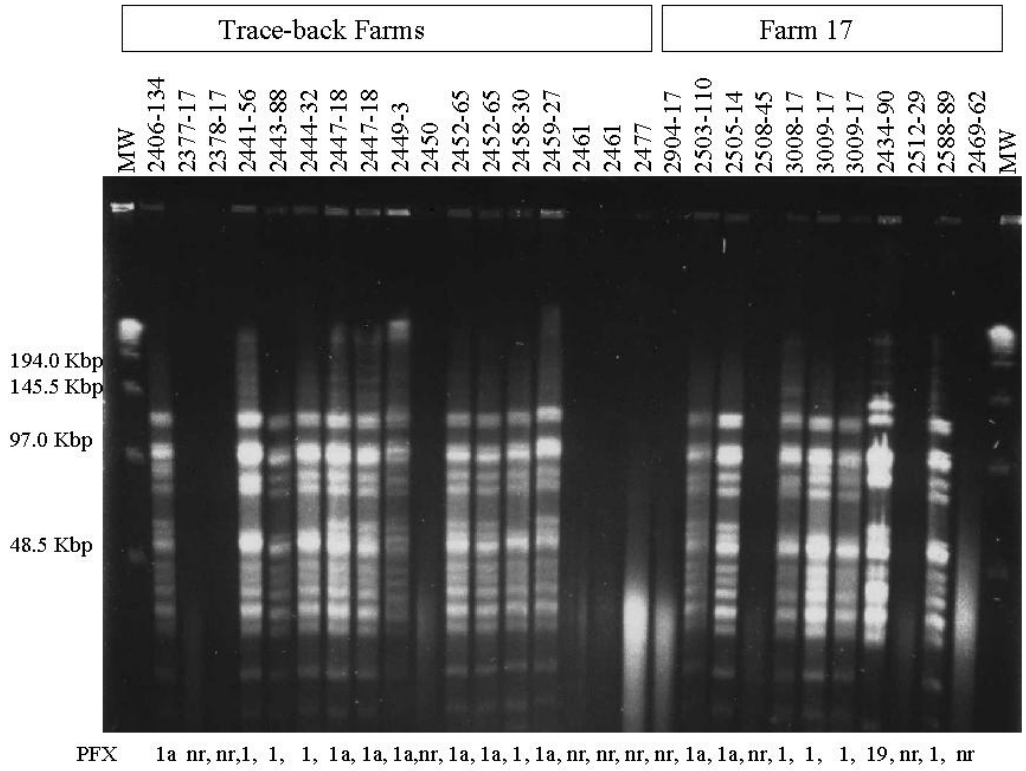


Figure 5-2. PFGE using *Xba*I for trace-back of a footrot outbreak from Farm 17

Kbp = kilobase pairs; MW = molecular weight marker; nr = no result; PFX = molecular type obtained using enzyme *Xba*I in the PFGE method. Numbers refer to Albany laboratory culture number (AC number) followed by farm identification number. Figure 5-2 shows the results obtained for isolates from the trace-back farms, and results for isolates from Farm 17.

The results for IRS-PCR using either primer PXT or primer PXG did not detect any further genetic differences in the isolates, and also confirmed the similarity and genetic identity of the isolates. With the exception of isolate AC 2434, all isolates that were genetically similar and clonal by the PFGE method (types PFA 7, PFA 7c, and PFX 1, PFX 1a), were genetically identical using IRS-PCR with either primer PXT (type IrsT 13) or primer PXG (type IrsG 8) (Figures 5-3 and 5-4). Isolate AC 2434 was IrsT 15 and IrsG 9, and was different to all the other isolates, which were IrsT 13 and IrsG 8 (Figure 5-3, Figure 5-4).

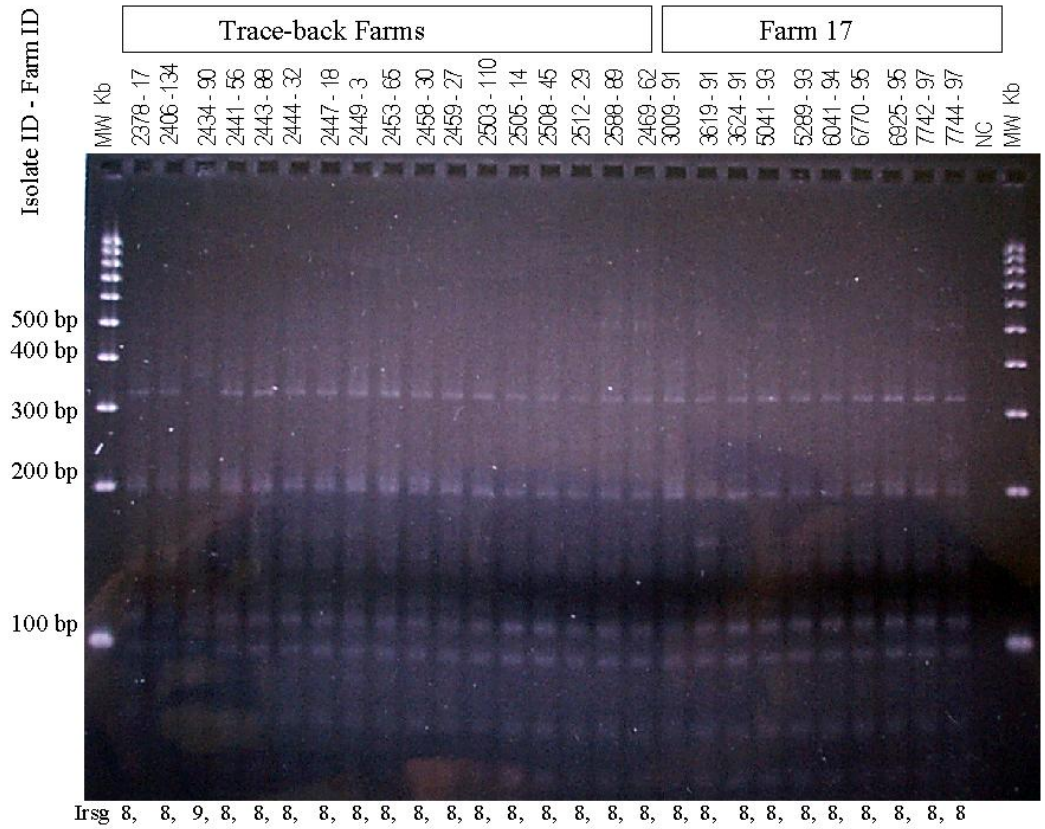


Figure 5-3. IRS-PCR using primer PXG for trace-back of footrot outbreak from Farm 17

Bp = base pairs; Isolate ID = Albany laboratory culture number (AC number) followed by farm identification number; IrsG = IrsG type obtained using primer PXG in the IRS-PCR method; MW = 100 base pair molecular weight marker. Figure 5-3 shows the results obtained for isolates from the trace-back farms, and results for isolates from Farm 17.

The amplified restriction bands obtained with primer PXG were faint (Figure 5-3), but agreed with the results obtained with primer PXT.

All four molecular methods indicated that isolate AC 2434 from Farm 90 was a different molecular type to the other isolates tested in the footrot infection trace-back from Farm 17. All other isolates were the same as those present on the source farm.

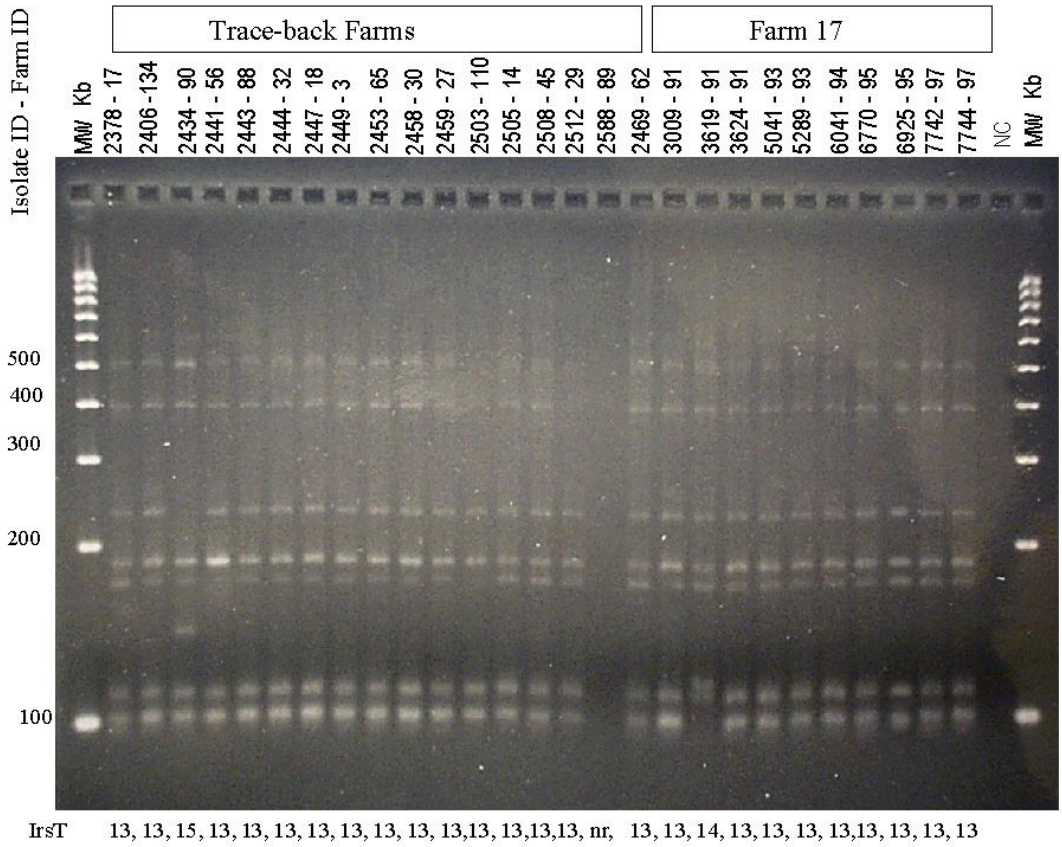


Figure 5-4. IRS-PCR using primer PXT for trace-back of footrot outbreak from Farm 17

Bp = base pairs; ID = identification number; IrsT = IrsT type obtained using primer PXT in the IRS-PCR method; MW = 100 base pair molecular weight marker. Numbers refer to Albany laboratory culture number (AC number) followed by farm identification number. Figure 5-4 shows the results obtained for isolates from the trace-back farms, and results for isolates from Farm 17.

5.3.2 Case Study 2. Molecular typing of epidemiologically related isolates

Six individual, epidemiologically related events were examined to determine genetic relatedness of the isolates involved.

In Event 1, all three isolates (AC 857, AC 830, AC 968) from all three farms (77, 107 and 106) were identical by PFGE, being types PFA 11 and PFX 12, respectively (Table 5-2, Figure 5-5). Typing of the isolates using the IRS-PCR and primer PXT showed that all three isolates from all three farms were type IrsT (Table 5-2, gel results not shown). The gelatin-gel test showed that all isolates were protease thermostable (S strains) and all had the zymogram profile S1.

Table 5-2. Investigation of six individual epidemiological events

Epidemiological Event	Year	Farm ID	Isolate ID	Zym	PFA	PFX	IrsT
1	1985	77	857	S1	11	12	2
1	1985	107	830	S1	11	12	2
1	1985	106	968	S1	11	12	2
2	1988	69	1572	S1	7	1	13a
2	1989	127	1685	S1	66	2	29c
3	1992	47	4223	S1	11	10	2
3	1993	75	4953	S1	11m	10a	2
4	1992	35	3798	S1	59	17	13a
4	1993	131	4851	S1	59a	17	6b
5	1993	73	5113	S1	11	12a	2
5	1993	83	5260	S1	11	12b	2
6	1994	13	6095	S1	11a	15	2
6	1994	92	6386	S1	11a	10	2

Farm ID = identification number; Isolate ID = Albany laboratory culture number (AC number); Zym = zymogram pattern; PFA = molecular type using restriction enzyme *Apa*I in the PFGE method; PFX = molecular type using restriction enzyme *Xba*I in the PFGE method; IrsT = molecular type using primer PXT in the IRS-PCR method.

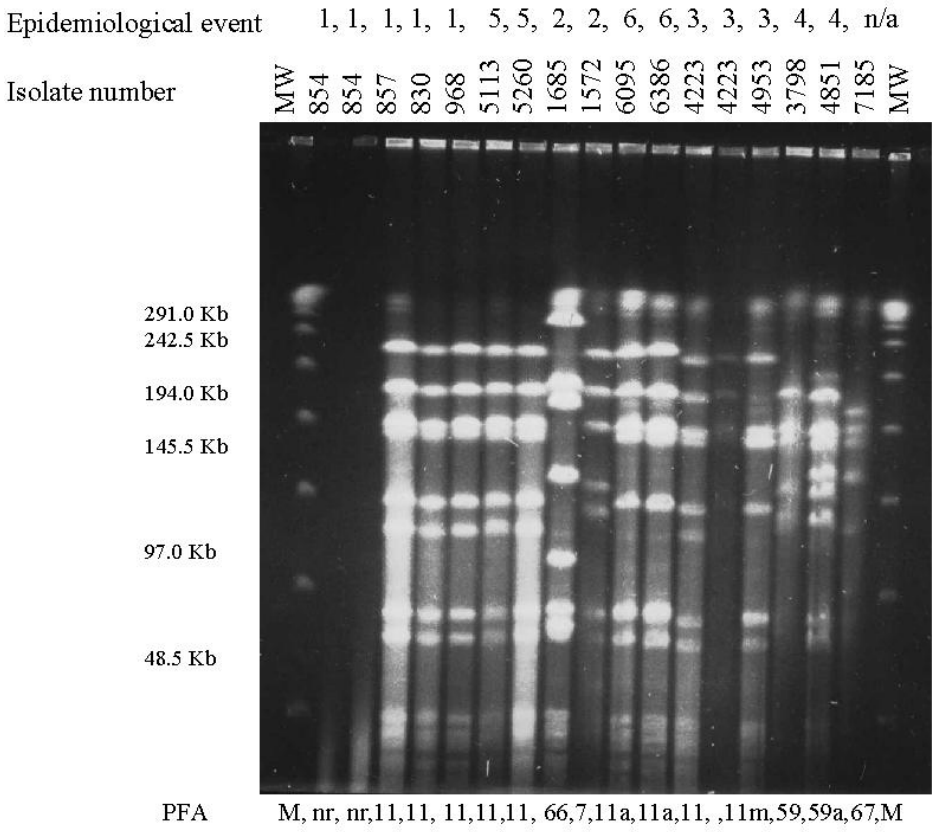


Figure 5-5. Results of PFGE using *Apa*I on isolates from epidemiologically related events

Isolate number refers to the Albany laboratory culture number (AC number); Kb = kilobase; M = marker; MW = molecular weight marker; n/a = not applicable; nr = no result; PFA = molecular type using restriction enzyme *Apa*I in the PFGE method. Isolate AC 7185 was used as a restriction control.

Events 2 to 6 were discrete epidemiological events in different geographical locations. Each event was composed of suspected transmission between neighbouring farms where footrot was found. These results are presented in Table 5-2 and Figures 5-5, and 5-6. The gel results for the IRS-PCR are not shown.

Event 2 comprising Farm 69 and Farm 127 showed that the two isolates were genetically different when tested by both PFGE and IRS-PCR (Table 5-2).

In Event 3, the two isolates from the neighbouring farms were identical when tested by IRS-PCR using primer PXT, but were classified as closely related when tested by PFGE using both *Apa*I and *Xba*I restriction enzymes.

In Event 4, the two isolates (AC 3798 and AC 4851) from Farms 35 and 131, respectively, were identical using PFGE with enzyme *Xba*I, however were classified as

genetically similar when tested using enzyme *Apa*I, and with the IRS-PCR method using primer PXT (Table 5-2).

In Event 5, the two isolates that were tested from the neighbouring farms (Farms 73 and 83) were identical when tested by PFGE using enzyme *Apa*I and IRS-PCR using primer PXT (Table 5-2). When tested using enzyme *Xba*I, there was a one-band difference between the two isolates indicating that they were closely related.

In Event 6, the two isolates (AC 6095 and AC 6386) were identical by PFA and IrsT, but were genetically dissimilar in PFX type.

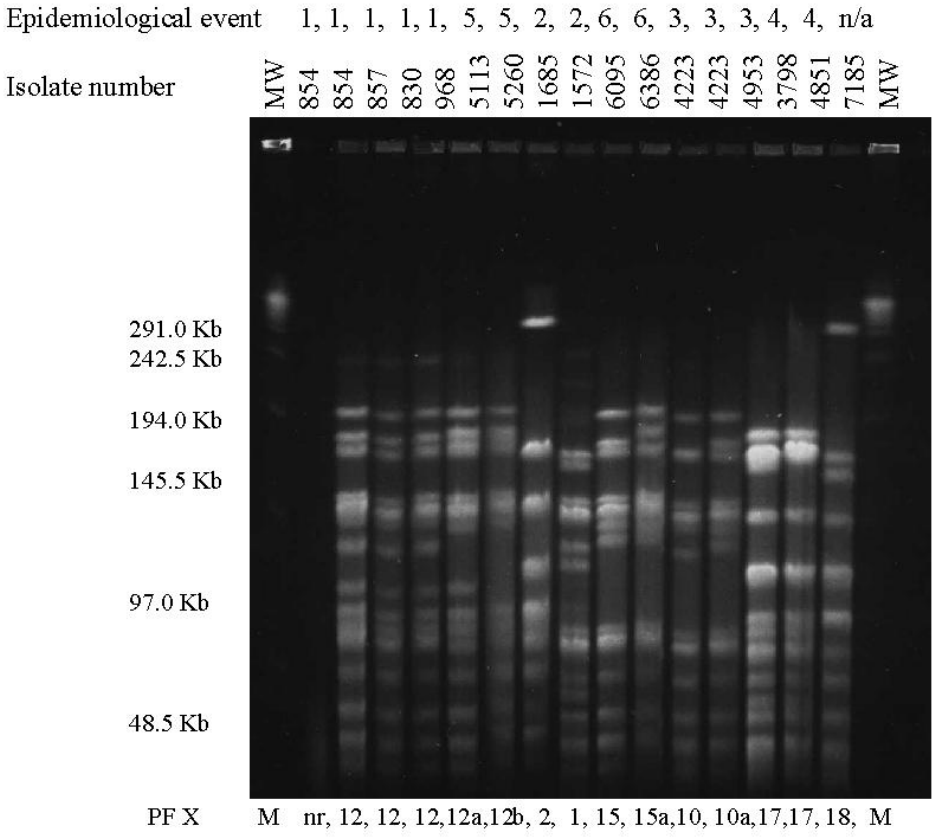


Figure 5-6. Results of PFGE with *Xba*I on isolates from epidemiologically related events

Isolate number = Albany laboratory culture number (AC number); Kb = kilobase; M = marker; MW = molecular weight marker; n/a = not applicable; nr = no result; PFX = molecular type using restriction enzyme *Xba*I in the PFGE method. Isolate 7185 was used as a restriction control.

5.3.3 Case Study 3. Examination of isolates from sheep infected with footrot over a prolonged period

Investigation of isolates from Farm 100

Analysis of the 29 isolates using the PFGE method identified three PFA clonal groups (PFA 9, 21 and 25), with types represented in each clonal group. PFA 9 clonal group had two types (PFA 9c, 9c+), PFA 25 had type 25 and subtype 25a, and PFA 21 clonal group was the most predominant clonal group with types 21, and subtypes 21a, 21e, and 21d. The IRS-PCR method identified 3 clonal groups (IrsT 1, 2, 22) and 1 subtype (IrsT 2d) (Table 5-3). According to the database of isolates tested in this study (chapter 4), the molecular types PFA 21, PFA 21a, PFA 21d and PFA 21e were not detected on any other properties in WA apart from this farm (100) and four neighbours.

Table 5-3. Examination of 29 molecular types of *D. nodosus* isolates from Farm 100 isolated over a 10-year period from 1992 - 2002

Year	No. of isolates	PFA	IrsT
1992	3	21	2
1992	1	21	nt
1994	1	25	nt
1995	1	25a	22
1995	1	9c	1d
1997	1	25a	22
1998	1	21	2d
2000	1	21d	2d
2000	1	21	2d
2001	1	9c+	nt
2001	5	21a	nt
2001	2	21a	2d
2001	2	21a	nt
2001	1	90	2d
2001	1	21	2d
2001	2	21	nt
2001	2	21e	2d
2002	1	21	2d
2002	2	21a	2d
2002	1	21a	nt

nt = not tested; PFA = molecular type using restriction enzyme *Apa*I in the PFGE method; IrsT = molecular type using primer PXT in the IRS-PCR method.

The PFA type 21 was detected on the property from 1992 until it was last tested in 2002. Over this time, subtypes 21a, 21b, 21d and 21e had developed. The subtypes by definition were considered to be genetically similar because they differed from each

other by a difference of up to three restriction bands. These genetic changes occurred over a ten-year period. No subtypes were detected on the property in 1992, but were detected from 2000 onwards. An investigation of such genetic changes in *D. nodosus* isolates is presented in chapter 7.

The main clonal group on Farm 100 (PFA 21 and IrsT 2) persisted on the farm for 10 years despite the fact that the virulent form of the disease was not seen in some years, and was assumed to have been eradicated. An isolate with an equivocal gelatin-gel result and termed a "T" strain, which is thought only to cause a benign infection was detected on the property, but was not subjected to eradication. This isolate typed as PFA 21, 21a and IrsT 2. These results are discussed further in chapter 6, which investigates the relationship between the gelatin-gel results, zymogram profile and molecular type.

Table 5-4. Genetic relationship between *D. nodosus* isolates from Farm 100 and neighbours

Year	Farm ID	Isolate ID	PFA	IrsT
1992	100	4290	21	2
2000	100	505-78	21d	2d
2000	100	505-78	21	2d
2000	195	444-581-1	21b	2
2000	195	444-580	21	28
2001	26	480-576	21	16
2001	157	452-481	21	2d
2002	176	549-517	21a	2

Farm ID = identification number; Isolate ID = isolate identification number; PFA = molecular type using restriction enzyme *Apa*I ain the PFGE method; IrsT = molecular type using primer PXT in the IRS-PCR method. Table 5-4 lists the isolates in ascending order according to year isolated.

Sheep from four neighbouring properties (Farms 26, 157, 176, 195) were examined, and three of these (Farms 157, 176, 195) had isolates from the same clonal groups as isolates from Farm 100 (PFA 21, 21a and IrsT 2, 2d) (Table 5-4). Farms 26 and 195 had identical PFA types (PFA 21) to the source farm (Farm 100), but had different IrsT types, IrsT 16 and IrsT 28, respectively. An isolate from Farm 195 had a PFA 21b subtype that was not seen on Farm 100 amongst the isolates tested in this study, however the IrsT type was common to Farm 100. A fifth neighbour (Farm 1) had no strains in common with Farm 100 (data not shown).

Investigation of isolates from Farm 17

Farm 17 was continually infected with footrot from 1990 until 1997, and 11 isolates were examined. The same PFA type was detected on the farm for four years between 1990 and 1993. In 1994 and 1995 subtype PFA 7c was detected, and then in 1997 PFA 7 was again detected on the property (Table 5-5).

With the exception of isolate AC 3619, all isolates that were genetically similar and clonal by the PFGE method (types PFA 7, PFA 7c, PFX 1 and PFX 1a) were all genetically identical by IRS-PCR using either primer PXT (type IrsT 13) or primer PXG (type IrsG 8) (Figures 5-3 and 5-4). Isolate AC 3619 showed a slight genetic change with primer PXT, being type IrsT 14 as indicated by the absence of a restriction fragment at 100 bp, and the presence of band at approximately 112 bp (Figure 5-3).

Table 5-5. Molecular types of 11 isolates of *D. nodosus* from Farm 17 over a seven year period (1990 – 1997)

Farm ID	Isolate ID	Year Isolated	PFA	IrsT	IrsG
17	2378	1990	7	13	8
17	3009	1991	7	13	8
17	3619	1991	7	14	8
17	3624	1991	7	13	8
17	5041	1993	7	13	8
17	5289	1993	7	13	8
17	6041	1994	7c	13	8
17	6770	1995	7c	13	8
17	6925	1995	7c	13	8
17	7742	1997	7	13	8
17	7744	1997	7	13	8

Farm ID = identification number; Isolate ID = isolate identification number; PFA = molecular type using restriction enzyme *Apa*I in the PFGE method; IrsT = molecular type using primer PXT in the IRS-PCR method; IrsG = molecular type using primer PXG in the IRS-PCR method.

5.3.4 Case Study 4. Epidemiology within a hoof

Where multiple isolates of *D. nodosus* were cultured from the same foot, in some cases different molecular types were found. Up to three different molecular types were detected in individual hooves on 6.1% (n= 15) of the 247 WA farms (Table 5-6). This

occurred in 26 (3.7%) of the 709 sheep tested. The isolates were either genetically similar (belonging to the same clonal group), or were completely different molecular types, and even different strains according to protease thermostability and zymogram profile. In four instances both virulent (thermostable) and benign (heat labile) strains occurred in the same hoof.

Table 5-6. Multiple molecular types detected in an individual hoof on 15 farms in WA

Farm ID	Isolate ID	Animal ID	Zym	PFA	IrsT
7	96-22-78a	78	U	12c	nt
7	96-22-78b	78	U	mixed	nt
41	97-373-638-1	638	S1	7	13h
41	97-373-638-2	638	S1	19g	29c
44	97-352-530-1	530	U2	9d	nt
44	97-352-530-2	530	U2	9d	5
44	97-352-530-3	530	U2	41	nt
44	97-352-530-3	530	U2	41	5c
44	97-352-533-1	533	U2	9i	nt
44	97-352-533-2	533	U2	92	nt
49	97-328-419-A	419	U1	9d	25
49	97-328-419-B	419	S1	11e	2
51	91-923-11	11	U6/U4	39	1a
51	91-923-11-B	11	U6/U4	39a	1a
51	91-923-11 C	11	U6/U4	39c	1a
52	97-128-98-B	98	U1	29	1
52	97-128-98-C	98	U1	29a	1
82	95-572-33	33	S	11f	28
82	95-572-33-A	33	S	11e	28
82	95-572-33-B	33	S	11g	28
82	95-572-33-B8	33	S	11i	28
82	95-572-26-1	26	S	11f	28
82	95-572-26-2	26	S	11g	28
98	97-103-5i	103	U4	19	18
98	97-103-5ii	103	U4	19a	18
98	97-118-49	49	S2	19a	18a
98	97-118-49B	49	S2/U4	19b	18
98	97-143-5A	143	E/U4	19b	18
98	97-143-6B	143	E/U4	19e	19
101	97-369-626-1	626	S	9d	12
101	97-369-626-1i	626	S	61	29a
115	98-467-1	115	U5	18	1g
115	98-467-2	115	U5	8	29
115	00-186-755-1	755	U5	8a	nt
115	00-186-755-2	755	S1	4b	nt
115	00-203-832-1	832	U5	18b	nt
115	00-203-832-2	832	U5	8	nt
115	00-203-833-1	833	U5	18b	nt

Table 5-6, continued

Farm ID	Isolate ID	Animal ID	Zym	PFA	IrsT
115	00-203-833-2	833	U5	8a	nt
115	00-203-845-1	845	U5	8	nt
115	00-203-845-2	845	S	4e	nt
115	00-203-845-3	845	U+	8a, 18	nt
115	00-203-846-1	846	U+	8a	nt
115	00-203-846-2	846	U+	18	nt
115	00-203-861-1	861	U5	8	nt
115	00-203-861-2	861	U5	mixed	nt
142	00-237-981-1	981	S1	11f	14
142	00-237-981-2	981	S1	71	8
176	02-549-518	518	S1	7	13a
176	02-549-518b	518	S1	7a	13a
195	00-444-579-1	579	S1	24b	17
195	00-444-579-2	579	S1	9	27
195	00-444-584-1	584	S1	24c	60
195	00-444-584-2	584	S1	81	28a
195	00-444-585-1	585	S1	24	2
195	00-444-585-2	585	S1	24d	60
239	V18i	18	U5	9a	13g
239	V18ii	18	S1	52	50
303	00-186-755-1	210 LH	U5	8a	nt
303	00-186-755-2	210 LH	S1	4b	nt
303	00-193-786-1	16 RF	S1	4b	nt
303	00-193-786-2	16 RF	U5	mixed	nt
303	00-193-795-1	125 LF	U+	18g	nt
303	00-193-795-2	125 LF	S1	4h	nt
303	00-193-796-1	125 RF	U6	4b	nt
303	00-193-796-2	125 RF	S1	4b	nt
303	00-203-861-1	387 LH	U5	mixed	nt
303	00-203-861-2	387 LH	U5	8	nt

Animal ID = animal identification number; Farm ID = identification number; Isolate ID = isolate identification number; mixed = Inconsistent DNA bands indicating that the culture was not pure; nt = not tested; PFA = molecular type using restriction enzyme *Apa*I in the PFGE method; IrsT = molecular type using primer PXT in the IRS-PCR method; Zym = zymogram profile.

Twenty-four of the isolates were tested more than once by PFGE and in each case the results were the same. They were also subcultured to ensure the purity of the culture.

In three cases where the DNA was mixed, the resulting fingerprint could be seen as a combination of two other fingerprints from isolates from sheep on the same property. This could only be discerned when a number of isolates were tested and other fingerprints clearly established and distinguished. A mixed culture could also be seen as a combination of lighter and brighter DNA bands in the fingerprint.

The results of typing the isolates obtained from Farm 44 are presented in detail to demonstrate that different molecular types may be found in a single hoof. These results are presented in Table 5-6 and Figure 5-7. Three single colonies (#1, #2, #3) from the hoof of animal number 530 were selected, subcultured and tested for molecular type. They were all identified as U2 in the zymogram test, however two different molecular types were identified, PFA 9d and PFA 41. Visual inspection suggested that these two fingerprints had three of ten restriction bands in common (Figure 5-7). The hoof from animal 533 on the same farm also demonstrated two different molecular types (PFA 9i and PFA 92) in the hoof (Figure 5-7 and Table 5-7). Molecular types PFA 9d and PFA 41 were identified as being closely related by the IRS-PCR method.

Table 5-7. Results of typing *D. nodosus* isolates from Farm 44

Farm ID	Isolate ID	Animal ID	Zym	PFA	IrsT
44	530-1	530	U2	9d	nt
44	530-2	530	U2	9d	5
44	530-3	530	U2	41	nt
44	530-3	530	U2	41	5c
44	532	532	U2	41	5c
44	533-1	533	U2	9i	nt
44	533-2	533	U2	92	nt

Animal ID = animal identification number; Farm ID = identification number; Isolate ID = isolate identification number; nt = not tested; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; IrsT = molecular type using primer PXT and IRS-PCR method; U = heat labile protease in the gelatin-gel test; U2 = zymogram profile; Zym = zymogram profile. The case number for these isolates was 97-352.

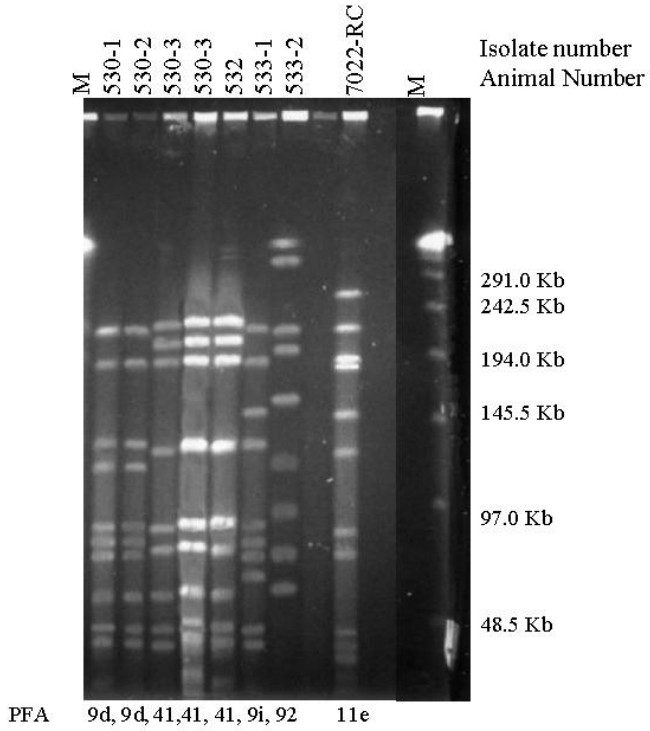


Figure 5-7. Different molecular PFA types of *D. nodosus* isolates in a single hoof in sheep from Farm 44

Kb = kilobase; M = molecular weight marker; PFA = molecular type using restriction enzyme *Apa*I in the PFGE method; RC = restriction control.

5.3.5 Genetic diversity on farms

Of the 247 farms tested in WA, 133 (53.8%) had more than one isolate tested by PFGE using the restriction enzyme *Apa*I. Three classifications of farms were identified based on the genetic similarity of the isolates found in infected sheep on the farm. These classifications were; (1) farms that had sheep infected only with genetically identical isolates, (2) farms that had sheep infected only with genetically similar isolates, that is, clonal isolates, and (3) farms that had sheep infected with isolates that were genetically dissimilar. In the first grouping, 41 farms (30.8 %) had identical PFA molecular types present on the farm (Table 5-8). However, from 73.2 % of these farms that had identical PFA types on the farm, only two isolates were tested. The remaining farms had three or more isolates tested. Four farms had seven to nine isolates tested and these were all identical on the respective farms.

There were 118 or 47.8% of farms that had more than one isolate of *D. nodosus* detected and subsequently typed by IrsT. When IrsT types were assessed, 31 farms

(26.3%) had genetically identical isolates, which was in agreement with the results of PFGE typing (Table 5-8).

The second grouping of farms were those that only had isolates that were genetically similar, and constituted a clonal group as seen by the presence of subtypes of the parent strain. The detection of genetically similar isolates occurred on 20.3% of farms when the isolates were tested by both PFGE and IRS-PCR.

The third grouping of farms were those that had genetically dissimilar isolates or a diverse range of *D. nodosus* strains on the property. The percentage of farms that had sheep infected with a diverse range of molecular types was 48.9% when isolates were typed using PFGE, and 53.4% of farms when tested by IRS-PCR (Table 5-7). On 44.6% (n = 29) of these farms clonal groups were seen in one or more of the genetic groups on the farm using data from PFGE typing.

Table 5-8. Distribution of isolates on 133 WA farms according to genetic similarity based on molecular type

	Typing Method	
	Number of Properties (Percentage)	
	PFA	IrsT
Genetic classification of isolates on farms	n (%)	n (%)
Genetically identical isolates	41 (30.8)	31 (26.3)
Genetically similar isolates (clonal)	27 (20.3)	24 (20.3)
Genetically dissimilar isolates	65 (48.9)	63 (53.4)

For PFA n = 133 farms; for IrsT types n = 118 farms; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; IrsT = molecular type using primer PXT in the IRS-PCR method. Data taken from 133 farms where more than one isolate was selected for molecular typing.

5.4 DISCUSSION

The epidemiological scenarios presented in this chapter represent a range of situations that may be investigated following a disease outbreak. Knowledge of how the organism reacts in its environment is vital for accurately interpreting the results from each scenario, and understanding the disease in general. However, confidence in the interpretation of results from an epidemiological investigation can only be achieved once a large mass of data has been accumulated. The data amassed from the study of a large number of organisms (chapter 4, molecular typing) was used to assist with interpretation of results in the epidemiological scenarios presented in this chapter.

From the results in chapter 4 where a large number of isolates were typed from WA, a genetic diversity of 1:4 was established. This means that, in general terms, one in every four randomly selected isolates is likely to be genetically different when tested by PFGE using enzyme *Apa*I. Therefore, to investigate an epidemiological link between isolates from an infected property, at least four isolates need to be tested to accurately assess this link. The six individual epidemiologically related events (Case study 2) were tested before the large database of isolates was established. Therefore, based on these limited results where only one isolate was tested from each farm, and each was genetically different, as in event 2, it would be difficult to say conclusively that a link did not exist between the two farms. Typing more isolates from each farm may well have found identical or similar molecular types common to both farms. Considering the statistics that 48.9% of farms contained isolates that were genetically diverse, then testing only one isolate from each farm does not adequately establish the lack of linkage between farms. On the other hand, if the same type was present on two epidemiologically related farms, as in event 1, 5 and 6 when tested by PFGE, then transmission between the farms was likely. The likelihood of transmission between two farms would increase when the isolates belonged to the more uncommon PFA or IrsT types in the State.

In events 3, 4, 5 and 6 in Case Study 2, where it was determined that the isolates involved were clonal by either PFGE or IRS-PCR, it may be difficult to establish a definite epidemiological link. In two of these events the isolates were tested in different years, and, although the exact month within each year was not noted, the infection probably was detected in the spring/summer of 1992/93. Therefore, it can be assumed

that there was a delay of at least a few months before footrot was detected on the second farm and when isolates were collected from infected sheep. Analysis of the results from the large database of isolates indicated that 20.3% of farms had genetically similar or clonal isolates when tested by both PFGE and IRS-PCR. These results indicate that isolates need to be tested as soon after an outbreak of footrot as possible because it appears the organism has a high rate of genetic change. However, testing by a number of molecular typing methods gives confidence in establishing an epidemiological link between isolates that are classified as genetically similar by one or more methods.

This case study where only one isolate was tested from each farm thought to have an epidemiological link, highlights the need to fully understand the information generated from molecular (or any other) techniques, so that an informed interpretation can be made. As only one isolate from each of these farms was stored in the culture collection, typing of a larger number of isolates could not be undertaken.

In Case Study 1 where Farm 17 sold infected rams to 16 farms, epidemiological tracing and collection of isolates was done within a short time frame after detection of footrot on the source farm. Very little genetic variation existed between the isolates, and three types (PFA, IrsT and IrsG) were identical for the majority of isolates. Only PFX types showed slight genetic variation. Therefore, it could be demonstrated through molecular typing that the source of the infection was almost certainly the rams purchased from Farm 17. Seven isolates tested by PFX were shown to be a subtype (PFX 1a) of the isolate from the source farm. However, the fact that these isolates were the same molecular type by three other methods showed that the likely source was from infected sheep purchased from Farm 17. The fact that there was little genetic change between the isolates was consistent with the relatively short time frame between the sale of the rams from Farm 17, and the detection of disease transmission. One isolate from Farm 90 was of a different molecular type, and it is unlikely that this isolate came from the source farm. However, as previously noted, more than one isolate should be tested to ensure that all genetic types that may be present on a farm are detected. If a number of isolates are not available for testing, then the use of a number of different molecular typing methods can more clearly establish the relatedness of the isolates that are tested.

In Case Study 3 a number of isolates from two farms were available for typing from different years, and genetically similar or clonal molecular types were identified. In later

years when footrot was discovered on farms neighbouring Farm 100, some clonal types were identified on neighbour farms that were not detected on the source farm. Although a likely epidemiological link was established because of genetically identical types present on the neighbour farm and the source farm, typing the organisms by a number of different methods strengthened the case for there being genetic similarity between the isolates.

As shown by Case Study 1, the use of PFGE using two restriction enzymes and IRS-PCR with two primers produced strong evidence for genetic similarity or difference when all methods confirmed the genetic difference or similarity. Genetic shifts that are detected by one technique but not another may be due to detection of molecular change in different regions of the genome, and/or may be indicative of minor change, or non-permanent change.

An outbreak of an infection often has a single source, and strains will evolve from the outbreak strain to form clonally related strains. These clonally related strains share common characteristics such as biochemical factors, virulence factors and genetic factors. Methods used to type and classify strains in an outbreak must be capable of differentiating and distinguishing isolates from different sources, yet clearly demonstrate the relationship of strains involved in the outbreak that originate from the same source (Olive and Bean, 1999). In this investigation of *D. nodosus* it was apparent that different outbreaks of infection began from single sources and that the “outbreak” strains evolve into genetically similar or clonal strains. In this study 20.3% of farms contain sheep infected with clonal strains, as identified by both typing techniques. This was found in the Case Study 3 that investigated isolates on two different properties that had footrot infection over a ten-year period. On both farms the original isolates evolved to produce molecular subtypes that were classified as genetically similar or clonal. On one farm (Farm 17) that had the PFA 7 type only, this evolved into one subtype; PFA 7c. On the other farm (Farm 100) the original isolate was PFA 21, and this evolved into three subtypes over the same timeframe. However on the farm that produced more subtypes from the original isolate, there were also other molecular types present. Determining whether the number of different molecular types present on a farm is a factor in the development of further subtypes was beyond the scope of this investigation. The subject of genetic diversity and the rate of genetic change is analysed in chapter 7.

Another factor that needed to be assessed in an epidemiological investigation was how common were the molecular types detected on a farm in relation to the types that were common throughout WA. In Case Study 2, Event 1, it could be established that the isolates on all three farms were identical by all three typing methods. Considering that the PFA 11 clonal group is one of the three major clonal groups present in WA, with 21.6% of all isolates belonging to this clonal group, then establishing a conclusive link between the three farms could be difficult to establish. Because all three molecular typing methods indicated the same molecular type, then it became highly likely that the transmission link between the three farms was correct. In the case of Farm 100 that had isolates belonging to clonal group PFA 21, no other unconnected WA farms were found to possess this clonal group. When footrot infection occurred in sheep on the neighbouring property, and a new clone of this group was detected then it provided strong evidence for transmission having occurred between the two farms.

When the genetic diversity that is present on farms was investigated, it was found that farms could be divided into three different categories depending upon the molecular types found on that farm. As previously discussed isolates could either be identical, similar, or diverse in molecular type. Results for PFGE typing and IRS-PCR typing respectively estimated that 48.9% and 53.4% of farms had isolates of *D. nodosus* that were genetically diverse. These farms could have obtained their infections either from different sources or from sheep or a flock of sheep that were infected with more than one molecular type. Some farms bought and sold sheep on a regular basis, and therefore potentially bought their sheep from a number of different farmers. The large amount of trading of sheep from different geographical areas could account for the greater genetic diversity of *D. nodosus* isolates found on these farms.

The results of this study show that multiple molecular types of *D. nodosus* may be present in a single hoof. Some isolates were genetically closely related and belonged to the same clonal group, whilst others were quite different and belonged to different clonal groups. A mix of virulent (thermostable) and benign (heat labile) strains also were identified in the same hoof in some cases. These results have implications for the eradication program as it highlights the importance of there being skilled staff available to perform the laboratory tests to isolate and identify virulent strains, so that false results associated with isolation of benign strains can be prevented. At the primary culture stage, individual colonies must be examined carefully to determine if different colony

morphology types are present, or if there is a difference in rate of growth of the colonies. It was these features that led staff at the Australian Reference Laboratory for Ovine Footrot Laboratory to select more than one colony for subculture from the primary inoculum, which were subsequently tested by gelatin-gel, zymogram profile and molecular typing.

The finding that different molecular types, and in particular benign and virulent types, can coexist in an individual hoof indicates that the organism can be spread from sheep to sheep, and that the source of the infection may be more than one infected sheep. In essence what is happening in regard to distribution of genetic types in the flock is also happening within the hoof. Highly virulent strains have been shown to have a higher rate of transmission after an extended length of time when non-infected sheep were mixed with experimentally infected sheep. In a trial where sheep were experimentally infected with four strains of *D. nodosus* (virulent S1, benign U1, benign U5, virulent U5), the highly virulent strain A198 was detected in 50% (136 of 271) of new lesions when sheep were sampled at 49 to 59 weeks following the introduction of clean sheep into the experimentally infected flock. The virulent U5 strain was detected at a higher rate (57%) compared to strain A198 (20%) in new infections when lesions were sampled between three to 13 weeks following the introduction of clean sheep (Jelinek and Depiazzi, 2003). The benign U1 strain (C305) was not detected in sheep at 49 weeks, and was only found in 0.7% of lesions overall, whereas the virulent U5 strain and the benign U5 strain were detected in 21% and 26%, respectively, of new infections at 49 weeks, and was detected in 28% and 31% of lesions overall. The virulent strain A198 was detected in 39% of lesions overall. The environment has been indicated as a factor in severity and persistence of virulent footrot. A virulent strain was recovered from experimentally infected sheep that had been held in a geographical area not normally conducive to the expression of footrot (Depiazzi *et al.*, 1998). Unfortunately, infection with a benign strain was not tested for a direct comparison with the virulent strain. It is likely that the virulent strains, which produce thermostable protease, cause more damage to the hoof, and can thereby penetrate the deep tissues of the hoof so that the organism is protected from the drying effects of the environment. Research showed that highly virulent strains are more difficult, if not impossible, to eradicate by footbathing with zinc sulphate compared to benign strains (Jelinek and Depiazzi, 2003).

The results of the molecular typing can help establish factors that may be considered as disease transmission risks. In Case Study 2, event 1where anecdotal evidence suggested that the truck was the common epidemiological link between infected properties, this likelihood was supported by the molecular types of the strains involved. Unfortunately, in this study the evidence of the truck as an agent for disease transmission was not confirmed because samples were not tested directly from the truck, only from the sheep that had been transported on the truck. There is no data to establish the timeframe between each usage of the truck, as this would also play a part in establishing the risk factor for truck transportation. It has been shown that *D. nodosus* does not survive for longer than 2 weeks in the environment, therefore if the truck was used within a limited timeframe between transporting infected sheep, then disease transmission could occur. Further work needs to be done to firmly establish contaminated transportation as a risk factor for footrot transmission.

This study showed that the PFGE and IRS-PCR methods could be applied to *D. nodosus* to provide high discrimination between unrelated isolates, yet clearly distinguish clonal types that originated from a single isolate. The PFGE results agree with results from work done in Malaysia, where the technique was used to differentiate *D. nodosus* isolates involved in an outbreak of footrot in that country. Using three different restriction enzymes (*Apa*I, *Sfi*I and *Sma*I), the authors showed that 12 isolates of *D. nodosus* from different epidemiologically unrelated farms had different molecular types. They concluded that the resulting eight clonal types probably had more than one source on the farms that were tested (Zakaria *et al.*, 1998). The results showed that there was a high genetic diversity in the *D. nodosus* population. The results presented in this thesis show that on some farms there is low genetic diversity, whereas on other farms there is high genetic diversity amongst *D. nodosus* strains, possibly associated with greater between-farm sheep movement.

In conclusion, it was established that to investigate an epidemiological link between isolates obtained from infected sheep on different farms, then at least four isolates from each farm should be tested to reliably detect the common genetic types present on the farm. After prolonged infection, clonal groups (genetically similar) of *D. nodosus* will evolve, and therefore epidemiological investigations should be undertaken relatively quickly to positively identify a link between infected properties. All available data, such

as sale yard and farmer records, should be used in conjunction with molecular typing when undertaking epidemiological investigations into footrot.

CHAPTER 6

AN INVESTIGATION OF *D. NODOSUS* MOLECULAR TYPES AND THEIR RELATIONSHIP TO PROTEASE THERMOSTABILITY

CHAPTER 6

6. An investigation of *D. nodosus* molecular types and their relationship to protease thermostability

6.1 INTRODUCTION

A great deal of work has been undertaken to investigate the difference between virulent and benign strains of *D. nodosus*. In particular, efforts have been made to find tests that can distinguish those strains that are capable of producing a virulent foot lesion compared to those strains that only cause a benign lesion, even under ideal conditions. The eradication campaign in WA is directed at removing virulent strains from the State. It is now well established that isolates that produce thermostable protease (S strains) are capable of causing a severe lesion in the hoof under ideal environmental conditions (Depiazzi *et al.*, 1998). Strains that only produce a heat labile protease (U strains) do not tend to cause virulent lesions, and hence do not need to be eradicated.

This situation is complicated by the fact that there are a small number of isolates that do not produce thermostable protease, yet cause severe lesions. These strains have been tested and confirmed to have virulence potential in pen trials. They have a zymogram profile of U5, even though most U5 types only cause benign hoof lesions. The strains that have a U5 zymogram profile, yet produce virulent lesions are in a minority.

Strains with one or other of two other zymogram profiles are also problematic from a diagnostic perspective. These are the U6 type and the T zymogram types. U6 types only show one protease band in the zymogram gel. This band is fast moving under electrophoretic conditions and is common to all strains (Figure 2-3). The T strains also have the fast moving band, but in addition have weakly expressed bands normally seen in S1 strains. Both U6 and T strains are negative in the gelatin-gel test, indicating they produce a thermolabile protease. The reason for their unusual profiles is not well understood, and the relationship of such strains to those with more usual profiles requires investigation.

These considerations led to the current investigation to determine relationships between protease thermostability, zymogram profile, and molecular type of *D. nodosus* strains.

6.2 MATERIALS and METHODS

6.2.1 Bacterial strains

6.2.1.1 Investigation of molecular type related to protease thermostability

All isolates used for assessment of molecular type (Table B-1, Appendix B) and for studying epidemiology (chapter 5) were used in the analysis of relationships between protease thermostability and molecular type. For an overall assessment of protease thermostability and zymogram type compared to molecular type, all 735 isolates from 247 farms in WA were used (Table 2-1). The majority (n = 516, 70.2%) were S strains, and the remainder (n = 219, 29.7%) were U strains. Of the 735 isolates, 364 (49.5%) were cultured from the year 2000 onwards, with 320 (43.5%) of these being S strains and 44 (5.9%) being U strains. One isolate (AC 7676) from Farm 98 gave inconclusive or equivocal results in the gelatin-gel test, and it was also difficult to assign a zymogram type to the isolate. It was designated “E” for equivocal result in the gelatin-gel test, and designated a S2/U4 zymogram profile. The molecular type of the isolate was investigated.

6.2.1.2 Investigation of molecular type related to zymogram type

Investigation of molecular type related to zymogram type U5

For the investigation of U5 strains, 47 isolates from 27 farms in WA were analysed. Strain details are presented in the results of molecular typing of U5 strains as shown in Table 6-3. A dendrogram was constructed using one representative molecular type from each farm, which involved 27 isolates from 21 farms. Five isolates could not be used in the dendrogram because either the DNA bands were too faint to be read by the GelCompar II program, or the molecular weight markers did not separate on the electrophoresis gel. The molecular types of these isolates were interpreted visually.

Investigation of molecular type related to zymogram type U6

Of the 735 isolates, 14 isolates from 12 farms had a U6 zymogram type. Three isolates from Farm 51 also were included as they could not be adequately differentiated by the zymogram and were classed as U6/U4 profile. In addition there were four isolates from a trial involving sheep artificially infected with the Type strain, A198 (ATCC 27521) to investigate the ability of footbathing to eradicate highly virulent strains

(Jelinek and Depiazzi, 2003). These were isolates 00-151-547, 00-167-480, 00-167-507 and 00-193-796#1. The U6 strains are described in the results section as shown in Table 6-6.

Investigation of molecular type related to zymogram type T

The molecular type of T strains was investigated using 12 isolates from four farms (Farm 67, 100, 115 and 177). One S strain from each of Farms 115 and 177, and three S strains from Farm 100 also were included in the analysis. There were no S strains (or any other strains) in the culture collection from farm 67. The T and S strains used are shown in the result section (Table 6-8).

Investigation of molecular type related to zymogram type on Farm 100

Farm 100 had footrot on the property for over ten years (1992 to 2002). During this time, S1, U5, U6 and T strains were detected and these were used to analyse the relationship between molecular type and zymogram type. Strain details are presented in the results of molecular typing of isolates from Farm 100 (Table 6-9).

6.2.1.3 Investigation of molecular type related to pen-tests for virulence

Results from two pen test trials were used to investigate a relationship between molecular type and virulence rating according to pen trial results. One pen trial formed the basis of an interstate research project (CHP94) headed by the Australian CSIRO (Commonwealth Scientific Research Organisation), and funded by Australian Wool Innovation (AWI). Molecular typing by PFGE and IRS-PCR was not part of the AWI project. Permission to use the pen trial results was granted by Dr Scott Williams, Project Manager, Animal Health and Exotic Diseases, AWI. Eighteen isolates from the National Culture Collection (NCC) comprising PFA clonal groups PFA 7, 11, 9 and 18 were examined for a relationship between virulence and molecular type (Table 6-10). These isolates were pen-tested for virulence by two laboratories (CSIRO, Victoria, and Department of Primary Industries, South Australia) under standardized conditions according to the protocol designed for the CHP94 project.

The second pen trial was conducted in 2001 by the Department of Agriculture WA (DAWA) as part of an investigation into virulence assessment of selected strains. Molecular typing of these strains was not a formal part of that project. Permission to use these results in this thesis was granted by Dr Ashley Mercy, Manager Animal Health

Program, DAWA. Seven isolates were pen tested for virulence as outlined in Table 6-11. The severity of the lesions was graded according to mean total weighted footscore (MTWFS) (Whittington and Nicholls, 1995a). Initially, lesions were scored as 0 (no lesion), score 1 and 2 (mild lesions), score3a, 3b, 3c (intermediate severity), and score 4, 5 (virulent). The method of MTWFS scores lesions of grade 1 and 2 as score 1 and 2; score 3a, 3b and 3c lesions are re-scored as 9; score 4 and 5 lesions are re-scored as 16. The sum of the highest score of each foot of each sheep is calculated. The isolate AC 4369 was not pen tested, but the lesion score in the field was recorded in the clinical notes (Table 6-11). Isolate 97-60-115 was pen tested, but only lesion score was recorded, not MTWFS.

The Type virulent strain, A198 (VPI 5731-1, ATCC 27521, originally isolated by J.R. Egerton from Wollogorang Station, Goulburn, New South Wales), and the benign strain, C305 (CSIRO, Melbourne), were used as controls in each pen test trial.

6.2.2 Molecular typing methods

All isolates were typed by PFGE using restriction enzyme *Apa*I and by IRS-PCR using primer PXT as detailed in chapter 2, General Materials and Methods. The molecular type of four U5 strains was investigated by PFGE using enzyme *Xba*I, and five U5 strains were investigated by IRS-PCR using primer PXG. The DNA of a subset of 16 U6 strains were cut with restriction enzyme *Xba*I and used in PFGE, and a subset of five isolates were typed using primer PXG and the IRS-PCR method.

6.2.3 Genetic relatedness of strains

The genetic relatedness of strains was analysed using Simpson's index of diversity, a simple diversity ratio, and using dendrograms prepared by the GelCompar II program as described in 2.8.4. Dendrograms were prepared for the major PFA clonal groups that were identified in WA. A dendrogram was prepared for isolates belonging to PFA 7 clonal group using 46 isolates from 31 properties; for PFA 11 clonal group using 73 isolates from 46 properties; for PFA 9 clonal group using 40 isolates from 28 properties; for clonal group PFA 19 using 15 isolates from 11 properties; and for clonal group PFA 8 using 11 isolates from 11 properties. Some PFA molecular types were represented by more than one strain.

6.3 RESULTS

6.3.1 Investigation of molecular type related to protease thermostability

Protease thermostable (S) strains

A total of 387 isolates in WA were found in the three most common *D. nodosus* clonal groups (PFA 7, 9, 11), with the remaining 348 isolates found at rates of less than 5% in one of the other 64 PFA clonal groups (chapter 4). 94.8% and 89.2% of all isolates in PFA 7 and PFA 11, respectively were S strains. Of all 516 S strains tested, 24.8% (n = 128) occurred in clonal group PFA 7, whilst 27.3% (n = 141) occurred in clonal group PFA 11 (Figure 6-1, Table 6-1).

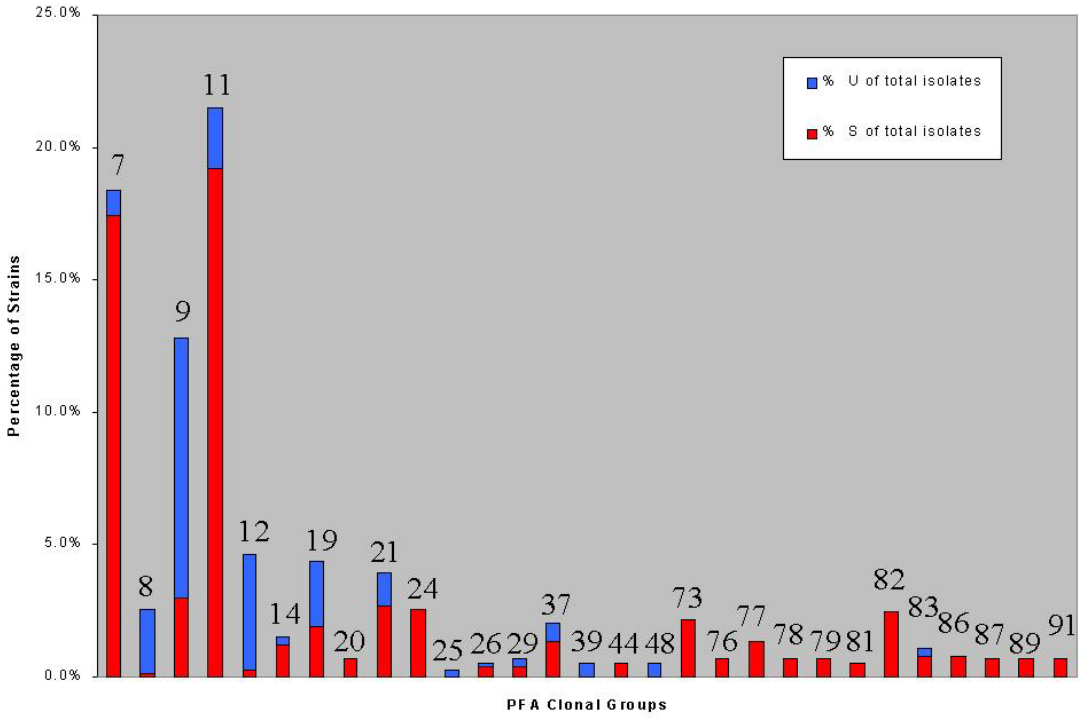


Figure 6-1. Distribution of S and U strains in WA according to PFA molecular type, amongst the 29 clonal groups having four or more isolates. The red colour represents the proportion of virulent strains in each clonal group, and the blue colour represents the proportion of benign strains

The third highest concentration of S strains occurred in clonal group PFA 9, with 4.3% (n = 22) of all S strains tested being found in this clonal group. Of the 29 most common clonal groups, 14 groups consisted of 100% S strains. Three (21.4%) of these clonal groups contained S strains that were detected in years both before and after the year 2000, but the majority (78.6%, n = 11) of these clonal groups had S strains that had only been detected from 2000 onwards. Overall, these 14 clonal groups containing 100%

S strains were composed of small numbers, at 3.9% or less, of the total number of S strains that were tested in WA (Figure 6-1, Table 6-1).

Table 6-1. Distribution of S and U types amongst the 29 most common clonal groups of *D. nodosus* in WA

PFA Clonal Type	Isolates n (%)	S strains in clonal gp n (%)	% S of all S strains	U strains in clonal gp n (%)	% U of all U strains	No. of Props n (%)
7	135 (18.4)	128 (94.8)	24.8	7 (5.2)	3.2	70 (28.3)
8	19 (2.6)	1 (5.3)	0.2	18 (94.7)	8.2	12 (4.8)
9	94 (12.8)	22 (23.4)	4.3	72 (76.6)	32.9	49 (19.8)
11	158 (21.5)	141 (89.2)	27.3	17 (10.7)	7.8	63 (25.5)
12	34 (4.6)	2 (5.9)	0.34	32 (94.1)	14.6	10 (4)
14	11 (1.5)	9 (81.8)	1.7	2 (18.2)	0.3	6 (2.4)
19	32 (4.4)	14 (2.7)	2.7	18 (56.3)	8.2	16 (6.5)
20	5 (0.7)	5 (100)	1.0	0	0	1 (0.4)
21	29 (3.9)	20 (68.9)	3.9	9 (31.0)	4.1	5 (2)
24	19 (2.6)	19 (100)	3.7	0	0	3 (1.2)
25	2 (0.3)	0	0	2 (100)	0.3	1 (0.4)
26	4 (0.5)	3 (75)	0.4	1 (25.0)	0.5	3 (1.3)
29	5 (0.7)	3 (60)	0.6	2 (40)	0.9	3 (1.3)
37	15 (2.0)	10 (66.7)	1.9	5 (33.3)	2.3	7 (2.8)
39	4 (0.5)	0	0	4 (100)	1.8	1 (0.4)
44	4 (0.5)	4 (100)	0.8	0	0	2 (0.8)
48	4 (0.5)	0	0	4 (100)	1.8	1 (0.4)
73	16 (2.2)	16 (100)	3.1	0	0	5 (2)
76	5 (0.7)	5 (100)	1.0	0	0	2 (0.8)
77	10 (1.4)	10 (100)	1.9	0	0	5 (2)
78	5 (0.7)	5 (100)	1.0	0	0	3 (1.3)
79	5 (0.7)	5 (100)	1.0	0	0	1 (0.4)
81	4 (0.5)	4 (100)	0.8	0	0	1 (0.4)
82	18 (2.4)	18 (100)	3.3	0	0	5 (2)
83	8 (1.1)	6 (75)	1.2	2 (25)	0.9	4 (1.6)
86	6 (0.8)	6 (100)	1.2	0	0	2 (0.8)
87	5 (0.7)	5 (100)	1.0	0	0	3 (1.2)
89	5 (0.7)	5 (100)	1.0	0	0	1 (0.4)
91	5 (0.7)	5 (100)	1.0	0	0	2 (0.8)

gp = group; n = number; Props = properties; Isolates = number of isolates in each clonal group and the percentage that it represents of all 735 isolates; S strains in clonal group = the number and percentage of strains in the clonal group that were protease thermostable (S); % S of all S strains = the percentage of all 516 S strains that occurred in that clonal group; U strains in clonal group = the number and percentage of strains in the clonal group that were protease heat labile (U); % U of all U strains = the percentage of all 219 U strains that occurred in that clonal group.

In this collection of isolates there was a bias towards S strains that were typed from the year 2000 onwards because of a policy set at that time to predominantly type strains that were detected on farms in quarantine. The distribution of S and U strains in the three most common clonal groups (PFA 7, 9, 11) compared to the number of strains present in the remaining 64 clonal groups in WA, before and after the year 2000 is shown in Table 6-2.

Table 6-2. Numbers of S and U strains in clonal groups PFA 7, 9, 11 compared to remaining clonal groups before and after the year 2000

Clonal group	PFA 7	PFA 11	PFA 9	Other
No. S strains				
Prior 2000	58	64	7	70
Year 2000	31	39	7	74
Year 2001	26	25	6	58
Year 2002	13	13	2	16
Total S strains 2000-02	70	77	15	158
No. of U strains				
Prior 2000	5	15	69	83
Year 2000	0	0	20	23
Year 2001	1	0	1	2
Year 2002	1	0	1	2
Total U strains 2000-02	2	2	3	37
Total strains				
Prior 2000	63	79	76	153
2000-02	72	79	18	195

No. = number; PFA = PFA clonal group using *Apa*I enzyme and PFGE method; S = protease thermostable strains; U = protease heat labile strains; Other = all other PFA clonal groups.

Protease thermostable (S) strains predominated in clonal groups PFA 7 and PFA 11 both before and after the year 2000. Before the year 2000, 92.1% (n = 58) of the 63 strains that were identified as belonging to clonal group PFA 7 were S strains. From the year 2000 onwards there were 72 strains that were identified as belonging to clonal group PFA 7, and 97.2% of these were S strains. Prior to the year 2000, 81.0% (n = 64) of the 79 strains identified as belonging to clonal group PFA 11 were S strains. After the year 2000, 97.5% of the 79 strains that were found in clonal group 11 were S strains.

Protease heat labile (U) strains

Protease heat labile (U) strains occurred predominantly in clonal group PFA 9, with 32.9% of all 219 U strains tested in WA, from 49 (19.8%) properties, belonging to this clonal group (Table 6-1, Figure 6-1). The U strains comprised 76.6% (n = 72) of all strains found in PFA 9 clonal group. 12.8% (n = 94) of all strains tested in WA belonged to clonal group PFA 9 (chapter 4). Prior to the year 2000, 76 strains were identified as belonging to clonal group PFA 9, and 90.8% (n = 69) of these were U strains (Table 6-2). From the year 2000 onwards, predominantly S strains were tested for molecular typing. A total of 18 isolates were found to belong to clonal group PFA 9 during this time, and 20% (n = 3) were U strains compared to 83.3% (n = 15) S strains.

Clonal group PFA 12, present on 4% (n = 10) of properties, contained the next highest number of U strains. In total, 94.1% (n = 32) of these were U strains, and this represented 14.6% (n = 32) of all U strains found in WA. Two clonal groups (PFA 8 and PFA 19) both contained 8.2% of all 219 U strains tested.

Clonal group PFA 7 had seven U strains (5.2%), which represented 3.2% of all U strains tested. Clonal group PFA 11 contained 17 (10.7%) U strains, which represented 7.8% of all U strains tested in WA. Prior to the year 2000, 15 (18.9%) strains in clonal group PFA 11 were U strains, whereas from the year 2000 onwards U strains comprised 2.5% (n = 2) of all strains in clonal group PFA 11. The remaining U strains were distributed amongst the other 62 clonal groups (total = 67) in low numbers.

Clonal group PFA 7

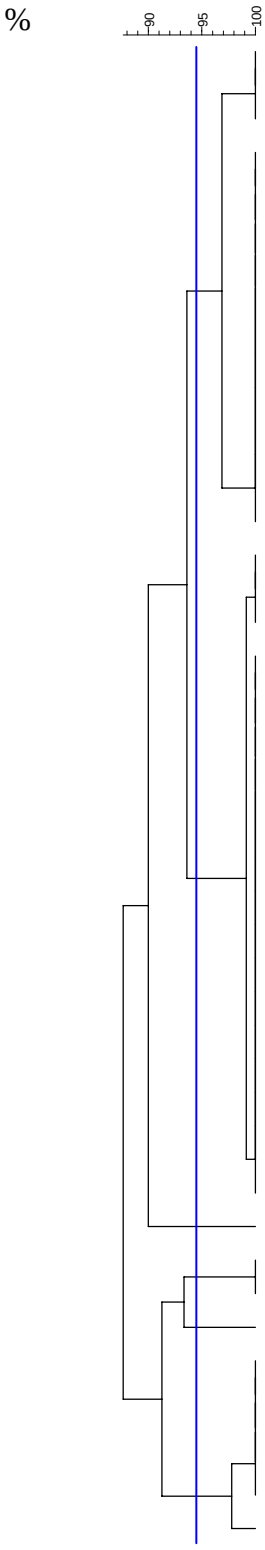
Clonal group PFA 7 contained 18.4% of all 735 isolates tested, and these consisted of 94.8% (n = 128) S strains and 5.2% (n = 7) U strains (Table 6-1). A dendrogram prepared from 45 molecular types within PFA 7 clonal group found on 31 properties in WA showed that the PFA molecular types were related at between 89% to 100% (Figure 6-2). The dendrogram contained five isolates that were protease heat labile and the remaining 40 isolates were S1 zymogram profile. Six clusters were identified at a level of 95% relatedness. Cluster I contained 15 strains from 12 farms that were related to each other at 97% similarity (Figure 6-2). This cluster contained three PFA types (PFA 7, 7b and 7f), with PFA 7 predominant (60%, n = 9).

Eight isolates in cluster I had corresponding IrsT types belonging to clonal group IrsT 13. Isolates of molecular type PFA 7 had corresponding IrsT types of IrsT 13, 13a or 13h, with one isolate having a corresponding IrsT type of 14 (Figure 6-2). One T strain occurred in cluster I and was PFA 7 and IrsT 13a. This isolate was 100% related to an S1 isolate by both PFA and IrsT type.

Cluster II harboured 20 strains from PFA 7a, 7h, 7i and 7N molecular types, including two subtypes of PFA 7a (PFA 7a-1 and PFA 7a-2) (Figure 6-2). PFA 7a, including the two subtypes, was the predominant molecular type with 65% (n = 13) of strains belonging to this molecular type. All three PFA 7h types that occurred on three different farms had corresponding IrsT types of IrsT 28c and were identified as being 100% related. Isolates that were identified as being PFA 7a had one of a possible five IrsT types (IrsT 2, 13, 13g, 13h or 28a). Strains in cluster II were 99% related to each other.

Clusters III, IV and V harboured two, two and one isolate each, respectively. Cluster VI harboured six strains with 83.3% (n = 5) of all strains belonging to molecular type PFA 7c, and one isolate of type PFA 7e.

The Simpson genetic diversity index for PFA 7 clonal group was 0.80 where N = 135, N(N-1) = 18090, $\Sigma nj(nj\text{-}1) = 3590$. The simple diversity ratio was 1:10.



Case no.	Isolate no.	Location	Zym	PFA	IrsT	Farm ID	
97-184	640	Toodyay	S1	7b	13d	79	
AC 1572	88-179	Kojonup	S1	7	13a	69	
AC 7369	96-133-578	Wokalup	T	7	13a	115	
97-170	576s	York	S1	7f	14	37	
AC 5042s	AC 5042	Mt Barker	S1	7	13	17	
97-184s	645	Toodyay	S1	7b	13d	79	
00-471	678	Mt Barker	S1	7	13h	175	I
02-16	780	Albany	S1	7	ND	208	
01-506	673	Katanning	U6	7	9	1	
02-247	784	Mt Barker	S1	7f	ND	311	
01-226	746	Mt Barker	S1	7b	28	26	
97-396	714	Mt Barker	S1	7	14	17	
01-14	4	Mt Barker	S1	7	13a	181	
01-478	573	Mt Barker	S1	7	13	1	
02-644	850	Mt Barker	S2	7f	ND	148	
02-75	992	Busselton	U6	7i	2	207	
97-209s	826	York	S1	7i	28c	37	
01-449	470	Youngs Siding	S1	7a	13g	169	
97-333s	443	Mt Barker	S1	7a-1	13	104	
97-170s	579	York	S1	7h	28c	37	
AC 2127s	2127	Collie	S1	7a	2	105	
00-471	677	Mt Barker	S1	7a	13h	175	
00-471	676	Mt Barker	S1	7a	13g	175	
00-473	684	Mt Barker	S1	7a	13h	165	
00-492s	745	Mt Barker	S1	7N	13h	26	II
01-315	19	Dardenup	S1	7a	2	222	
00-438s	562	Katanning	S1	7a-2	14d	132	
02-36	852	Manjimup	S1	7a	NR	223	
01-451	479	Kalgan	S1	7a	13h	202	
01-506	674	Katanning	S1	7a	13	1	
98-9	122	Katanning	S1	7h	28c	226	
01-15	8	Boyup Brook	S1	7i	2	171	
00-514	825	Albany	S1	7a	13g	227	
00-307	211	Manjimup	S1	7a	28a	156	
AC 7755	97-266-158a	York	S1	7h	28c	60	
97-204s	801	Donnybrook	S1	7L	2e	2	III
AC 6488s	95-146-122.	unknown	S1	7g	6a	119	IV
00-441s	569	Bunbury	S1	7m	28	184	
AC 3264s	3264	Cranbrook	U5	7k	13g	5	V
00-422s	515	Bunbury	S1	7c	13c	184	
97-333s	441	Mt Barker	U4	7e?	1e	104	
01-311	9	Mt Barker	S1	7c	13g	178	VI
6041	97-396-722	Mt Barker	S1	7c	13	17	
00-513	822	Bunbury	S1	7c	13g	184	
00-441	568	Bunbury	S1	7c	28	184	

Figure 6-2. Dendrogram of 46 isolates representing 14 molecular types within PFA 7 clonal group from 31 properties in WA

ID = identification number; IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; ND = not done; NR = no result; no. = number; Zym = zymogram profile. I-VI = clusters identified.

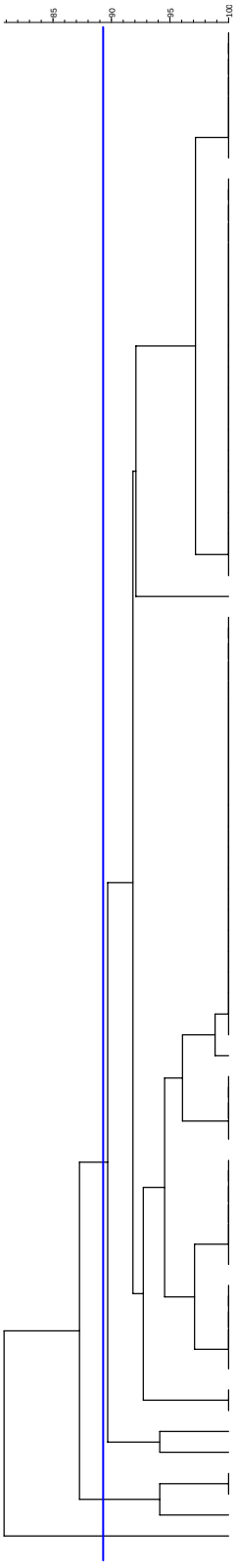
Clonal group PFA 11

Clonal group PFA 11 contained 21.5% (n = 158) of all 735 isolates tested, and these consisted of 89.2% (n = 141) S strains and 10.7% (n = 17) U strains (Table 6-1). The dendrogram prepared from 73 molecular types (from 46 properties) identified within clonal group PFA 11, indicated that isolates were related at a level of 81% to 100% (Figure 6-3). Four clusters (I - IV) were identified at a level of 90% relatedness.

Cluster I contained 67 isolates from 45 farms that were related at a level of 92% similarity. These comprised isolates from PFA 11c, 26.9% (n = 18); from PFA 11e, 17.9% (n = 12); from PFA 11, 16.4% (n = 11); from PFA 11N, 11.9% (n = 8), with the remaining ten isolates from eight other PFA types (Figure 6-3). The 18 isolates of molecular type PFA 11c were related at a level of 97% to six isolates that were PFA 11a, marked as Group A on the dendrogram (Figure 6-3). Fourteen of the isolates of PFA 11c molecular type had a corresponding IrsT type that belonged to IrsT 13 clonal group. These isolates came from nine properties.

The remaining three clusters (II, III and IV) harboured two, three and one isolate, respectively. One isolate (01-39-110) in cluster II was PFA 11N. All other isolates of this type were found in cluster I (Figure 6-3). Although this isolate was run on the same gel as eight of the isolates of PFA 11N in cluster I, and on the gel, visually appeared to belong to PFA 11N, the software located this isolate in a different cluster. All fingerprints were checked visually and the marking of the bands for analysis in the software program was also re-checked with no change in the result.

The Simpson genetic diversity index for PFA 11 clonal group was 0.87. (N = 158, N(N-1) = 24806, $\Sigma nj(nj\text{-}1) = 3184$). The simple diversity ratio = 1:9.



Case no.	Isolate no.	Location	Zym	PFA	IrsT	Farm ID	
02-19	792	Mt Barker	S1	11a	ND	50	
AC 3357	AC 3357c	Boyup Brook	U1	11a	2	20	
01-423	411	Katanning	S2	11a	2	167	
00-531	876	Mt Barker	S1	11h-b	28	176	
01-12	990	Mt Barker	S1	11c	13g	177	
01-12	991	Mt Barker	T	11c	13g	177	
97-60	115a	Boyup Brook	U5	11a	2	20	
97-214s	876	Newlands	S1	11a	28	19	
AC 7381s	96-136-621	Northam	S1	11h	13e	34	
AC 7760	97-304-286	Toodyay	S1	11c	13a	34	
97-333	442	Mt Barker	S1	11c	13c	104	
97-316s	372	Toodyay	S1	11c	13a	34	
01-399	332	Manypeaks	S1	11c	2	205	
00-431	542	Mt Barker	S1	11c	13c	26	
96-147	741	Boyup Brook	S1	11c	13h	99	
AC 8539	00-230-967	Kendenup	S1	11c	13	143	
02-20	839	Mt Barker	S1	11c	13g	177	
02-20	795	Mt Barker	S1	11c	13g	177	
02-73	989	Mt Barker	S1	11c	13	224	
02-140	234a	Bruce Rock	S1	11c	28	232	
AC 9246	02-140-235	Bruce Rock	S1	11c	28	232	
AC 9250s	02-151-274	Tenterden	S1	11L	13c	233	
00-521	849	Kendenup	S1	11c	13g	188	
00-521	852	Kendenup	S1	11c	13g	188	
98-67	394	Pemberton	U5	11c	1d	225	
97-214	876a	Newlands	S1	11a	28	19	
96-135	615a	Moora	S1	11c	13	39	
97-341	491	Toodyay	T	11La	13	67	
00-487	731	Manypeaks	S1	11N	2	205	
AC 830	85-540a	Broomehill	S1	11	2	107	
00-499	777	Mt Barker	S1	11N	14b	26	
00-498s	773#1	Kendenup	S1	11N	2	159	
AC 7754	97-263-148	Donnybrook	S1	11	28	74	I
97-300s	268	Busselton	S1	11K	17	96	
97-328	419B#1	Narrikup	S1	11e	2	49	
01-39	109#1	Darkan	S1	11N	2e	172	
AC 8802	01-39-109#2	Darkan	S1	11N	2e	172	
AC 8808	01-46-141	Darkan	S1	11N	2	172	
02-37	861	Boyup Brook	S1	11e	28	220	
AC 6346	97-145-340	Narrikup	S1	11e	3	112	
AC 9252	02-151-277	Tenterden	S1	11N	13c	233	
01-399	332	Manypeaks	S1	11e	2	205	
AC 9253	02-151-278	Tenterden	S1	11N	13c	233	
02-442	260	Mt Barker	S1	11e	ND	312	
01-222	737	Busselton	S1	11	ND	210	
00-528	866	Mt Barker	S1	11N+	13g	176	
AC 7117	97-124-25	Narrikup	S1	11e	2	57	
AC 5336	97-145-341	Narrikup	S1	11e	14	112	
AC 7890	AC 7890	Wannamal	S1	11	20	98	
97-328s	424#1	Narrikup	S1	11e	2	49	
01-222	738	Busselton	S1	11K	17	208	
01-459	502	Williams	S1	11	28	198	
02-25	820	Denmark	S1	11	ND	186	
01-436	435	Boyup Brook	S2	11	2	130	
95-572s	33	Williams	S1	11f	28	82	
AC 7022	AC 7022c	Narrikup	U6	11e	14	112	
97-57	85	Mt Barker	S1	11e	ND	113	
02-247	733	Mt Barker	S1	11e	ND	311	
97-400	735	Bow River	S1	11e	14	81	
AC 6613	97-124-22b	Donnybrook	U6	11e	14	72	
97-263s	148	Donnybrook	S1	11	28	74	
AC 7748s	97-235-0	Donnybrook	S1	11d	28	8	
97-300	268	Busselton	S1	11K	17	96	
95-572s	33B8	Williams	S1	11i	28	82	
AC 968	85-842	Broomehill	S1	11	2	106	
00-199	824	Kojonup	S1	11	28	140	
00-199	825	Kojonup	S1	11	28	140	II
01-39	110	Darkan	S1	11N	14	172	
01-515	697	Mt Barker	S1	11h	28a	173	
02-140	234b	Bruce Rock	S1	11c-1	28	232	
97-400	739	Bow River	S1	11c-1	14b	81	III
00-467s	664	Plantagenet	S1	11h-a	28a	173	
95-572s	33B5	Williams	S1	11g	28	82	IV

Figure 6-3. Dendrogram of 73 strains from PFA 11 clonal group from 46 properties in WA

IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; No. = number; Zym = zymogram profile. I-IV = clusters identified; A = group identified within cluster I.

Clonal group PFA 9

Clonal group PFA 9 contained 12.8% (n = 94) of all 735 isolates tested, and these consisted of 76.6% (n = 72) U strains and 23.4% (n = 22) S strains (Table 6-1). The dendrogram prepared from 40 isolates (from 28 properties) identified within clonal group PFA 9 indicated that isolates were related at a level of 84% to 100% (Figure 6-4).

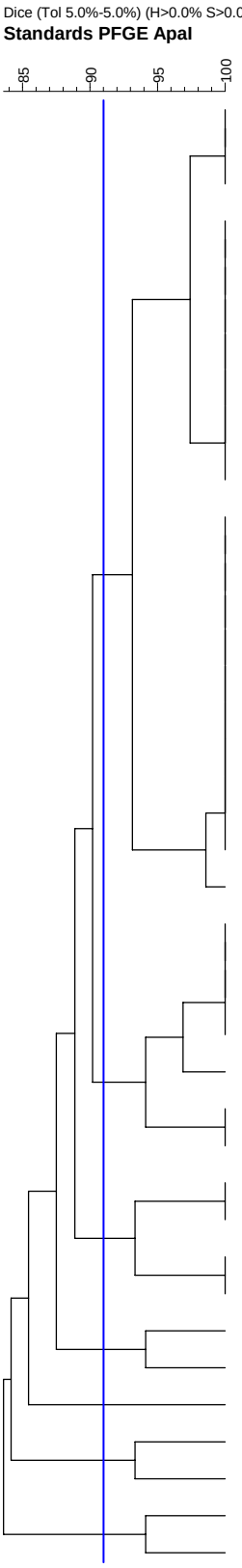
Seven clusters were identified at a level of 89% relatedness. Cluster I harboured 22 isolates from 19 properties that were related to each other at a level of 93% similarity. Eight PFA molecular types were present in this cluster. Molecular type PFA 9a was predominant with 22.7% (n = 5) of isolates having this type, followed by PFA 9 (18.2%), with types PFA 9b, PFA 9c and PFA 9d each comprising 13.6% of isolates.

Cluster II contained seven isolates, and these were from molecular types PFA 9e (57.1%), PFA 9m (28.6%), with one isolate of PFA 9k. The relatedness of these isolates was 94%.

Cluster III contained four isolates, with the remaining seven isolates occurring in either clusters IV, V, VI or VII (Figure 6-4). The four isolates in cluster III typed as either IrsT 1c or IrsT 1d, however these IrsT types also were found in clusters I, II and VI.

Isolate AC 6047 was molecular type PFA 9a-1 and was located in cluster III on the dendrogram (Figure 6-4). Two other isolates were type PFA 9a-1 and these were located together in cluster I. All isolates were checked visually on the gels, and in the GelCompar II software, but the location of the isolates on the dendrogram did not change.

The Simpson genetic diversity index for PFA 9 clonal group was 0.90. (N = 94, N(N-1) = 8742, $\Sigma nj(nj\text{-}1) = 874$). The simple diversity ratio = 1:5.



Case no.	Isolate no.	Location	Zym	PFA	IrsT	Farm ID	
97-432s	930s	Boyup Brook	U5	9d	5	6	I
97-352	530-2a	Kendenup	U2	9d	5	44	
97-328s	419A	Narrikup	U1	9d	25	49	
AC 7164s	96-25-994	Boyup Brook	U1	9a	21	63	
97-327s	416#2	Donnybrook	U1	9b	1d	74	
01-38	103	Darkan	S1	9a	21a	172	
01-38	104	Darkan	S1	9b	21	172	
01-15	7	Boyup Brook	S1	9a	1d	171	
AC 6904	97-145-332	Perillup	U1	9b	1d	100	
AC 6624	AC 6624b	Boyup Brook	U1	9a	1	20	
AC 6065	94-618a	Boyup Brook	U1	9a	1	20	
97-175s	599	Capel	U1	9n	21	9	
97-174s	597	Narrikup	U1	9f	32a	84	
AC 7204s	96-67-153	Young	U1	9	1	42	
00-444	579 #2	Plantagenet	S1	9	27	195	
00-377	407	Kendenup	S1	9c	1d	159	
AC 6171	94-710-45	Serpentine	U1	9a-1	1a	126	
01-32	69	Belmont	S1	9a-1	27a	154	
AC 6	76-656	Tenterden	U1	9c	1	70	
AC 2647	90-529-707	Bridgetown	S1	9	13	59	
AC 6476s	97-124-17	Mt Barker	U6	9c	1	97	
AC 6412	97-124-16a	Mt Barker	U1	9	1a	97	
97-383s	673	Denmark	U1	9k	1d	55	II
96-130	513	Boyup Brook	U1	9e	1d	99	
96-130	511	Boyup Brook	U1	9e	ND	99	
AC 7165	97-124-27a	Albany	U6	9e	1	63	
97-386s	680	Williams	U1	9e	1	24	
00-439	565	Boyup Brook	S1	9m	21	171	
00-439s	566	Boyup Brook	S1	9m	21	171	
02-146	253	Tenterden	S1	9L	1c	233	III
02-146	251	Tenterden	S1	9L	1c	233	
AC 6047	94-593-715	Tenterden	U1	9a-1	1d	122	
00-295s	165	Darkan	S1	9u	1d	154	
97-174	596	Narrikup	U	9p	32	84	IV
AC 6205	94-730-126	Boyup Brook	U3	9b	1e	136	
97-369	626#1	Donnybrook	S1	9d	12	101	V
97-180s	628s	Boyup Brook	U5	9o-1	5	6	VI
02-29s	834	Rocky Gully	U5	9o	1c?	52	
97-369s	623a	Donnybrook	U6	9d-1	11	101	VII
AC 8231s	00-189-65	Bridgetown	U5	9s	21	139	

Figure 6-4. Dendrogram of 40 strains from PFA 9 clonal group from 28 properties in WA

IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; no. = number; Zym = zymogram profile. I - VII = clusters identified.

Clonal group PFA 19

Clonal group PFA 19 contained 4.4% (n = 32) of all 735 isolates tested, and these consisted of 56.3% (n = 18) U strains and 2.7% (n = 14) S strains (Table 6-1). The dendrogram prepared from 15 isolates (from 11 properties) identified within clonal group PFA 19 indicated that isolates were related at a level of 88% to 100% (Figure 6-5).

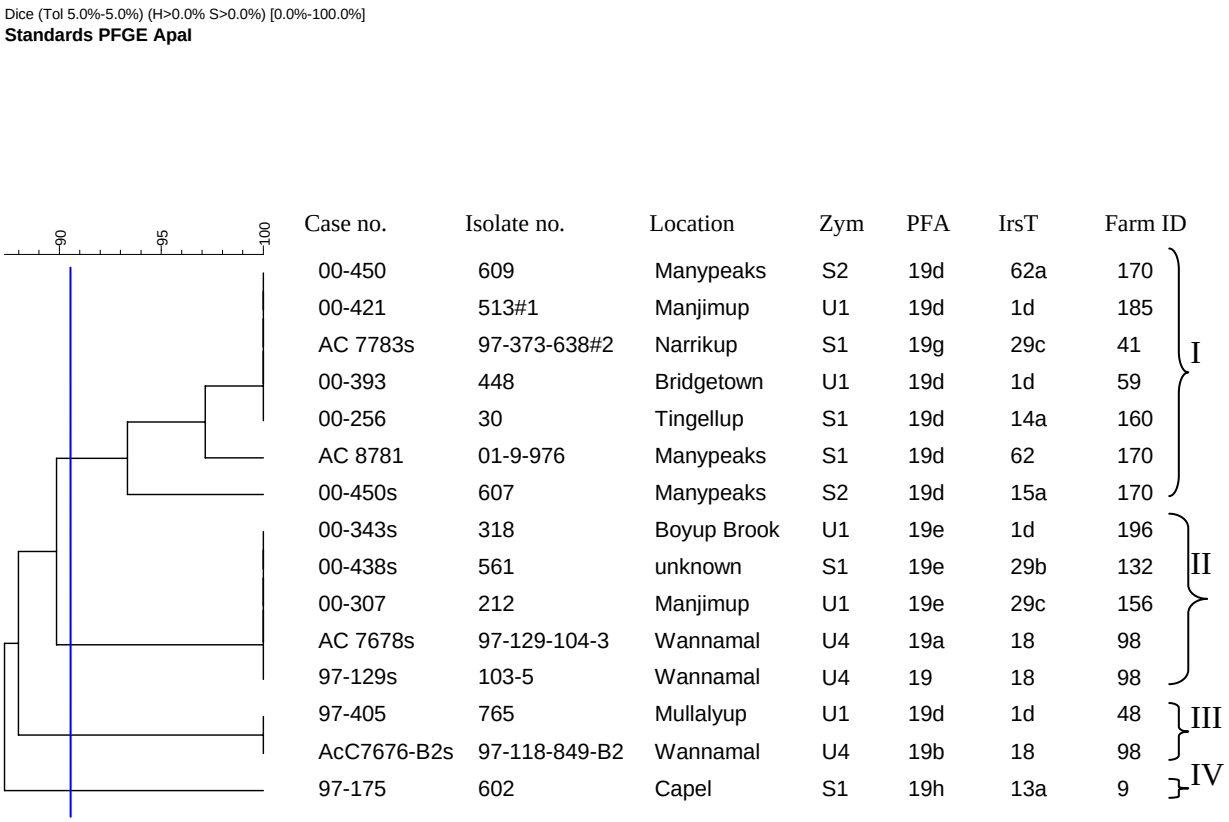


Figure 6-5. Dendrogram of 15 strains from PFA 19 clonal group from 11 properties in WA

IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; no. = number; Zym = zymogram profile. I-IV = clusters identified.

Four clusters (I, II, III and IV) were identified at a level of 90% relatedness (Figure 6-5). Cluster I contained seven isolates at 93% similarity. Six (85.7%) isolates from four farms were of molecular type PFA 19d. One isolate of type PFA 19d was identified as zymogram type U1, while all other PFA 19d types were identified as either S1 or S2 (Figure 6-5). Cluster II contained five isolates that were identified by the dendrogram as being 100% related. These isolates were genetically similar by PFA, and were predominated by isolates from PFA 19e molecular type (60%, n = 3) with one isolate identified as PFA 19 and another isolate identified as PFA 19a. Clusters III and IV

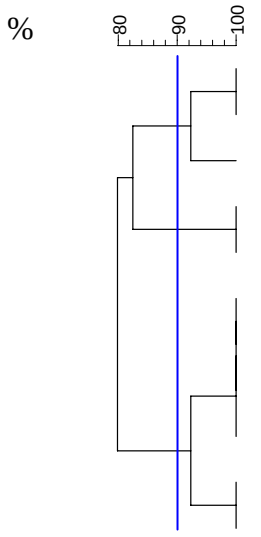
contained two and one isolate, respectively. One isolate (97-405-765) was identified as having molecular types PFA 19d and IrsT 1d and was located in cluster III of the dendrogram (Figure 6-5). One other isolate that had identical PFA and IrsT types was located in cluster I. Both fingerprints were checked visually on the electrophoresis gels and in the GelCompar II software.

Strains identified as protease heat stable (S strains) were located in clusters I, II and IV, whereas U strains were located in clusters I, II and III.

Due to the small number of isolates the diversity index was not calculated.

Clonal group PFA 12

Clonal group PFA 12 contained 4.6% (n = 34) of all 735 isolates tested, and these consisted of 94.1% (n = 32) of all U strains tested, and 5.9% (n = 2) of all S strains tested (Table 6-1). The dendrogram prepared from 11 isolates from eight properties indicated that isolates were related between 80% to 100% (Figure 6-6).



Case no.	Isolate no.	Location	Zym	PFA	IrsT	Farm ID	
00-468s	666	Kojonup/Leasd.	U5	12e	5	138	I
AC 8777	01-9-974	Manypeaks	S1	12e?	1b	170	I
00-269	67	Boyup Brook	S1	12f	59	99	I
96-22s	978	Boddington	U1	12c	1	7	II
00-530	874	Albany	U5	12c	1d	302	II
AC 5944s	5944	Kojonup	U5	12	5	11	III
00-450	610	Manypeaks	S2	12	18c	170	III
AC 8572	00-287-13.	NK	U5	12	5	151	III
AC 6361	AC 6361	Kojonup	U5	12	5	11	III
97-210s	864	Coomberdale	U1	12a	5b	38	III
AC 8537	00-209-889	Kojonup	U5	12	5	138	III

Figure 6-6. Dendrogram of 11 strains from PFA 12 clonal group from eight properties in WA

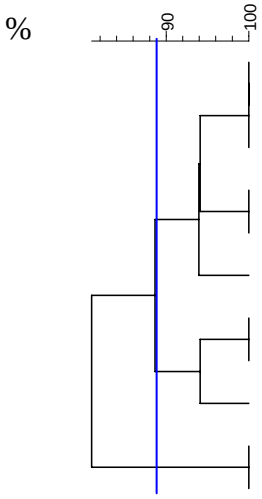
AC = Albany culture number; IrsT = molecular type obtained using primer PXT and IRS-PCR method; NK = not known; no. = number; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; s = denotes standard molecular type; Zym = zymogram profile. I-III = clusters identified.

Three clusters were identified at a level of 90% relatedness. Cluster I contained three isolates from three farms comprising molecular types PFA 12e and PFA 12f, which were 92% similar (Figure 6-6). All isolates had different IrsT types. Cluster II contained two

isolates, both of which were PFA 12c. Cluster III harboured six isolates of which five were PFA 12 and one isolate was PFA 12a, which were related at a level of 92% similarity. Five of the isolates in cluster III had corresponding IrsT types of either IrsT 5, or IrsT 5b. U5 strains were present in each cluster, but the majority (66.7%, n = 4) were found in cluster III. One S2 strain was found in cluster III, which had a molecular type of PFA 12 (as per all the isolates in cluster III), but had a different IrsT type of IrsT 18c.

Clonal group PFA 8

Clonal group PFA 8 contained 2.6% (n = 19) of all 735 isolates tested, and these consisted of 94.7% (n = 18) U strains and 5.3% (n = 1) S strains (Table 6-1). The dendrogram prepared from 11 isolates (from 11 properties) identified within clonal group PFA 8, indicated that isolates were related at a level of 82% to 100% (Figure 6-7).



Case no.	Isolate no.	Location	Zym	PFA	IrsT	Farm ID	
9055s	01-362-181	Mt Barker	U5	8e	29b	157	I
9054	01-361-177	Rocky Gully	U5	8e	29b	199	
98-9	124	Katanning	S4?	8e	10	226	
AC 7825s	98-30-177	Mt Barker	U5	8	29b	64	
00-203-843#2	#2	WRS	U5	8	29	115	
00-303s	198	unknown	U5	8c	29a	230	
00-143s	306iii	WRS	U5	8b	29d	137	II
AC 8060	98-483-5326	Albany	U5	8b	29c	85	
00-300	190#2	Mt Barker	U5	8a	29a	163	
00-336s	292	unknown	U5	8d	29a	218	III
01-192	657	Boyup Brook	U5	8d	29a	221	

Figure 6-7. Dendrogram of 11 strains from PFA 8 clonal group from 11 properties in WA

IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; no. = number; Zym = zymogram profile. I-III = clusters identified.

Three clusters (I, II, III) were identified at a level of 90% relatedness (Figure 6-7). Isolates (n = 6) from molecular types PFA 8, 8c or 8e were present in Cluster I, and were related at a level of 94% similarity. Isolates from molecular types PFA 8a and 8b were present in cluster II, and both isolates in cluster III were PFA 8d. Ten (90.9%) of the 11 isolates in the dendrogram had a corresponding IrsT type from the clonal group IrsT 29, and all ten isolates were U5 strains. One isolate had the zymogram profile of S4 and this had a different IrsT type being IrsT 10 (Figure 6-7).

These findings will be expanded in section 6.3.2: relationship of molecular type to zymogram type.

6.3.2 Investigation of molecular type related to zymogram type

The association between molecular types and zymogram types is summarised in Table 6-3, using the clonal groups that contained multiple isolates. The zymogram types of U5, U6 and T are discussed in detail, separately. As stated previously, the majority of S strains were found in either clonal group PFA 7 or PFA 11, with only 4.3% of all 516 S strains found in clonal group PFA 9 (Table 6-3).

Table 6-3. Summary of relationship between molecular type and zymogram profile in the six most common clonal groups

Phenotype	Isolates	PFA 7	PFA 11	PFA 9	PFA 12	PFA 19	PFA 8
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Total Isolates	735	135 (18.4)	158 (21.5)	94 (12.8)	34 (4.6)	32 (4.3)	19 (2.6%)
Total S	516 (70.2)	128 (24.8)	141 (27.3)	22 (4.3)	2 (0.3)	14 (2.7)	1 (5.3)
Total U	219 (29.8)	7 (3.2)	17 (7.8)	72 (32.9)	32 (14.6)	18 (8.2)	18 (94.7)
Zymogram Types		N	N	N	N	N	N
S1	497 (96.3)	125	136	22	2	7	0
S2	19 (3.7)	3	5	0	0	6	0
S3	1 (0.2)	0	0	0	0	0	0
S4	1 (0.2)	0	0	0	0	0	1
S2/U4	1 (0.2)	0	0	0	0	1	0
U1	115 (52.5)	1	3	46	25	13	1
U2	9 (4.1)	0	0	5	0	0	0
U3	8 (3.6)	0	0	6	0	1	0
U4	11 (5.0)	1	0	1	0	4	0
U5	47 (21.5)	1	5	10	7	0	17
U6	14 (6.4)	3	5	4	0	0	0
U6/U4	3 (1.4)	0	0	0	0	0	0
U6 (A198)	4	0	0	0	0	0	0
T	12 (5.5)	1	4	0	0	0	0

Isolates = number of isolates with each zymogram profile and the percentage that it represents of all 735 isolates; N = number; PFA 7 etc = molecular type using restriction enzyme *Apa*I and PFGE method; S = heat stable protease; S1-S4 = Zymogram profile; T = T strains; U = heat labile protease; U1-U6 = zymogram profile; U6 (A198) = U6 strains associated with isolate A198. The percentage of zymogram types was calculated from the total number of S strains (n = 516), or total number of U strains (n = 219).

The most common isoenzyme profile for S strains was S1, which comprised 96.3% (n = 497) of S strains in the database. Of the six main clonal groups (PFA 7, 9, 8, 11, 12, and 19), S1 isolates were found in all groups, but the majority of S1 isolates were found in groups PFA 7 and PFA 11 (Figure 6-2). There were 3.7% (n = 19) of S2 strains and

these occurred in clonal groups PFA 7, PFA 11 and PFA 19. There was one each of S3 and S4 strains, but none of these occurred in the major clonal groups. The isoenzyme type S2/U4 (n = 1) occurred in clonal group PFA 19 (Table 6-3).

In clonal group PFA 11 there were 17 (10.7%) U strains. These were composed of five U5 strains, five U6 strains, four T strains, and three U1 strains. Clonal group PFA 7 contained one isolate each of U1, U4, U5 and T, with three U6 isolates, and together these represented 3.2% of all U strains. The interpretation of the molecular type in relation to these zymogram profiles is discussed in each of the next specific sections.

The majority (32.9%) of U strains were found in clonal group PFA 9 (Figure 6-1, 6-8).

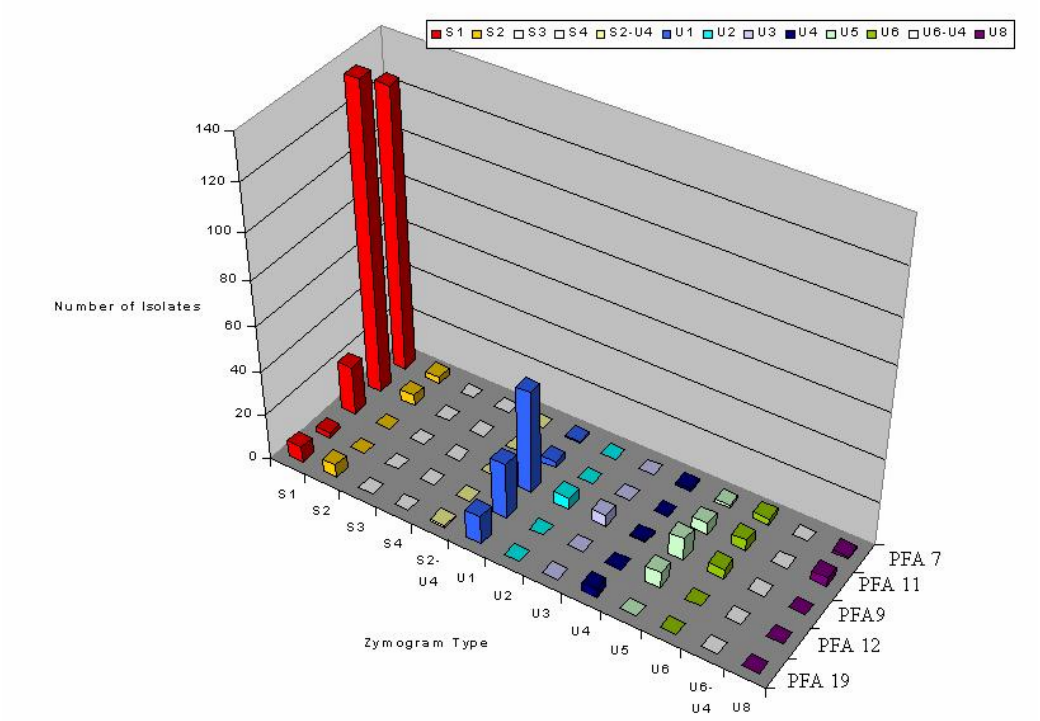


Figure 6-8. Distribution of zymogram types among five selected PFA clonal groups

In this figure, U8 = T strains.

Nine isoenzyme profiles were recognised in the zymogram method for U strains, and amongst the 219 U isolates, 52.5% were U1 and 21.5% were U5 (Table 6-2). U1 strains were predominant in clonal groups PFA 9 (n = 46), PFA 12 (n = 25) and PFA 19 (n = 13) (Figure 6-2). One U1 strain and three U1 strains were found in PFA 7 and PFA 11, respectively.

The U1 strain that had molecular type PFA 7c was found on Farm 6. Only U strains were found on this property, however it was noted in the clinical notes that 1% of infected sheep had lesion score 4 or 5. U5 strains were also detected on this property. The U5 isolates typed as PFA 9d or PFA 9o. No lesion score was recorded for the U1 isolate with PFA 7c.

Of the three U1 isolates that were in clonal group PFA 11, two were PFA 11a from Farm 20, and one was type PFA 11e from Farm 112. In all, 18 isolates from 10 farms typed as PFA 11a. The U1 and U5 isolates of PFA 11a came from Farm 20, which also had an S1 isolate with PFA 11a type. The U5 (PFA 11a) isolate from Farm 20 had been pen-tested for virulence and was identified as a virulent strain (Table 6-11).

The S1 strains that were found in clonal group PFA 9 were composed of nine subtypes (PFA 9, 9a, 9b, 9c, 9d, 9L, 9L+, 9m and 9u). These subtypes also were recorded for U strains. All S strains of clonal group 9 were found on 14 properties. Three of these (Farms 38, 39 and 40) were owned by the same company, with the farms located within the same Shire but separate from each other. The S strains on these properties were identical in PFA type and IrsT type to U strains found on these properties. On the other properties, only S strains were selected for molecular typing and, therefore, it was not known if U strains also occurred on the same property. Farm 120 had two S1 strains that both typed as PFA 9d and IrsT 1d. In the clinical notes the lesion scores were rated mild and scored 2 and 3a. One other property (Farm 101) had two S1 strains of type PFA 9d. The strains were IrsT 11a and IrsT 12. There was no record of the lesion score for these isolates. Eighteen isolates had the PFA 9d type, and 14 of these were U strains being U1 (n = 6), U2 (n = 3), or U5 (n = 4). The IrsT types varied. There was no record of the clinical expression or lesions score seen in sheep with these isolates.

One isolate detected in 1990 (AC 2467) on Farm 59, gave the molecular type PFA 9a and IrsT 13 and was identified as protease heat labile in the gelatin-gel test, but S1 according to the zymogram test. An isolate detected on this property in the year 2000 was identified as having an equivocal result in the gelatin-gel test, and was identified as a U1 in the zymogram profile and was typed as PFA 19d, IrsT 1d. The sheep infected with this isolate were rated as having mild foot lesions in the clinical notes, with a comment of the possibility that the lesions were due to foot scald. In the clonal group PFA 19 there were 56.2% (n = 18) U strains and 43.7% (n = 14) S strains. U strains included U1 (n = 13),

U4 (n = 4) and U3 (n = 1). Of the U4 isolates, two gave an equivocal result in the gelatin-gel test.

One isolate that produced anomalous results in the gelatin-gel test and zymogram profile proved to belong to the clonal group PFA 19. This isolate (AC 7676 from Farm 98) was equivocal in the gelatin-gel test and was assigned a zymogram type of S2/U4. Six subcultures of the isolate all produced the same results for all tests.

The PFA 19 clonal group had a very similar visual banding pattern to PFA 12 clonal group. The predominant zymogram type in clonal group PFA 12 was U1 (n = 25), followed by U5 (n = 7). Two S1 strains occurred in clonal group PFA 12 (Table 6-3).

Clonal group PFA 8 comprised 19 strains, which was 2.6% of the entire collection of isolates. PFA 8 clonal group contained 18 U strains (8.2% of the total U strains), and of these 17 were U5 zymogram type. Also included in clonal group 18 was one U1 strain and one S4 strain (Table 6-3).

Isolate AC 3107, which was subcultured six times and gave a zymogram profile of U6/U4 is discussed under the U6 isolates, section 6.3.2.2.

6.3.2.1 Molecular type of U5 strains in WA

Amongst the U5 strains, some had been detected in lesions showing a severe or virulent form of footrot. A total of 47 U5 isolates occurred in the collection of 735 isolates (6.4%), and these were found on 27 farms (10.9%). Twenty-four PFA types were identified amongst the U5 strains, and these grouped into 11 clonal groups. The results with the IRS-PCR indicated there were 17 IrsT types and these grouped into nine clonal groups. These results are presented in Table 6-4.

The highest percentage of isolates occurred across four clonal groups, these being PFA 8, PFA 9, PFA 12 and PFA 11. The greatest number of U5 strains belonged to clonal group PFA 8 (36.2%; n = 17), and were found on 10 properties. This was followed by clonal group PFA 9, which contained 21.3% (n = 10) of U5 strains and occurred on six properties (Table 6-4). Clonal groups PFA 12 and PFA 11 contained 14.9% and 10.6% of isolates, respectively.

Table 6-4. Distribution and molecular types of U5 strains of *D. nodosus* from WA

Isolate Number	Farm ID	Zym	PFA	PFX	IrsT	IrsG
3264	5	U5	7k	nt	13g	1
8058	5	U5	37a	nt	1d	nt
97-180-624	6	U5	9d	nt	5	nt
97-180-628	6	U5	9o	nt	5	nt
97-432-930	6	U5	9d	nt	5	nt
97-432-933-1	6	U5	9d	nt	5a	nt
97-432-936	6	U5	9d	nt	5a	nt
5944	11	U5	12	nt	5	5
6361	11	U5	12	nt	5	5
6971	20	U5	11a	nt	2	nt
97-060-114	20	U5	11a	nt	nt	nt
97-060-115	20	U5	11a	10	2	nt
02-029-834	52	U5	9o	nt	1c	nt
6161	64	U5	8	nt	6	nt
7825	64	U5	8	nt	29b	nt
98-467-205-2	64	U5	8	28	29	nt
5679	68	U5	18	11	1g	5
8060	85	U5	8b	nt	29c	nt
8061	85	U5	8b	nt	29b	nt
4307	95	U5	9r	nt	30	nt
02-048-896	100	U5	21a	nt	2d	nt
97-106-679	115	U5	9e	nt	1d	4
00-143-306	137	U5	8b	29	29d	nt
00-143-308	137	U5	8b	nt	nt	nt
00-143-310	137	U5	8b	nt	nt	nt
00-143-314	137	U5	8b	nt	nt	nt
8536	138	U5	64a	nt	1d	nt
8537	138	U5	12	nt	5	nt
00-468-666	138	U5	12e	nt	nt	nt
00-468-669	138	U5	12	nt	5	nt
8231	139	U5	9s	nt	21	nt
8572	151	U5	12	nt	5	nt
8586	153	U5	8a	nt	29b	nt
9055	157	U5	8e	nt	29b	nt
00-300-190	163	U5	8a	nt	29a	nt
8870	174	U5	37	nt	5	nt
9054	199	U5	8e	nt	29b	nt
00-336-292	218	U5	8d	nt	29a	nt
01-192-655	221	U5	8d	nt	nt	nt
01-192-657	221	U5	8d	nt	29a	nt
7836	225	U5	11a	nt	1d	nt
98-067-394	225	U5	11a	nt	1d	nt
00-303-198	230	U5	8c	nt	29a	nt

Table 6-4 (continued)

Isolate Number	Farm ID	Zym	PFA	PFX	IrsT	IrsG
8005	235	U5	52	nt	28	nt

8015A	239	U5	9a	nt	13g	nt
00-530-873	302	U5	1a	nt	29b	nt
00-530-874	302	U5	12c	nt	1d	nt

Farm ID = Farm identification number; IrsG = molecular type using primer PXG and IRS-PCR method; IrsT = molecular type using primer PXT and IRS-PCR method; nt = not tested; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; PFX = molecular type using restriction enzyme *Xba*I and PFGE method; U5 = heat labile protease, zymogram profile 5; Zym = zymogram profile.

The 17 molecular types identified amongst the U5 isolates using the IRS-PCR method and primer PXT could be grouped into nine clonal groups (Table 6-4, Table 6-5).

Table 6-5. Distribution of 11 PFA clonal groups amongst 47 U5 strains on 27 farms in WA

Clonal Group	No. of isolates	% of Isolates	No. of Farms	% of farms overall
PFA 8	17	36.2	10	4.0
PFA 9	10	21.3	6	2.4
PFA 12	7	14.9	4	1.6
PFA 11	5	10.6	2	0.8
PFA 37	2	4.3	2	0.8
PFA 1	1	2.1	1	0.4
PFA 7	1	2.1	1	0.4
PFA 18	1	2.1	1	0.4
PFA 21	1	2.1	1	0.4
PFA 52	1	2.1	1	0.4
PFA 64	1	2.1	1	0.4

Number of farms with U5 strains = 27; number of farms overall = 247.

Five U5 strains were typed by IRS-PCR using primer PXG, and amongst these there were three molecular types. Two isolates from Farm 11 (AC 5944, AC 6361) had the same PFA (PFA 12), IrsT (IrsT 5) and IrsG (IrsG 5) molecular types. One isolate from Farm 68 also had IrsG 5 molecular type, but had different PFA and IrsT types (Table 6-4).

The greatest percentage of U5 strains (78%, n = 32) occurred in one of three clonal groups, these being groups IrsT 29, IrsT 5 and IrsT 1 (Table 6-6). Of these the highest number (31.7%, n = 13) of isolates were found in clonal group IrsT 29, and these occurred on 11 properties in WA (Table 6-6).

Table 6-6. Distribution of nine clonal groups identified by IrsT molecular types amongst 41 U5 isolates from 27 properties in WA

Clonal Group	No. of isolates	% of Isolates	No. of Farms	% of farms overall
IrsT 29	13	31.7	11	4.5
IrsT 5	11	26.8	5	2.0
IrsT 1	8	19.5	7	2.8
IrsT 2	3	7.3	2	0.8
IrsT 13	2	4.9	2	0.8
IrsT 6	1	2.4	1	0.4
IrsT 21	1	2.4	1	0.4
IrsT 28	1	2.4	1	0.4
IrsT 30	1	2.4	1	0.4

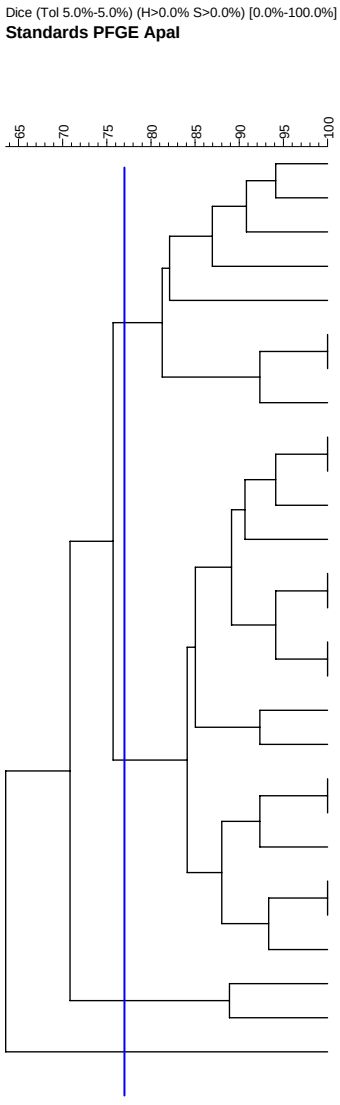
Number of U5 isolates typed by IRS-PCR = 41; number of farms with U5 strains typed by IRS-PCR = 27; number of farms overall = 247.

Zymogram U5 strains were isolated from virulent lesions in infected sheep on three properties in WA; Farms 6, 20 and 68 (Table 6-4). On Farm 6, PFA types 9d and 9o were identified and these were either IrsT 5 or IrsT 5a. Amongst infected sheep on the farm, 1% of the foot lesions were classified as virulent lesions, being score 4 or 5. Also on Farm 6, which had 1% of lesions classified as virulent, one isolate was identified as U1 and typed as PFA 7c and IrsT 5a. Type PFA 7c included 20 isolates collected from seven farms over the years 1991 to 2001, and all except the isolate from Farm 6 had a zymogram profile of S1.

Farm 68 had one U5 isolate (AC 5679) with molecular types PFA 18 and IrsT 1g that was associated with a virulent lesion. This occurred in infected sheep that had been purchased from the eastern States of Australia. The farm subsequently underwent an eradication program, and no other isolates of this type have been found since in WA.

Farm 20 also had sheep with virulent lesions from which U5 strains were isolated, and these had types PFA 11a and IrsT 2. No S strains were detected on this farm. Eighteen isolates in the collection were molecular type PFA 11a and these were isolated from nine farms including Farm 20 over the years 1991 to 2002. They were composed of 11 S1 strains, five U5 strains and two U1 strains. The U5 and U1 strains came from Farm 20. As stated previously in section 6.3.2 the U5 strain (PFA 11a) from Farm 20 produced virulent lesions in a pen trial.

The remaining U5 isolates have never been classified as virulent. On Farm 64 only U strains were detected. The farmer had purchased an infected ram in 1994 from the eastern States of Australia. The lesions in this case of footrot were always mild, which was confirmed by pen-testing, and eradication was not required. The PFA type was PFA 8. Strains of subtypes of PFA 8, 8a, 8b, 8c, 8d and 8e all isolated since 1998 were identified on eight farms in WA, including Farm 64. None of these infections resulted in virulent lesions, and the clinical notes have always indicated a benign lesion. Isolates from two of these farms (Farm 85 and 137) were pen-tested and were benign.



Case no.	Isolate no.	Location	Zym	PFA	IrsT	Farm ID	
97-180s	628s	Boyup Brook	U5	9o-1	5	6	I
00-530	874	Albany	U5	12c	1d	302	
97-432s	930s	Boyup Brook	U5	9d	5	6	
02-29s	834	Rocky Gully	U5	9o	1c?	52	
8870	01-64-189	Cranbrook	U5	37	5	174	
AC 5944s	5944	Kojonup	U5	12	5	11	
AC 8572	00-287-135D	NK	U5	12	5	151	
AC 8537	00-209-889	Kojonup	U5	12	5	138	
00-143s	306iii	WRS	U5	8b	29d	137	II
AC 8060	98-483-5326	Albany	U5	8b	29c	85	
00-300	190#2	Mt Barker	U5	8a	29a	163	
00-530	873	Albany	U5	1a	29b	302	
9055s	01-362-181	Mt Barker	U5	8e	29b	157	
9054	01-361-177	Rocky Gully	U5	8e	29b	199	
AC 7825s	98-30-177	Mt Barker	U5	8	29b	64	
98-467	205#2	Mt Barker	U5	8	29b	64	
00-468s	666	Kojonup/Leas.	U5	12e	5	138	
AC 8058s	98-481-317#2	Cranbrook	U5	37a	1d	5	
00-336s	292	unknown	U5	8d	29a	218	
01-192	655	Boyup Brook	U5	8d	29a	221	
AC 3264s	3264	Cranbrook	U5	7k	13g	5	
98-67	394	Pemberton	U5	11c	1d	225	
97-60	115a	Boyup Brook	U5	11a	2	20	
00-303s	198	unknown	U5	8c	29a	230	
AC 8231s	00-189-65	Bridgetown	U5	9s	21	139	III
AC 5679s	5679	Kojonup	U5	18	1g	68	
AC 8536s	00-209-888	Kojonup	U5	64a	1d	138	IV

Figure 6-9. Dendrogram showing genetic relatedness by PFA typing of 27 U5 strains from 22 farms in WA

IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; no. = number; Zym = zymogram profile. I-IV = clusters identified.

Farm 5 had a combination of U1, U5 and S1 strains and all had different PFA types. The U5 isolates on this farm were typed as PFA 37a (IrsT 1d) and PFA 7k (IrsT 13g).

The Simpson diversity index for the U5 isolates was 0.95, where N = 47, N(N-1) = 2162, $\Sigma nj(nj\text{-}1) = 100$, and D = 1-(100/2162). The simple diversity ratio for the 24 molecular types found amongst the 47 isolates was 1:1.9.

A dendrogram of 27 strains from 22 properties showed that the U5 strains were related between 64% and 100% (Figure 6-9). At a level of 76% relatedness, four clusters (I – IV) were recognised (Figure 6-9). Cluster I harboured eight strains from seven farms, and consisted of six PFA types that were related at 91% similarity. Six strains (75%) in cluster 1 were IrsT 5 molecular type. One IrsT 5 type was identified in cluster II. Cluster II harboured the majority of isolates, which were related at 76%, and comprised 16 from 14 farms. Twelve PFA types occurred in cluster II and ten (62.5%) of these belonged to clonal group PFA 8. Ten of the isolates belonged to IrsT 29 clonal group, and all but one of these had a corresponding PFA clonal type of PFA 8. Clusters III and IV harboured two and one isolate, respectively.

6.3.2.2 Molecular type of U6 strains

The U6 strains (n = 21) constituted 2.8% of all isolates tested in this study. Thirteen different molecular types from 7 clonal groups (clonal groups PFA 4, 7, 9, 11, 21, 39, 83), were identified in this group of isolates (Tables 6-7 and 6-8).

Table 6-7. Distribution of seven clonal groups amongst 21 U6 strains on 14 farms in WA

Clonal Group	No. of Isolates	% of Isolates	No. of Farms	% of Farms Overall
PFA 11	5	23.8	4	1.6
PFA 9	4	19.0	3	1.2
PFA 4	4	19.0	1	0.4
PFA 7	3	14.3	3	1.2
PFA 39	3	14.3	1	0.4
PFA 21	1	4.8	1	0.4
PFA 83	1	4.8	1	0.4

% of isolates = percentage of U6 strains where n = 21; No. of farms = number of farms on which a U6 strain was detected; % of farms overall = percentage of farms overall where U6 strain was detected where total number of farms = 247.

U6 isolates were identified on 14 (5.7%) farms.

The most common molecular type was PFA 11e, with five U6 strains of this type (Table 6-7). Over the whole WA collection, 29 isolates had molecular type PFA 11e (3.9%) and this group also contained 23 isolates with S1 zymogram profile, and one isolate of U1. The U1 isolate came from Farm 112, which also contained S1 isolates with the molecular type PFA 11e (Table 6-8).

Table 6-8. Distribution and molecular types of 21 U6 strains compared to other strains on WA farms

Isolate Number	Farm ID	Zym	PFA	PFX	IrsT	IrsG
6251	57	U6	11e	6	3a	3
7117	57	S1	11e	5	2	nt
6612	72	U6	11e	7	14	2a
6613	72	U6	11e	7	nt	nt
6502	76	U6	11e	7	14	2a
7022	112	U6	11e	7	14	2a
7136	112	S1	11e	7	14	2a
6476	97	U6	9c	8	1	nt
6412	97	S1	9	8	1a	nt
7165	63	U6	9e	4	1	nt
96-025-995	63	U6	9e	nt	1a	nt
7781	101	U6	9d-1	9	11	nt
7782	101	S1	9d	21	11a	nt
4821	100	U6	21	nt	nt	nt
4288	100	S1	21	nt	2	nt
5136	129	U6	7	22	2	nt
01-506-673	1	U6	7	nt	9	nt
01-478-573	1	S1	7	nt	13	nt
02-075-992	207	U6	7i	nt	2	nt
02-075-993	207	S1	7e	nt	cont	nt
9249	233	U6	83c	nt	13c	nt
02-146-257	233	S1	83c	nt	13c	nt
3107	51	U6/U4	39	23	1a	1
3107	51	U6/U4	39c	23	1a	nt
3107	51	U6/U4	39a	23	1a	nt
3109	51	U1	39b	24	1a	nt
00-151-547	303	U6	4b	25	26	nt
00-167-480	303	U6	4b	25	26	nt
00-167-507	303	U6	4b	25	26	nt
00-193-796#1	303	U6	4b	nt	26	nt
00-164-445	303	S1	4b	25	26	nt

Isolates grouped according to Farm number and PFA type. Cont = contaminated; Farm ID = Farm identification number; IrsG = molecular type using primer PXG and IRS-PCR method; IrsT = molecular type using primer PXT and IRS-PCR method; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; PFX = molecular type using restriction enzyme *Xba*I and PFGE method; nt = not tested; S = thermostable protease; U = heat labile protease; Zym = zymogram profile.

Isolates of *D. nodosus* on three of the four farms (Farms 72, 76 and 112) that had U6 isolates of type PFA 11e had identical molecular types overall (PFA 11e, PFX 7, IrsT 14 and IrsG 2a) (Table 6-8). An isolate from Farm 57 also was of PFA 11e, but had different PFX, IrsT and IrsG types. No data were available to determine if these farms had traded sheep with each other. All farms were in different geographical areas. All U6 types with the molecular type of PFA 11e were isolated in 1994 and 1995.

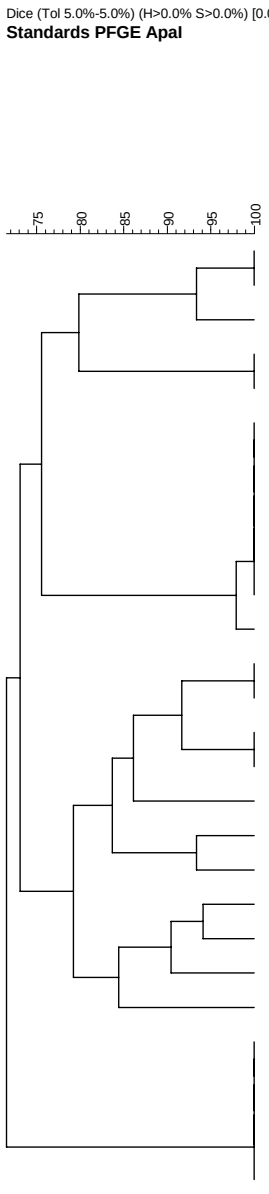
Nine properties, including the property on which a trial to assess the difficulty of the eradication of isolate A198 was undertaken (Jelinek and Depiazzi, 2003), had both an S1 strain and a U6 strain detected. On six of these farms (Farms 1, 57, 112, 100, 233, 303) the U6 strains had the same PFA type as an S1 strain on the same farm (Table 6-8). On three of these farms (Farms 112, 233, 303) the S1 and U6 isolates on the same farm had the same IrsT type, and on Farms 112 and 303 the U6 and S1 strains also had the same PFX type. The U6 isolate on Farm 112 had the same molecular type by all four methods as the molecular types obtained for the S1 strain on that farm. On three other farms (Farms 97, 101, 207) the U6 strain on the individual farm had a PFA molecular type that was a subtype of the molecular type obtained for the S1 strain. This result was obtained on Farm 207 where the S1 strain was molecular type PFA 7e and the U6 strain was molecular type PFA 7I (Table 6-8). The U6 and S1 isolates on Farm 97 had the same PFX type (PFX 8) but had molecular types PFA 9c and PFA 9, respectively.

All strains from Farms 63, 72, and 76 were U6 strains, and therefore no other strains were available for comparison.

One isolate (AC 3107) from Farm 51 was subcultured three times. The isolate and the subcultures were difficult to type according to the zymogram profile and were subsequently classified as U6/U4 (M. Palmer, Australian Reference Laboratory for Ovine Footrot, pers comm). The three subcultures of isolate AC 3107 had PFA molecular types of PFA 39, 39a, and 39c, but all had the same PFX type (PFX 23) and the same IrsT type (IrsT 1a). All molecular subtypes of isolate AC 3107 came from a single hoof in an infected animal. These results are also discussed under hoof epidemiology in Chapter 5 (section 5.3.4).

Farm 51 also had a U1 strain, AC 3109, and this typed as PFA 39b, PFX 24 and IrsT 1a. It was identical by IrsT type to the subcultured isolates from AC 3107, but had a one-band difference and therefore was genetically similar by PFA and PFX type (Table 6-8).

Four U6 isolates on Farm 303 that were detected following artificial infection of sheep with isolate A198 had the same PFA, PFX and IrsT molecular types as the S1 strain (Table 6-8). These results are discussed in detail in chapter 7.



Case no.	Isolate no.	Location	Zym	PFA	IrsT	Farm ID
AC 6412	97-124-16B	Mt Barker	U1	9	1a	97
AC 6476s	97-124-17	Mt Barker	U6	9c	1	97
AC 7165	97-124-32	Boyup Brook	U6	9e	1	63
AC 9249	02-146-255	Tenterden	U6	83c	13c	233
02-146s	257	Tenterden	S1	83c	13c	233
AC 6251	97-124-15	Narrikup	U6	11e	3a	57
AC 6612	97-124-21	Donnybrook	U6	11e	14	72
AC 6613	97-124-22b	Donnybrook	U6	11e	14	72
AC 6502	97-124-18	Helena Valley	U6	11e	14	76
AC 5336	97-145-341	Narrikup	S1	11e	14	112
AC 7022	AC 7022c	Narrikup	U6	11e	14	112
AC 7117	97-124-25	Narrikup	S1	11e	2	57
AC 3107s	97-124-11B1a	Donnybrook	U6/U4	39a	1a	51
AC 3107s	97-124-11B2b	Donnybrook	U6/U4	39	1a	51
02-118	123	Mt Barker	U6	21	2	100
01-516	707	Mt Barker	S1	21	ND	100
AC 3107s	97-124-11c	Donnybrook	U6/U4	39c	1a	51
97-369s	623a	Donnybrook	U6	9d-1	11	101
AC 3109s	97-124-11c	Donnybrook	U6/U4	39b	1a	51
02-75	992	Busselton	U6	7i	2	207
01-506	673	Katanning	U6	7	9	1
02-75	993	Busselton	S1	7e	NR	207
97-369	626#1	Donnybrook	S1	9d	12	101
00-167-498s	ex A198	WRS	S1	4b	26	115
00-167-507	ex A198	WRS	U6	4b	26	115
00-151-547	ex A198	WRS	U6	4b	26	115
00-167-480	ex A198	WRS	U6	4b	26	115
00-193	796#1	WRS	U6	4b	26	115

Figure 6-10. Dendrogram showing genetic relatedness of U6 strains and corresponding zymogram types on the same farm

IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; no. = number; Zym = zymogram profile.

A dendrogram was constructed from the U6 strains and the other strains of different zymogram type but identical or similar molecular type that were isolated from the same farm (Figure 6-10). The U6 strains were related at a level of between 67% to 100%.

The Simpson index of diversity for U6 PFA types was 0.91, where N = 21, N(N-1) = 420, $\Sigma nj(nj\text{-}1) = 36$ and D = 1-(36/420) = 0.91. The simple diversity ratio was 1:1.6.

The Simpson index of diversity for U6 IrsT types was 0.89, where N = 20, N(N-1) = 380, $\Sigma nj(nj\text{-}1) = 42$, to give D = 0.89 (1-42/380). There were nine IrsT molecular types amongst the U6 strains. The simple diversity ratio for U6 strains with IrsT types was 1:2.

6.3.2.3 Molecular type of T strains

Of the isolates tested in this study, 12 (1.6%) had a T zymogram profile. Details of the molecular types obtained by PFGE and IRS-PCR methods are presented in Table 6-9.

Table 6-9. Molecular typing results for 12 T strains detected on four farms in WA in comparison to S strains on the same farm

Isolate Number	Farm ID	PFA	IrsT	Zym
97-341-488	67	11L	13	T
97-341-491	67	11L	nt	T
01-012-991	177	11c	13g	T
01-012-992	177	11c	13g	T
01-012-994	177	11c	13g	S
98-341-735	100	21	2d	T
00-505-788	100	21d	2d	T
00-505-789	100	21	2d	T
01-323-040	100	21a	nt	T
01-466-523	100	21a	2d	T
01-466-524	100	21e	2d	T
01-516-706	100	21e	2d	T
4288	100	21	2	S
4289	100	21e	2	S
9152	100	21a	2d	S
96-133-578	115	7	13a	T
7343	115	7	13a	S

Isolates grouped according to Farm number and PFA type. Farm ID = Farm identification number; IrsT = molecular type using primer PXT and IRS-PCR method; nt = not tested; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; T = designated zymogram profile.

The 12 T strains consisted of seven different molecular types from three clonal groups (PFA 7, 11, 21) when typed using PFA. The IRS-PCR method showed four

molecular types from two clonal groups (IrsT 2 and IrsT 13) for the ten isolates that were tested (Table 6-9).

The Simpson index of diversity for T strains according to PFA molecular types was 0.92, where N = 21, N(N-1) = 132, Σ*nj*(*nj*-1) = 10, where gives D = (1-10/132) = 0.92. The simple diversity ratio with seven molecular PFA types from 21 strains was 1:3.

The Simpson index of diversity for T strains according to IrsT type molecular types was 0.64, where N = 10, N(N-1) = 90, Σ*nj*(*nj*-1) = 32, such that 1-32/90 gives D = 0.64. The simple diversity ratio was 1:2.5.

A dendrogram prepared from nine T strains and two S strains present on four properties indicated that the T strains were related at a level between 77% to 100% similarity.

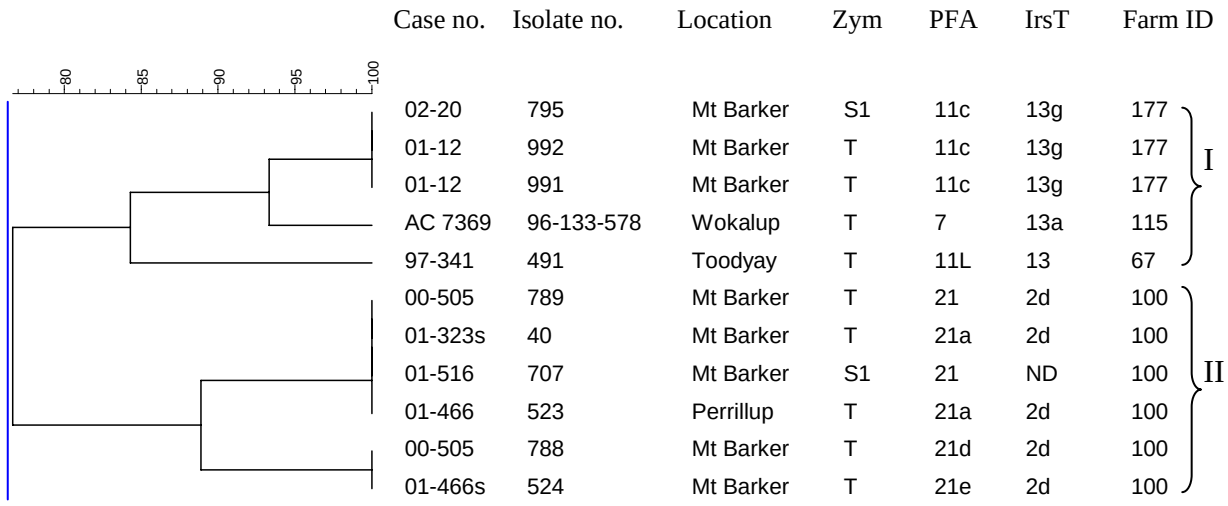


Figure 6-11. Dendrogram of nine T strains and two S strains from four farms in WA

IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; no. = number; Zym = zymogram profile. I, II = clusters identified.

Two main clusters were identified at 77% relatedness, with strains in cluster I being related to each other at 84%, and strains in cluster II being related to each other at 88% (Figure 6-11). Cluster I comprised four T strains and one S1 strain from Farms 67 and 177. Two T strains and one S1 strain from Farm 177 were 100% related on the dendrogram, and were molecular type PFA 11c and IrsT 13g. Isolate AC 7369 was PFA 7 and IrsT 13a from Farm 67 and was related to the other four isolates in cluster I at the 94% level. Cluster II comprised five T strains and one S1 strain from Farm 100. Three T strains were 100% related, according to the dendrogram, to the S1 strain. Two T strains

were identified as type PFA 21a, and IrsT 2d. One S strain and one T strain were identified as PFA 21. Isolates obtained from Farm 100 are discussed further in section 6.3.2.4.

6.3.2.4 Investigation of molecular type and zymogram type on Farm 100

Farm 100 had sheep infected with footrot at various times over a ten-year period. During this time isolates with zymogram profiles of S1, U5, U6 and T were detected. The predominant strain was S1. One U5, one U6 and seven T strains were detected. All the zymogram types belonged to the molecular clonal group PFA 21 as detected by PFGE and clonal group IrsT 2 as detected by IRS-PCR. Subtypes of PFA 21, 21a, 21d, 21e and subtypes IrsT 2, 2d were recognised (Table 6-10).

Table 6-10. Distribution of zymogram types and corresponding molecular types on Farm 100

Isolate Number	Farm ID	PFA	IrsT	Zym
92-1287-326	100	21e	2	S1
92-1287-325	100	21	2	S1
93-585-631	100	21	2	U6
98-341-735	100	21	2d	T
00-505-788	100	21d	2d	T
00-505-789	100	21	2d	T
01-323-040	100	21a	nt	T
01-466-523	100	21a	2d	T
01-466-524	100	21e	2d	T
01-516-706	100	21e	2d	T
02-164-311	100	21	2d	S1
02-026-823	100	21a	2d	S1
02-048-896	100	21a	2d	U5

Farm ID = Farm identification number; IrsT = molecular type using primer PXT and IRS-PCR method; nt = not tested; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; S = thermostable protease; U = heat labile protease; Zym = zymogram profile.

The S1 strains typed as PFA 21, PFA 21a and IrsT 2, IrsT 2d. The T strains also typed as PFA 21, 21a and 21d, but also had type PFA 21e that was not detected amongst the S strains that were identified. The T strains had the same IrsT molecular type as the S1 strains, which were identified as either IrsT 2 or IrsT 2d.

Examples of the PFA 21, 21a and 21e for S1 and T strains is shown in Figure 6-12. One U 5 strain was isolated in 2002 and was identified as molecular type PFA 21a, IrsT 2d. One U6 strain was isolated in 1993 and was identified as molecular type PFA 21 and IrsT 2.

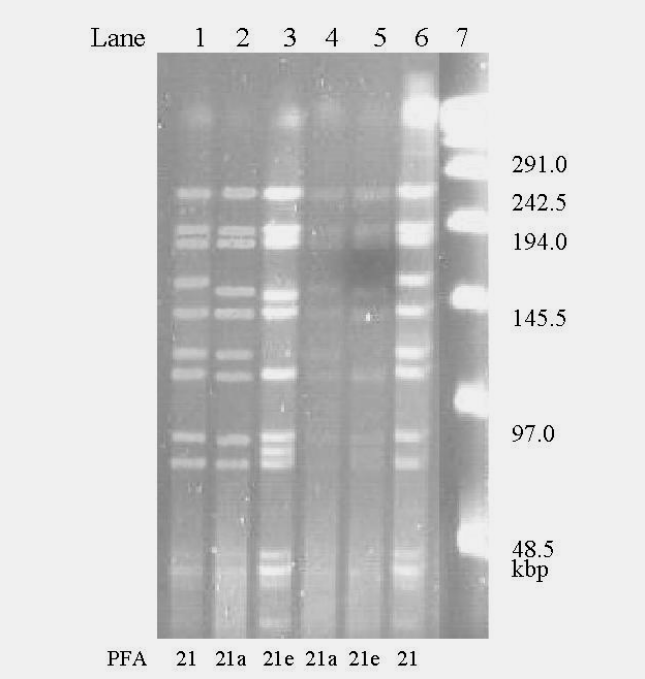
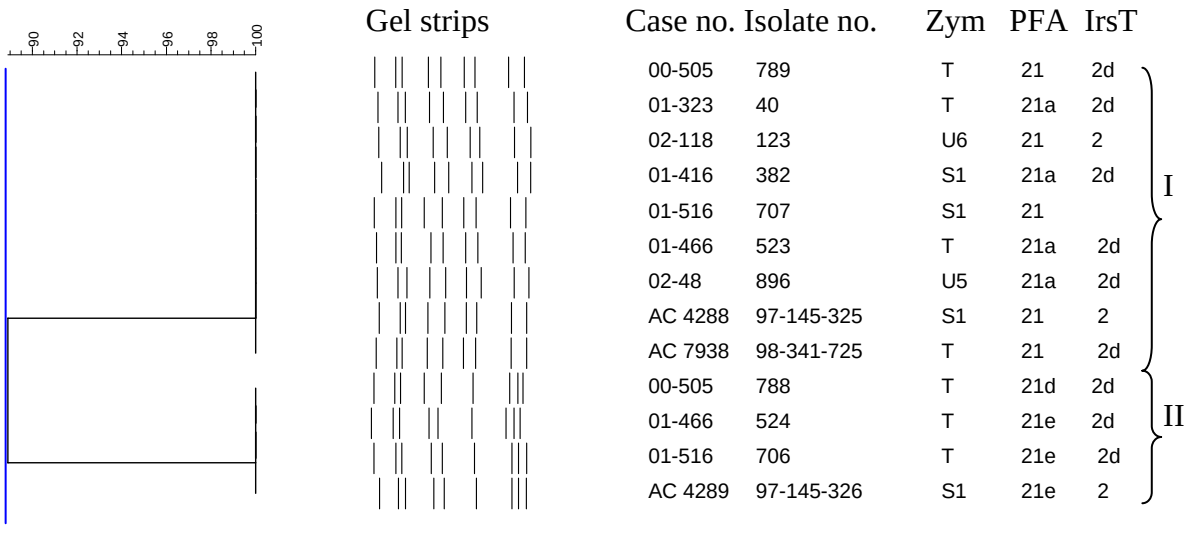


Figure 6-12. Three PFA molecular types from Farm 100

Lane 1 = AC 4288 (S1); Lane 2 = 01-466-523 (T); Lane 3 = 01-466-524 (T); Lane 4 = 02-26-823 (S1); Lane 5 = 01-516-706 (T); Lane 6 = AC 7938 (T); Lane 7 = lambda molecular weight marker.

A dendrogram prepared from 13 isolates representing PFA molecular types (PFA 21, 21a, 21d, 21e) and zymogram profiles S1, U5, U6 and T indicated that the isolates were 89% to 100% related, and formed two clusters at 89% similarity (Figure 6-13).



Case no.	Isolate no.	Zym	PFA	IrsT	
00-505	789	T	21	2d	I
01-323	40	T	21a	2d	
02-118	123	U6	21	2	
01-416	382	S1	21a	2d	
01-516	707	S1	21		
01-466	523	T	21a	2d	
02-48	896	U5	21a	2d	
AC 4288	97-145-325	S1	21	2	
AC 7938	98-341-725	T	21	2d	
00-505	788	T	21d	2d	II
01-466	524	T	21e	2d	
01-516	706	T	21e	2d	
AC 4289	97-145-326	S1	21e	2	

Figure 6-13. Dendrogram showing relatedness of 13 isolates detected on Farm 100

IrsT = molecular type obtained using primer PXT and IRS-PCR method; PFA = molecular type obtained using restriction enzyme *Apa*I and PFGE method; no. = number; Zym = zymogram profile. I, II = clusters identified. Gel strips showing digitised restriction fragments are indicated on the dendrogram.

The dendrogram identified PFA types 21 and 21a in cluster I as 100% related. The gel in Figure 6-12 indicated that fingerprint PFA 21 and PFA 21a are different from each other by a slight shift in band position at approximately 150 bp and 145.5 bp, respectively.

Cluster II contained PFA types 21d and 21e, which were 100% related to each other, but 89% related to the other PFA types in cluster I (Figure 6-12). Visually the fingerprint types PFA 21d and PFA 21e were different from each other, but this was not detected by the GelCompar II software. An example of PFA 21d is not shown.

6.3.3 Relationship between molecular type, and virulence as determined by pen tests

Eighteen isolates from the National Culture Collection were used to compare the results of molecular typing and virulence. The isolates were tested for protease thermostability, zymogram profile, PFGE, IRS-PCR, and virulence as assessed by pen testing, and field observations. The results for the Type strain (A198) were also included. These results are summarised in Table 6-11, with the results grouped according to PFA type.

Five isolates belonged to the PFA 7 clonal group and these were composed of three S1 types and two U1 types of which all S strains and one U strain were virulent by pen diagnosis, with the second U1 strain classed as benign by pen testing. Laboratory 2 designated one S strain (V36) as benign in the pen testing, whereas laboratory 1 rated this strain virulent. It was recorded as virulent in field diagnosis. In the records of the National Culture Collection, laboratory 2 rated strain V65 as both virulent and benign in pen tests, which may be a clerical error. This strain was rated virulent in pen diagnosis by laboratory 1, and intermediate in field diagnosis. Two strains were identified as type PFA 7L. One was S1 (V65) and the other was U1 (V19). They had different IrsT types. The U1 strain was rated benign by pen testing and by field diagnosis, whereas the S1 strain was rated virulent by pen tests and intermediate in field diagnosis.

Table 6-11. Comparison of molecular type and pen-test for virulence amongst 18 *D. nodosus* isolates from the National Culture Collection

Isolate ID	Protease	Zym	PFA	IrsT	PD 1	PD 2	FD
A198	S	S1	4a	26	V	V	V
V27	S	S1+	7	13	V	V	B
V32	U	U1	7d	13a	V	V	B
V36	S	S1+	7	2	V	B	V
V65	S	S1+	7L	18b	V	V/B	I
V19	U	U1	7L	1b	B	B	B
V30	S	S1+	11a	2	nt	V	V
V24	S	S1+	11a	2	V	V	V
V42	S	S3	11	2	nt	V	I
V40	S	S1+	11	2	nt	V	B
V73	U	U1	9a	1	nt	V	V
V45	U	U1	9d	25	V	V	B
C305	U	U1	9d	54	B	B	B
V38	U	U5	9d	nt	nt	B	B
V28	U	U1	9b	5c	B	V	B
V9	S	S3	9q	1a	nt	V	B
V4	U	U1	9e	9	nt	B	B
V57	S	S1+	9e	1b	nt	V	B
V54	U	U5	18	1g	B	B	B

B = benign; ID = identification; FD = field diagnosis; nt = not tested; PD 1 = pen diagnosis results from laboratory 1; PD 2 = pen diagnosis results from laboratory 2; IrsT = molecular type using primer PXT and IRS-PCR method; nt = not tested; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; S = thermostable protease; U = heat labile protease; V = virulent; Zym = zymogram profile.

Four strains from clonal group PFA 11, identified as S strains, were virulent in the pen test, with variable virulence results for clinical field diagnosis. All strains were IrsT 2. One strain was S3 (V42) in the zymogram profile.

Of the eight isolates from the PFA 9 clonal group, five were U1 strains, one was a U5 strain and two were S strains (S1+ and S3). Five isolates were classed as virulent in the pen test, these being three U1 strains and the two S strains. A discrepancy occurred with the classification of virulence by pen test for isolate V28, with laboratory 1 recording benign, and laboratory 2 recording a virulent classification. All strains, apart from one U1 strain, were classed as benign according to field clinical diagnosis.

Isolate V54, identified as PFA 18 and IrsT 1g, had an identical molecular type according to both methods to isolate AC 5679 from WA. Isolate AC 5679 was considered to be a virulent U5 strain because in pen tests in WA it had produced lesions with a high lesion score for virulence (Table 6-12).

Table 6-12. Pen test and field virulence ratings for eight isolates from WA farms

Isolate ID	Farm ID	Protease	Zym	PFA	IrsT	MTWFS	PD	FD
AC 5679	68	U	U5	18	1g	6.27	V	V
AC 8111	85	U	U5	8b	29b	1.13	B	B
AC 8586	153	U	U5	8a	29b	0.73	B	B
AC 8738	100	U	T	21a	2d	1.2	B	B
A198	118	S	S1	4a	26	11.17	V	V
C305	118	U	U1	9d	54	1	B	B
AC 4369	20	U	U1	11a	2	LS 4*	nt	V
97-60-115	20	U	U5	11a	2	LS 4, 5*	V	V

AC = Albany culture collection number; B = benign; ID = identification; FD = field diagnosis; IrsT = molecular type using primer PXT and IRS-PCR method; LS = lesion score; MTWFS = mean total weighted footscore; nt = not tested; PD = pen diagnosis; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; S = thermostable protease; U = heat labile protease; V = virulent; Zym = zymogram profile.

6.4 DISCUSSION

The analysis carried out in this chapter sought to establish the relationship between protease thermostability, zymogram profile and molecular type, and also sought to assess whether there was a relationship between virulence and molecular type. Results from chapter 4 established that some molecular clonal groups predominate in WA, and have done so for more than 20 years.

The investigation of the relationship between protease thermostability and molecular type revealed that the thermostable (S) strains and heat labile (U) strains predominated in different molecular groups, and this was evident for the three main clonal groups in WA, PFA 7, PFA 9 and PFA 11. Clonal groups PFA 7 and PFA 11 were dominated by S strains: 94.8% and 89.2%, respectively, whereas clonal group PFA 9 was dominated by U strains (76.6%). In the collection of isolates used for molecular typing there was a bias towards S strains from the year 2000 onwards. The dominance of S strains in clonal groups PFA 7 and PFA 11 was evident from the analysis of figures both for the total number of strains tested, and for the number of strains tested prior to the year 2000. Despite the program for the eradication of S strains, these strains continued to be

identified in clonal groups PFA 7 and PFA 11 with 45.9% (n = 147) of all S strains isolated between 2000 and 2002 being found in these two clonal groups.

The dominance of U strains in clonal group PFA 9 occurred prior to the year 2000, but from this time on S strains were dominant in clonal group PFA 9. It is highly likely that the bias toward selecting S strains for molecular typing resulted in this dominance of S strains in this clonal group, and that the results before the year 2000 are a truer reflection of the proportion of U strains in this clonal group in WA.

Very little difference was observed in the percentages of S strains dominating in the three different clonal groups prior to the year 2000, compared to the total figures for the collection of isolates. Thus, most of the virulent strains (as identified by protease thermostability) were found in one of two groups that have persisted in WA over a 20-year period. Therefore, clonal groups PFA 7 and PFA 11 could be considered to be major virulent endemic clones.

In WA 67 clonal groups were identified by PFGE, and as stated above, 52.6% of isolates occurred in one of the three main clonal groups (chapter 4). The remaining 47.3% of isolates were found amongst the other 64 clonal groups, with no more than 5% of the total isolates found in any one of these clonal groups. Despite more than 50% of isolates in WA belonging to one of the three main clonal groups, *D. nodosus* is genetically diverse (98%) as calculated by Simpson's diversity index, and also by a simple diversity ratio of the number of molecular types obtained for the number of isolates. Less diversity was seen in the three main clonal groups identified by PFGE, compared to the overall diversity. Isolates in clonal group PFA 9 were the most diverse with a value of 90%, whereas clonal groups PFA 11 and PFA 7 showed less diversity with values of 87% and 80%, respectively. These figures were reflected in the simple diversity ratios obtained with the three clonal groups. Isolates in clonal group PFA 9 had a ratio of 1:5 indicating that one of every five isolates had a different PFA 9 molecular type. Isolates in clonal group PFA 11 and PFA 7 had ratios of 1:9, and 1:10, indicating that only one of every nine or ten isolates would be a different molecular type within that clonal group. Numbers in the other clonal groups were too small to obtain a meaningful figure for genetic diversity.

Dendrograms constructed for the major clonal groups identified two or three main clusters of isolates that were related at approximately 90%. Generally, the different molecular types were scattered throughout the respective dendrograms, but in all major clonal groups (PFA 7, 9, 11) a cluster occurred that had closely related isolates dominated by one particular molecular type. These isolates came from a variety of farms, and therefore the genetic similarity was not due to isolates detected from the same property. Trace-back data was not available for the majority of isolates, therefore the likelihood of these genetically similar clusters being due to an infection outbreak with a common source farm is not known.

Some isolates in a clonal group had identical molecular types by PFA and IRS-PCR, but other isolates present in the same clonal group had identical PFA types, yet different IrsT types. These two types of isolates either occurred in the same cluster or were identified in other clusters. This probably is a reflection of the relative genetic distance between the two isolates, but results with the dendrograms were not consistent enough for this to be clearly determined.

Two other PFA clonal groups (PFA 8 and PFA 19) were examined using dendrograms, and isolates in both clonal groups were related between 82% to 100% for both PFA types. Particular PFA types predominated in some clusters, but there was an instance where one isolate identified as PFA 19d occurred in a cluster that was different to all other isolates that were identified as PFA 19d. A similar finding occurred in the dendrogram of PFA 9. The use of a dendrogram is a guide to determining how related one strain is to another, but careful visual examination of the gel fingerprints is required to determine if the dendrogram is able to distinguish between two genetically similar isolates. In many cases the dendrogram indicated that two fingerprints were identical, but visual examination indicated a band difference that was not detected by the GelCompar II software. Some isolates that were duplicated on different gels were not recognised as 100% identical by the GelCompar II software. In some of these cases no improvement of the alignment were obtained, despite re-assessment of the bands that were marked for the GelCompar II software, and despite the fact that all molecular weight markers from all the gels aligned at 100%.

The relationship between protease thermostability and molecular type was investigated by examining the division of isoenzyme profile (zymogram) according to

molecular type. The S1 strains predominated, and were a genetically diverse group as they occurred in many different clonal groups, whereas the other isoenzyme types (S2-S4) occurred less frequently. As expected from the results obtained for protease thermostability of strains, S1 strains predominated in clonal groups PFA 7 and PFA 11, followed by PFA 9. U1 strains predominated in clonal group PFA 8 followed by PFA 9, PFA 12 and PFA 19.

The division of S and U strains into different clonal groups was not clear-cut. Although clonal group PFA 9 was dominated by U strains, 23.4% of strains in this group were S strains, which comprised 4.3% of all S strains tested. All S strains in clonal group PFA 9 were zymogram profile S1. The S1 strains comprised nine PFA molecular types within this clonal group, and came from 14 farms of which three were geographically separated but owned by the same farmer. Sheep on these three farms also were infected with U strains that belonged to clonal group PFA 9, but the presence of U strains on the remaining six farms was unknown. This group of isolates that were protease thermostable (S), yet had molecular types more often associated with U strains is most likely a result of them being epidemiologically related. Unfortunately there is no data to support any infection linkages.

Although clonal groups PFA 7 and PFA 11 were predominated by S1 strains, U strains also were found in these clonal groups. The predominant U strain zymogram type was U6 (n = 3) in clonal group PFA 7, and U5 (n = 5) in clonal group PFA 11, with one U1 isolate being found in clonal group PFA 7, and three U1 strains being found in clonal group PFA 11. Considerable genetic diversity occurred within the U5, the U6 and the T strain zymogram groups. Most isolates within each individual zymogram group were genetically unrelated to the other isolates in that group, and were isolated from different properties.

A dendrogram constructed from the U6 isolates indicated that they were related between 67% to 100%, and the diversity index of 91% for PFA types showed that U6 strains were a highly diverse group. This was reinforced by the diversity ratio of 1:2 suggesting that every second U6 strain detected would be of a different molecular type. The U6 strains were found in seven different clonal PFA groups. Five U6 isolates were molecular type PFA 11e and these were isolated from three farms between 1994 and 1995. These findings may suggest an outbreak of footrot from a common source, but

there is no epidemiological data to support this. Despite these five isolates being of the same PFA molecular type, the remaining U6 isolates were not closely related to each other. The striking observation noted for the U6 strains was how unrelated they were to each other compared to their genetic relatedness to S1 strains (and to a lesser extent U1 strains) from the same property. The detection of U6 strains is uncommon in WA, as U6 strains comprised only 0.3% of all strains of *D. nodosus* isolated between 1997 and 2003 (M. Palmer, Australian Reference Laboratory for Ovine Footrot, Albany, unpublished data, 2003).

A similar pattern to the U6 observations was seen for the T strains. T strains are so named because they have the fast moving electrophoretic protein band that is present in all *D. nodosus* strains. U6 strains have this band only, whereas T strains have two slower moving bands in the same position as S1 strains, but the bands are weakly expressed. T strains (and U6 strains) are protease heat labile in the gelatin-gel test. On all farms where T strains were isolated, S1 strains also were isolated, and in all cases the T strains had the same molecular type as the S1 strain isolated on the same farm. The T strains were found to be genetically diverse, with a diversity index of 92% and a ratio of 1:3, and were comprised of seven PFA molecular types. The dendrogram of T strains showed that through their molecular type they were different from each other, but were identical to an S1 strain isolated on the same farm. On farms where T strains were found, S1 strains were predominant on those farms.

Strains having the U5 zymogram profile were isolated in the highest number after the U1 strains, and comprised 6.4% of total strains, and 21.5% of all U strains in the collection of isolates. The highest number (n = 17, 36.2%) of U5 strains occurred in clonal group PFA 8, and were found on ten farms. U5 strains were the dominant strain in this clonal group. It is hypothesised that the distribution of U5 strains belonging to clonal group PFA 8 is a result of an epidemiological link through sale of infected sheep to other farms. The original U5 strain had come from infected sheep purchased from the eastern States of Australia, and by pen testing was classed as a benign strain. No other isolates from other zymogram groups that possess this molecular type have been detected in WA. Of the 47 U5 strains in the collection, 21.2% (n = 10) occurred in clonal group PFA 9, which was predominated by U1 strains. One U5 strain and five U5 strains occurred in clonal groups PFA 7 and PFA 11, respectively, which were dominated by S1 strains. As

with the other zymogram groups, the U5 strains were found to be a genetically diverse group with a high diversity index of 95%, and a diversity ratio of 1:1.9.

As was found for the U6 and T strains, the U5 strains that occurred in the clonal groups PFA 7 and PFA 11, dominated by S1 strains had the same molecular type as an S1 strain on the same farm. It is apparent from these results that in the majority of cases the U5, U6 and T strains appear to have evolved from either an S or U strain on that same farm.

An investigation of isolates obtained from Farm 100, where footrot occurred for ten years, provided a good case study for examining the relationship between S1, U6, T and U5 strains. Following the initial outbreak of virulent footrot in 1992 with S strains of molecular types PFA 21 and 21e, the farm was declared free of virulent footrot after a successful eradication program. Sheep infected with the benign forms of the disease (isolates with zymogram profiles U6 and T) did require treatment, according to the official eradication policy in WA. In 2001 virulent footrot (S strain) was again detected on the farm. All S1, U5, U6, and T strains detected on the farm belonged to clonal group PFA 21. These molecular types have only been found on Farm 100, and after the second detection of virulent footrot on this farm, these isolates (S strains) also were found on three neighbouring farms. The re-appearance of virulent footrot of the same molecular type as the initial outbreak suggests that the re-infection was either from an undetected source of infected sheep, or had evolved from the protease labile strains that remained on the property after eradication of the virulent strains. Both the U6 and T strains that had been detected before eradication were identical in molecular type by both PFGE and IRS-PCR to the eradicated S strain. The U5 strain subsequently detected on the farm in 2002 was the same molecular type according to PFA and IrsT type as the S strain detected at the same time. T strains have continued to be isolated in 2001 and 2002 from infected sheep on this farm, and four PFA molecular types (21, 21a, 21d, 21e) have been detected. This suggests that the T strain on this property is undergoing constant genetic change. Two molecular types of the S strain have also been isolated: PFA 21 and 21a.

For a small number of isolates it proved difficult to assign a zymogram type. One isolate (AC 7676) gave anomalous results in the gelatin-gel test and the zymogram method. It was investigated for molecular type, and was found to belong to PFA 19 clonal group with zymogram profile S2/U4. Isolates from this clonal group appeared to

cause more anomalous results in the gelatin-gel and zymogram tests than other clonal types. Despite repeated subcultures to ensure cultural purity the results remained inconsistent in the zymogram test and were classed as S2, U4 or S2/U4. This may be a characteristic of this clonal group, and could be used to assist interpretation of difficult results in the gelatin-gel and zymogram tests. Thus, isolates that produce equivocal results in the gelatin-gel test may belong to a limited number of molecular types, such as PFA 19.

Two isolates and six subcultures of one of the isolates (AC 3107) that gave anomalous results in the zymogram method were investigated for molecular type, and found to produce a unique type of PFA 39 with two subtypes (39a and 39c). No other strains in the collection belonged to this clonal group, so it is not known whether or not the anomalous results in the zymogram were a feature of this particular clonal group.

The investigation of the relationship between molecular type, protease thermostability and zymogram profile was investigated by analysing the U5, U6, T strains, and other minor strains that produced anomalous results when assessing protease thermostability and isoenzyme profiles (zymogram). The results lead to the hypothesis that the atypical isoenzymes arise from changes in the isoenzymes of a parent strain. The discrepancies seen in either the gelatin-gel test or the zymogram profile may be due to changes in the protein such as differences in structure or protein folding. These changes may affect the thermostability of the protease, or result in different isoforms of the protease. Previous research indicated that a single amino acid change was responsible for the differences between a protease isoenzyme present in virulent isolates, and an isoenzyme present in benign isolates (Riffkin *et al.,* 1995). Other research indicated that protease thermostability of an enzyme was enhanced by a single amino acid substitution (Imanaka *et al.,* 1986). This may explain why U5, U6 and T strains have the same molecular type as S strains detected in infected sheep on the same property. The S strains are the parent strains, and the U5, U6 or T strains may arise because of an amino acid substitution that results in a structural change in the protease protein, which affects the thermostability of the protease. This hypothesis needs further investigation.

To investigate if a relationship existed between molecular type and virulence, particular groups of strains were used in the assessment. Strains that were pen-tested for virulence in a national project, strains that were pen-tested in WA, and strains that had

virulence ratings recorded according to field and clinical data, were typed by PFGE and IRS-PCR to determine if virulent strains belonged to a particular molecular type. It is generally accepted that isolates that produce a heat labile protease (U strains), as detected in the gelatin-gel test, are thought to only cause benign lesions (chapter 1). Thus all U strains, including U5, U6 and T strains, are considered not to be virulent strains.

In spite of this assumption for benign strains, clinical and field data from WA show that a small number of the U5 strains have caused a virulent lesion in the hoof. Overall, U5 isolates are in very low numbers, and between the years 1997 to 2003 U5 strains comprised only 1.4% of all *D. nodosus* isolated (M. Palmer, Australian Reference Laboratory for Ovine Footrot, Albany, unpublished data, 2003). One of the virulent U5 strains (PFA 18) had been introduced into the State by sheep imported from the eastern States of Australia. This strain was eradicated successfully from the farm and no further strains of this zymogram type, or molecular type have occurred in WA. The strain (AC 5679) produced virulent lesions when assessed for virulence potential by pen-testing.

On Farm 20, a virulent U5 isolate was detected with the same molecular type as an S1 strain (PFA 11a) present on the farm. The U5 strain (97-60-115) from this farm produced virulent lesions in pen-testing trials. A U1 strain also detected on the same farm had the same PFA 11a type, and in the field caused virulent lesions rated score 4. This strain was not pen-tested. On another occasion, a virulent U5 strain from Farm 5 had a molecular type of PFA type 7k. PFA clonal group 7 is more normally associated with S strains. Two S strains were typed from this farm, but they were of a different molecular type to the U5 strain. Without typing more S strains it is difficult to say whether the genetically related S strains might have been present on the farm. This could not be investigated because only a limited number of isolates from this farm were stored in the culture collection.

In WA, U5 strains with molecular type PFA 8a and 8b were found to produce only benign lesions in artificially infected sheep when pen-tested for virulence potential. The highest number of U5 isolates in the collection of isolates occurred in clonal group PFA 8. It is hypothesised that the spread of this clonal group had arisen through the sale of infected sheep, although strong epidemiological data does not exist to support this possibility.

A T strain (Farm 100) caused only benign lesions when pen-tested for virulence potential. Pen-testing of the benign Type strain (C305) has occurred a number of times including in pen tests both in WA and through the National Project, CHP94. In all cases the lesions were rated as benign only.

In the results for pen-testing for the assessment of virulence potential of isolates in the National Culture Collection, the results were inconclusive for establishing a relationship between molecular type and virulence. All protease thermostable strains were rated as virulent in pen-tests. The S strains were from clonal groups PFA 7, PFA 9 and PFA 11. One U1 strain (PFA 7d) was found to be virulent, whereas another U1 strain (PFA 7L) was found to be benign. Three U1 strains belonging to clonal group PFA 9 rated virulent in the pen test by both laboratories, whereas another two U1 strains and one U5 strain belonging to the same clonal group, tested benign.

These results suggest that, in some instances, molecular type may assist in determining the likely potential for virulence of an isolate. For example, further isolations of U5 strains from clonal group PFA 8 may be regarded as likely to be benign because of the information known about this clonal group. U6 and T strains may be regarded as benign despite having the same molecular type as an S strain on the same property because the heat labile status of the protease indicates that the isolate will not result in a virulent lesion. The finding from the National Project that some U1 isolates caused virulent lesions cannot be fully explained at this time. It might be that the presence and molecular type of S strains from the same farm needs to be established. Cultures need to be subcultured a number of times to ensure culture purity. Strains that are used in pen tests should be typed before their inoculation into the sheep hoof, and then re-typed on re-isolation from the infected hoof to ensure strain identity and to rule out the possibility of cross-contamination. The act of washing down pens with water to remove faeces etc might lead to contamination of sheep between pens.

Molecular type is more likely to indicate the genetic source of the isolate, and once a database of strains is established this information can be used to make assumptions about newly isolated strains that match with known molecular types. Isolates with either a benign zymogram profile, but a molecular type more normally associated with virulent strains, or isolates with a virulent zymogram profile, but a molecular type more normally associated with benign strains might be the intermediate virulent strains that some

researchers recognise. Currently, there is no *in-vitro* test that distinguishes these intermediate strains. The term intermediate strain is not strictly recognised in WA.

In conclusion, it was found that strains belonging to each zymogram group were genetically diverse within that group. Zymogram groups found on a farm were more genetically related to other strains from other zymogram groups found on that farm than they were to physically unrelated strains within the same zymogram group. It is likely that the U6 and U5 strains evolve from either a U or S strain on the same farm, and in the case of some U5 strains have the potential to carry the same virulence determinant if they have evolved from an S strain. This would suggest that there is an as yet unidentified virulence factor apart from thermostable protease. The T strains appear to evolve from an S strain on the same farm, but data suggests that they are not potentially virulent, and there is no evidence to suggest that T strains revert to virulent strains. If this were to happen it could have disastrous consequences for the eradication program. The fact that the numbers of farms in WA with sheep with S strains has decreased from more than 291 in 1974 to less than 77 in 2004 suggests that virulent strains do not arise from previously benign strains.

It is possible that the isoenzymes result as an anomaly in the protein due either to different composition, conformation or folding of the protein. Identification of strains with different isoenzymes but the same molecular type might help identify the original source of the isolate, and possibly its virulence potential. Thus, once it is known how a clonal group behaves in relation to virulence potential, then predictions can be made about isolates that are detected and found to belong to a known clonal group.

This current chapter, which investigated clonal groups in relationship to protease thermostability, protease isoenzymes and virulence, found that PFGE typing identified considerable genetic diversity among the S and U strains, as well as diversity among the different isoenzyme types. The results also showed that protease thermostable strains predominated in two main clonal groups in WA, whereas protease heat labile strains predominated in a separate clonal group. A combination of techniques may be useful to help establish the virulence potential of an isolate. Using the information gained from the analysis of a large number of isolates, predictions may be made about the potential virulence of an isolate. The reason why some protease heat labile strains have the ability to cause virulent lesions still needs further investigation.

CHAPTER 7

INVESTIGATION OF GENETIC DIVERSITY IN *DICHELOBACTER NODOSUS*

CHAPTER 7

7. Investigation of genetic diversity in *Dichelobacter nodosus*

7.1 INTRODUCTION

The results described in previous chapters of this thesis indicate that *Dichelobacter nodosus* is genetically diverse; that certain molecular clonal groups persist in Western Australia; that different zymogram profiles are genetically related on a farm; and that certain protease heat-labile isoenzyme profiles may evolve from strains that produce thermostable protease.

The work described in this chapter sought to investigate the mechanism underlying the observed genetic diversity and to determine the amount and rate of genetic change in *D. nodosus*. The rate of genetic change has implications for the eradication program when investigating trace-back and trace-forward sources in an attempt to locate the source of an infection outbreak.

Dichelobacter nodosus produces a number of proteases, but the most predominant is a serine protease that in some isolates is heat labile and in others is thermostable. Isoenzymes of the serine proteases are produced, and in some isolates anomalies occur between the production of thermostable protease and the expected isoenzyme profile. Research results from chapter 6 indicated that some strains that produced a heat labile protease (U6, U5, T) were the same molecular type as strains on the same property that produced thermostable protease. In contrast, isolates within the U6, U5 or T groups were unrelated in molecular type when there was no epidemiological link between them.

With time, isolates undergo genetic change; this can lead to the recognition of clonal groups of strains that have similar virulence potential based on production of thermostable protease. However, some strains within these clonal groups are genetically similar overall, yet phenotypically different due to a lack of production of thermostable protease. As a result, these anomalous strains were investigated to determine relationships between the genetic profile and protein profile, using PFGE, IRS-PCR and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Three case

studies were used, which involved strains from individual farms, and a fourth case study involved isolates from a research trial that sought to investigate how different strains of *D. nodosus* responded to eradication attempts. One of the strains used to artificially infect sheep in the research trial (Jelinek and Depiazzi, 2003) was the Type strain A198, and over the course of the infection U6 strains were detected. Selected strains were used to compare the genetic and protein differences and similarities within and between clonal groups.

The aims of this chapter were to analyse DNA fingerprints to investigate the mechanism for genetic change, and to provide a rough estimate of the rate of genetic change. The aim was also to determine to what extent protein profiles were conserved amongst strains of different genetic backgrounds by comparing whole cell protein profiles across clonal groups. The protein profiles of the genetic subtypes of strain A198 were compared to investigate whether there were detectable differences associated with changes in protease thermostability.

7.2 MATERIALS and METHODS

7.2.1 Bacterial strains

The 171 strains that were investigated came from four case studies. Case studies one, two and three were used to investigate the mechanisms for genetic diversity, and case study four was used to investigate conservation of whole cell protein profiles across clonal groups.

Case study 1

Two isolates (AC 7756 and AC 7757) were studied that had been obtained in 1997 from infected sheep on Farm 1.

Case study 2

Eight isolates from the culture collection were studied comprising AC 3107 and six subcultures taken from individual colonies (#1, #2, #3, #A, #B, #C), and AC 3109, all of which had been isolated in 1991 from the one infected hoof of a sheep on farm 51.

Case study 3

A selection of 136 strains of *D. nodosus* were re-isolated from sheep 12 to 18 months after they were artificially infected with one of the following four strains: the Type strain A198 (VPI 5731-1, ATCC 27521, originally isolated by J.R. Egerton from Wollogorang Station, Goulburn, New South Wales), the benign strain C305 (U1), the virulent strain AC 5679 (U5), and the benign strain 00-143-306 (U5). The research trial sought to investigate the effectiveness of a daily footbathing regime to eradicate *D. nodosus* strains S1, U1, and U5 (Jelinek and Depiazzi, 2003), and was unrelated to the work on molecular typing. The strains re-isolated from strain C305 (U1) were not used in this current analysis. The isolates are detailed in the result tables (Tables 7-2, 7-3 and 7-4).

Case study 4

Four isolates from different clonal groups and different zymogram groups were selected from the National Culture Collection (NCC). These were isolates V3 (S1, PFA 24, from WA), V8 (U5+, PFA 52, from WA), V23 (U1, PFA 53, from NSW) and V48 (S1, PFA 44b, from SA). Also tested from WA were AC 8058 (U5, PFA 37a) from Farm 5 and isolate AC 2127 (S1, PFA 7a) from Farm 105. Isolates V3, V8 and V48 were virulent by pen-test. Isolates V23 and AC 8058 were benign by pen test.

A subset of five S1 strains (detailed in case study three), with the molecular subtypes, PFA 4b, 4c, 4e, 4f, and 4g were investigated for whole cell protein profiles. In addition, one U6 isolate (PFA 4b) from the same trial, five strains from clonal group PFA 11, and five isolates from PFA types 24, 37a, 44b, 52, 53 were used, whilst isolate AC 2127 (PFA 7a) was included as a control on the protein gels.

7.2.2 Methods

7.2.2.1 Molecular typing

PFGE and IRS-PCR were used to fingerprint the isolates, using the methods described in chapter 2.

7.2.2.2 Whole cell protein profile

The whole cell protein content of the isolates was analysed by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method using the Phast mini system (Pharmacia) (Figure 7-1). Bacterial cells were grown for four days on TASH MM plates in an anaerobic atmosphere (section 2.2). Cells were collected into 600 µl of sterile normal saline in a 1.5 ml microfuge tube to an opacity of No. 6 McFarland nephelometer opacity standard. The cell suspension was mixed thoroughly and 40 µl was added to 100 µl of sample buffer containing bromophenol blue (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue). The cells were stored at –20°C until required. To prepare the proteins the cell suspension in sample buffer was boiled for three minutes and then cooled to room temperature. The suspension was centrifuged at 10,000 g (10,000 rpm) for 5 minutes. Two microlitres of supernatant was used to load the gel.

The instructions for the use of the Phast mini system were followed according to the methods manual. The “slow PAGE 3” program for SDS gels was used and two gels per run were electrophoresed for 1.5 hours. The gels were stained using the Phast silver staining kit. The silver-stained gels were soaked in 10% glycerol for 10 minutes and then placed between two sheets of cellophane before being dried using gel drying equipment from BioRad.

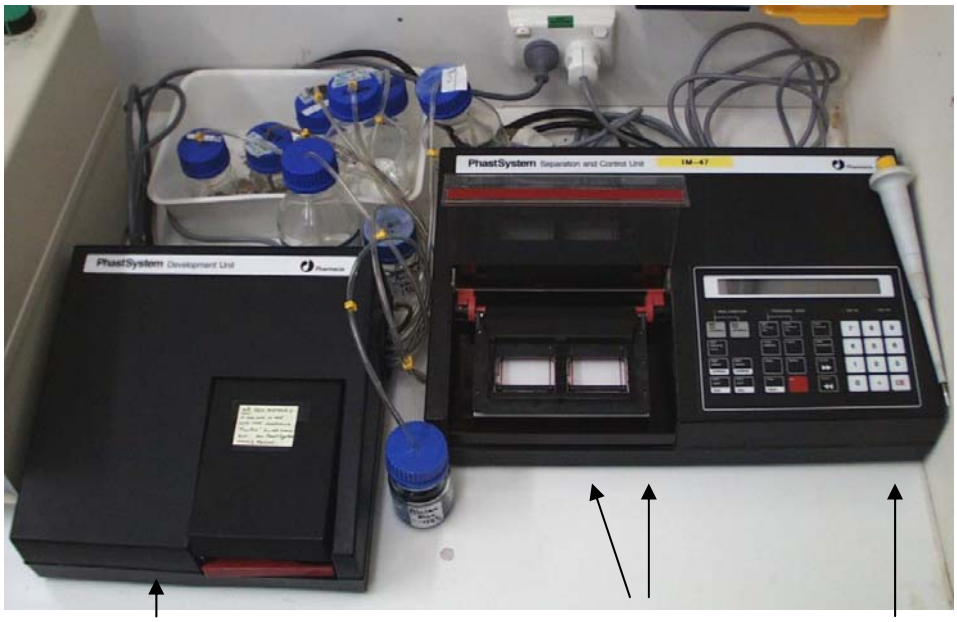


Figure 7-1. Photograph of Phast mini system for protein electrophoresis

7.3 RESULTS

7.3.1 Case Study 1

Two isolates (AC 7756, AC 7757) obtained from infected sheep on Farm 1 produced thermostable protease as detected in the gelatin-gel test, and were zymogram type S1. They were identified as molecular type PFA 24a and PFA 24, respectively, by PFGE using the restriction enzyme *Apa*I. The two isolates were similar in restriction banding pattern, with the exception of three bands (Figure 7-2).

PFA type 24 had a restriction fragment band at approximately 325 kb that was not present in molecular type PFA 24a. The latter type had two bands at 125 kb and 200 kb that were not present in molecular type PFA 24 (Figure 7-2). A point mutation in the 325 kb band from PFA 24 molecular type has resulted in the bands at 125 kb and 200 kb in PFA 24a.

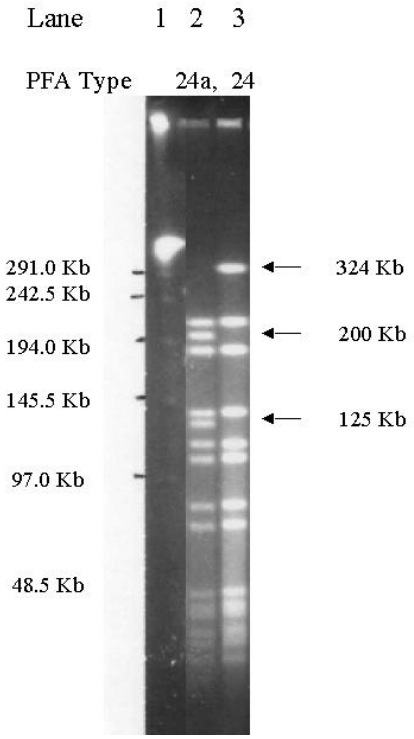


Figure 7-2. Observation of point mutation in PFGE patterns of isolates AC 7756 and AC 7757 from case study 1.

Lane 1 = molecular weight marker 48.5 kb ladder; Lane 2 = AC 7756 (PFA 24a); Lane 3 = AC 7757 (PFA 24).

7.3.2 Case Study 2

The PFGE method identified three PFA molecular types (PFA 39, 39a, 39c) from six subcultures of isolate AC 3107, which was detected in the hoof of an infected sheep on Farm 51. Isolate AC 3109, identified in the same hoof, was molecular type PFA 39b (Figure 7-3). All eight isolates had the molecular type of IrsT 1a using the IRS-PCR method (Table 7-1).

Three of the molecular PFA types (39, 39a and 39c) possessed a restriction band at 130 kb, but this band was absent in PFA 39b (Figure 7-3). PFA 39c had a restriction band at 230 kb that was not found in the other molecular types. Molecular type PFA 39a was different from PFA 39, 39b and 39c by the absence of a band at 82 kb and the addition of a band at 77 kb.

Table 7-1. Molecular types obtained with isolate AC 3109, isolate AC 3107 and six subcultures of this isolate

Isolate Number	Zym	PFA	IrsT
AC 3107	U6/U4	39	1a
AC 3107 A	U6/U4	39	1a
AC 3107 B	U6/U4	39	1a
AC 3107 C	U6/U4	39c	1a
AC 3107 #1	U6/U4	39a	1a
AC 3107 #2	U6/U4	39	1a
AC 3107 #3	U6/U4	39	1a
AC 3109	U6/U4	39b	1a

AC 3107 – designations A, B, C, #1, #2, #3 refer to subcultures; IrsT = molecular type using primer PXT and IRS-PCR method; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; U6/U4 = zymogram profile obtained for isolates. Described as a U6 profile with weakly expressed bands of a U4 profile; Zym = zymogram profile.

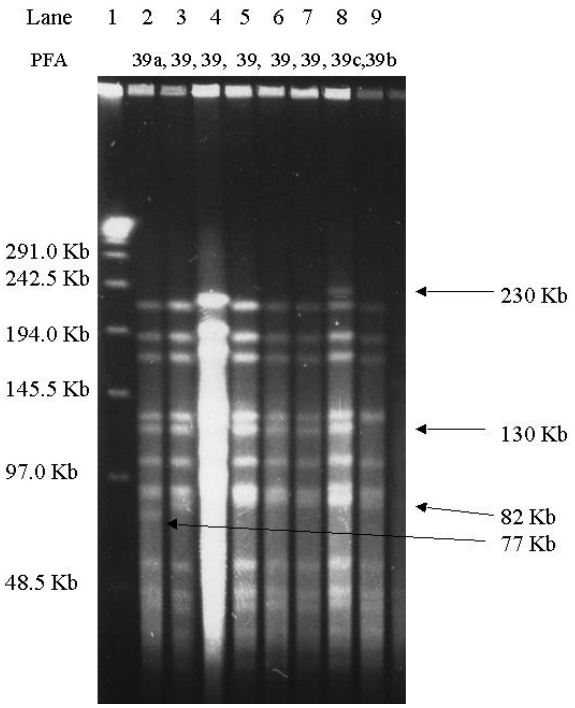


Figure 7-3. PFA molecular types obtained from subcultures of AC 3107, and isolate AC 3109 from case study 2

Lane 1 = DNA lambda ladder; Lane 2 = AC 3107 #1 (PFA 39a); Lane 3 = AC 3107 #2 (PFA 39); Lane 4 = AC 3107 #3 (PFA 39); Lane 5 = AC 3107 (PFA 39); Lane 6 = AC 3107 #A (PFA 39); Lane 7 = AC 3107 #B (PFA 39); Lane 8 = AC 3107 #C (PFA 39c); Lane 9 = AC 3109 (PFA 39b). Smear in Lane 4 indicates overload of DNA.

7.3.3 Case Study 3

7.3.3.1 Results of molecular typing of re-isolated strains from strain A198

Of the 136 strains typed by PFGE, 125 belonged to clonal group PFA 4. The original Type strain, A198, from the culture collection was PFA 4a, and a further six PFA 4 subtypes (PFA 4b, 4c, 4d, 4e, 4f, 4g) were identified from the re-isolated strains (Table 7-2). These subtypes comprised 12% (n = 15) of the 125 isolates in the clonal group. The most predominant molecular type was PFA 4b, as 88.0% (n = 110) of strains that belonged to clonal group PFA 4, were of this type. The zymogram profiles of the strains identified as PFA 4b were 107 S1 strains, and three U6 strains. The molecular type PFA 4e comprised the next largest group of isolates with 7.2% (n = 9) of isolates belonging to this group, and all were S1 strains.

Table 7-2. Distribution of subtypes within clonal group PFA 4 detected in 125 strains re-isolated from artificially infected sheep

PFA type	No. (%) of strains	No. (%) of S	No. (%) of U6
4b	110 (88.0)	107 (87.70)	3 (100.0)
4e	9 (7.20)	9 (7.38)	0 (0)
4c	2 (1.60)	2 (1.64)	0 (0)
4f	2 (1.60)	2 (1.64)	0 (0)
4d	1 (0.80)	1 (0.82)	0 (0)
4g	1 (0.80)	1 (0.82)	0 (0)

Total number of strains = 125; total number of S = 122; total number of U6 = 3. PFA = molecular type using restriction enzyme *Apa*I and PFGE method; S = protease thermostable; U6 = protease heat labile strains with U6 zymogram profile.

Two isolates belonged to each molecular type PFA 4c and 4f, and one isolate each belonged to PFA 4d and PFA 4g (Table 7-2). The results were not analysed to determine how many of these isolates came from individually re-sampled hoofs.

Amongst seven S1 strains representing the seven different PFA 4 molecular types and three U6 strains, there were three PFX molecular types (PFX 25, 25a, 25b), and three IrsT types (IrsT 26, 26a, 26b). All three U6 strains were identified as the same molecular type (PFA 4b, PFX 25, and IrsT 26) by all three methods (Table 7-3). An S1 strain (00-167-498) had the same molecular type (PFA 4b, PFX 25, IrsT 26) as the U6 strains. Two

S1 isolates (00-167-498, 00-186-744) identified as molecular types PFA 4b and 4g, respectively, had the same molecular type of PFX 25, but had different IrsT molecular types (IrsT 26 and 26a). Two other S1 isolates (00-167-486 and 00-181-692) were molecular types PFA 4d and PFA 4e, respectively, but had the same PFX type (PFX 25a) and the same IrsT type (IrsT 26).

A further two S1 isolates (00-167-488 and 00-181-696) had the same PFX type (PFA 25b), but different PFA types (PFA 4c and PFA 4f), and different IrsT types (IrsT 26 and 26b) (Table 7-3).

Table 7-3. Molecular types arising from nine strains re-isolated from sheep artificially-infected with strain A198

Isolate Number	Zym	PFA	PFX	IrsT	Hoof ID
A198 (ATCC 27521)	S1	4a	nt	26	n/a
00-186-744	S1	4g	25	26a	104 LH
00-167-498	S1	4b	25	26	210 LF
00-167-488	S1	4c	25b	26	155 RF
00-167-486	S1	4d	25a	26	125 LF
00-181-692	S1	4e	25a	26	125 RH
00-181-696	S1	4f	25b	26b	176 LF
00-151-547	U6	4b	25	26	nk
00-167-507	U6	4b	25	26	393 RH
00-167-480	U6	4b	25	26	16 RF

ID = identification number; IrsT = molecular type using primer PXT and IRS-PCR method; LF = left front; LH = left hind; n/a = not applicable; nk = not known; nt = not tested; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; PFX = molecular type using restriction enzyme *Xba*I and PFGE method; RF = right front; RH = right hind; S = protease thermostable; U = protease heat labile; Zym = zymogram profile.

The difference in molecular weight between PFA types 4a, 4b, 4c, 4d, 4e, 4f and 4g was estimated to be 35-45 kilobases (Figure 7-4). Of the isolates detected in the sheep artificially infected with strain A198, PFA 4b was the most common molecular type (data not shown). The original strain of A198 from the culture collection was molecular type PFA 4a. This was different to the molecular type PFA 4b that was identified in the majority of isolates detected in the artificially infected sheep. In PFA 4b a restriction band occurred at 160 kb, but this was not present in PFA 4a molecular type, whereas in

PFA 4a an extra band occurred at 125 kb (Figure 7-4, lanes 2 and 4). The molecular type PFA 4g was different to PFA 4b by the additional restriction band at 135 kb, and the absence of a band at 160 kb (Figure 7-4, lanes 3 and 4).

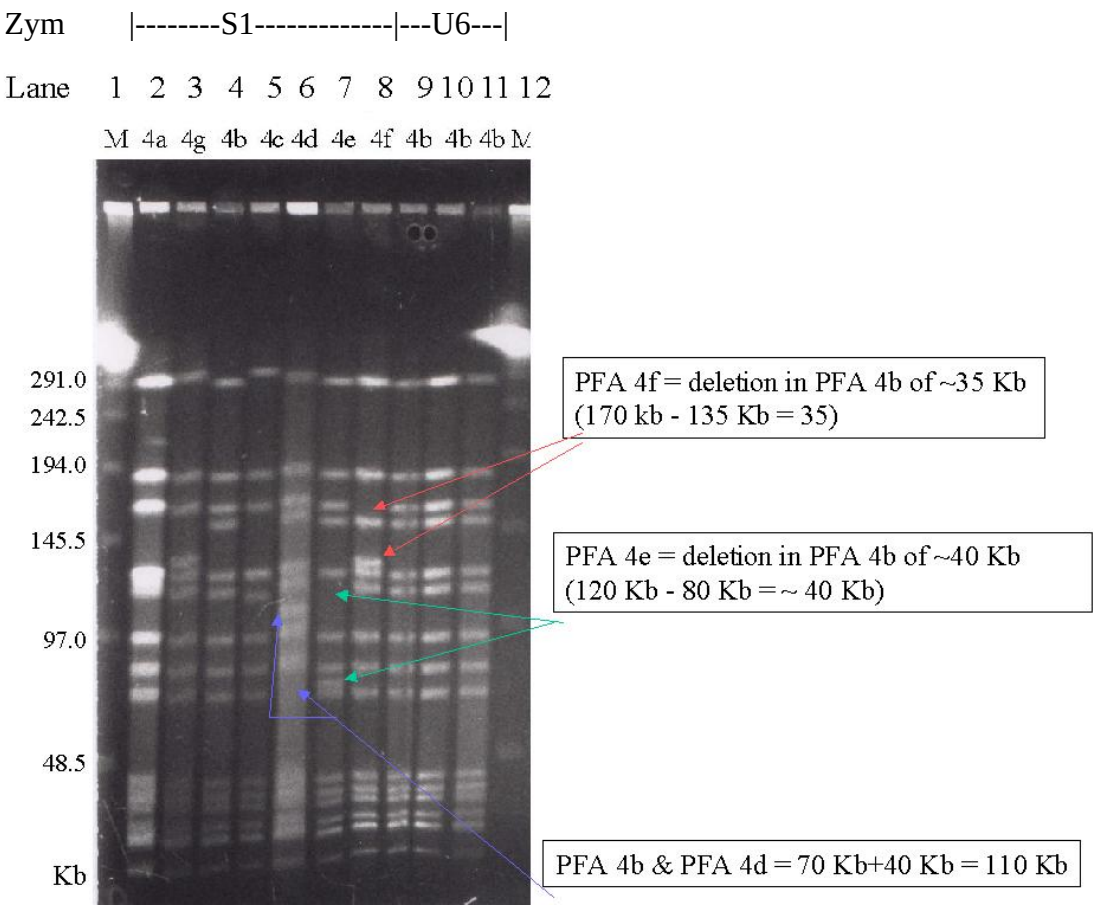


Figure 7-4. Molecular subtypes isolated from sheep artificially infected with isolate A198

Lane 1 = DNA lambda ladder; Lane 2 = A198 from culture collection; Lane 3 = 00-186-744; Lane 4 = 00-167-498; Lane 5 = 00-167-488; Lane 6 = 00-167-486; Lane 7 = 00-167-502; Lane 8 = 00-181-696; Lane 9 = 00-157-547; Lane 10 = 00-167-507; Lane 11 = 00-167-480; Lane 12 = DNA lambda ladder.

The difference between PFA 4c and PFA 4b was the absence of a restriction band at 160 kb and a band at approximately 300 kb, rather than at 291 kb as in PFA 4b (Figure 7-4, lanes 4 and 5). Molecular type PFA 4d was different to PFA 4b by the absence of a restriction band at 70 kb, and the presence of a band at 110 kb (Figure 7-4, lanes 4 and 6).

The molecular type PFA 4e was different from PFA 4b by the absence of a restriction band at 120 kb, and the presence of a band at 80 kb (Figure 7-4, lanes 4 and

7). Molecular type PFA 4f had a different banding pattern to PFA 4b by the absence of a restriction band at 170 kb, and the presence of a band at 135 kb that was not seen in PFA 4b (Figure 7-4, lanes 4 and 8).

7.3.3.2 Results of molecular typing of re-isolated strains from AC 5679

AC 5679 was one of the two U5 strains artificially inoculated into sheep in the same flock, and was molecular type PFA 18. Five subtypes (PFA 18a, 18b, 18c, 18d, 18e) were identified from the five re-isolated strains by PFGE using restriction enzyme *Apa*I (Table 7-4 and Figure 7-5). Four molecular types were identified by PFGE using restriction enzyme *Xba*I (PFX 11, 26, 27, 27a), whereas the IRS-PCR method using PXT primer identified four molecular types (IrsT 1g, 13, 55, 55a).

Table 7-4. Genetic diversity of isolate AC 5679 re-isolated from artificially infected sheep

Isolate Number	Zym	PFA	PFX	IrsT	Hoof
01-71-293 (AC 5679)	U5	18	11	1g	nk
00-179-676b	U+	18a	11	1g	230 LH
00-285-121	U5	18b	26	13	192 RH
00-285-117	U5	18c	26	55	116 RH
00-285-116	U5	18d	27	55a	116 LH
00-285-133	U5	18e	27a	55a	411 RH

IrsT = molecular type using primer PXT and IRS-PCR method; LF = left front; LH = left hind; nk = not known; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; PFX = molecular type using restriction enzyme *Xba*I and PFGE method; RF = right front; RH = right hind; U5 = protease heat labile with zymogram profile 5; U+ = protease heat labile with undesignated zymogram profile; Zym = zymogram profile.

The molecular type PFA 18a was different to the original molecular type PFA 18 by the absence of a restriction band at 125 kb (Figure 7-5, lanes 13 and 14). The restriction band at 145.5 kb was absent in molecular type PFA 18b, but bands were present at 150 and 140 kb. The bands at 125 kb and 107 kb were absent in PFA 18b, and an additional band occurred at 48.5 kb (Figure 7-5, lanes 13 and 15). Molecular type PFA 18c had additional restriction bands at 140, 128 and 48.5 kb, but did not possess bands at 125 or 107 kb, compared to PFA 18 (Figure 7-5, lanes 13 and 16). PFA 18d possessed additional restriction bands at 140 kb, and 65 kb, but lacked a restriction band at 117 kb that occurred in PFA 18 (Figure 7-5, lanes 13 and 17).

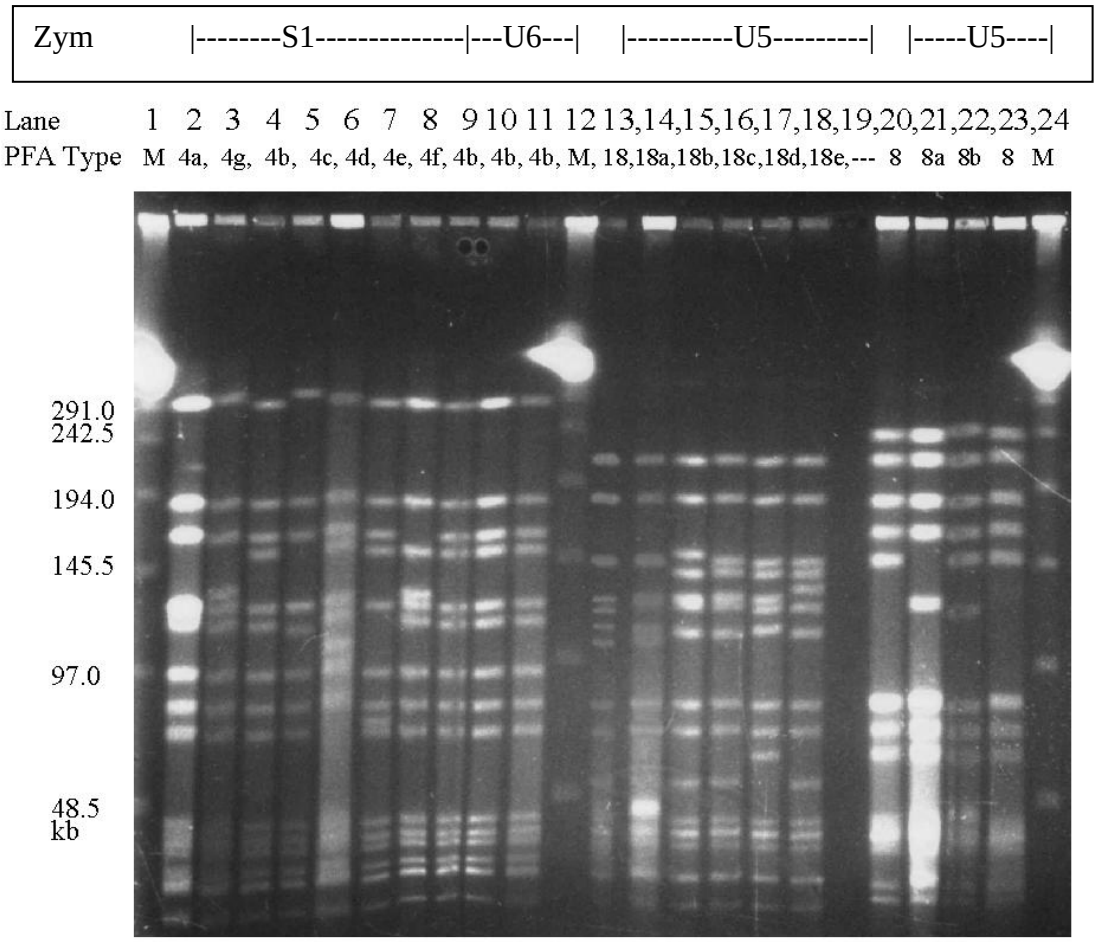


Figure 7-5. Detection of molecular subtypes following prolonged infection with known strains

Lane 1 = DNA lambda ladder; Lane 2 = A198 from culture collection; Lane 3 = 00-186-744; Lane 4 = 00-167-498; Lane 5 = 00-167-488; Lane 6 = 00-167-486; Lane 7 = 00-167-502; Lane 8 = 00-181-696; Lane 9 = 00-157-547; Lane 10 = 00-167-507; Lane 11 = 00-167-480; Lane 12 = DNA lambda ladder; Lane 13 = 00-143-307; Lane 14 = 00-179-676b; Lane 15 = 00-285-121; Lane 16 = 00-285-117; Lane 17 = 00-285-116; Lane 18 = 00-285-133; Lane 19 = DNA lambda ladder; Lane 20 = 98-467-205#2; Lane 21 = 00-186-755#1; Lane 22 = 00-143-306; Lane 23 = 00-203-843#2; Lane 24 = DNA lambda ladder.

Molecular type PFA 18e possessed additional restriction bands at 140, 135 and 48.5 kb, but did not possess bands at 130 and 117 kb, which were present in PFA 18 (Figure 7-5, lanes 13 and 18).

7.3.3.3 Results of molecular typing of re-isolated strains from AC 6161

A second U5 strain (AC 6161) used to artificially infect sheep was molecular type PFA 8. Two PFA 8 subtypes were detected, PFA 8a and PFA 8b (Table 7-5, Figure 7-5, lanes 20 to 23).

Table 7-5. Genetic diversity of isolate AC 6161 re-isolated from infected sheep

Isolate Number	Zym	PFA	PFX	IrsT	Hoof
98-467-205 #2	U5	8	28	29	nk
00-186-755 #1	U5	8a	28	29	201 LH
00-143-306	U5	8b	28a	29d	nk
00-203-843 #2	U5	8	28	29	75 RF

IrsT = molecular type using primer PXT and IRS-PCR method; LH = left hind hoof; nk = not known; PFA = molecular type using restriction enzyme *Apa*I and PFGE method; PFX = molecular type using restriction enzyme *Xba*I and PFGE method; RF = right front; U5 = protease heat labile with zymogram profile 5; Zym = zymogram profile.

Molecular type PFA 8a was different to PFA 8 by the absence of a restriction band at 145.5 kb and the presence of a band at 130 kb (Figure 7-5, lanes 20 and 21). Molecular type PFA 8b possessed a band at 125 kb that was not seen in PFA 8 (Figure 7-5, lanes 20 and 22).

7.3.4 Case Study 4

7.3.4.1 Comparison of whole cell protein profiles of seven re-isolated strains from A198

The whole cell protein profiles of S1 strains obtained from sheep artificially infected with strain A198 showed no difference to the whole cell protein profiles obtained for the U6 strains that were co-isolated (Figure 7-6, lanes 2 to 7, and lane 8).

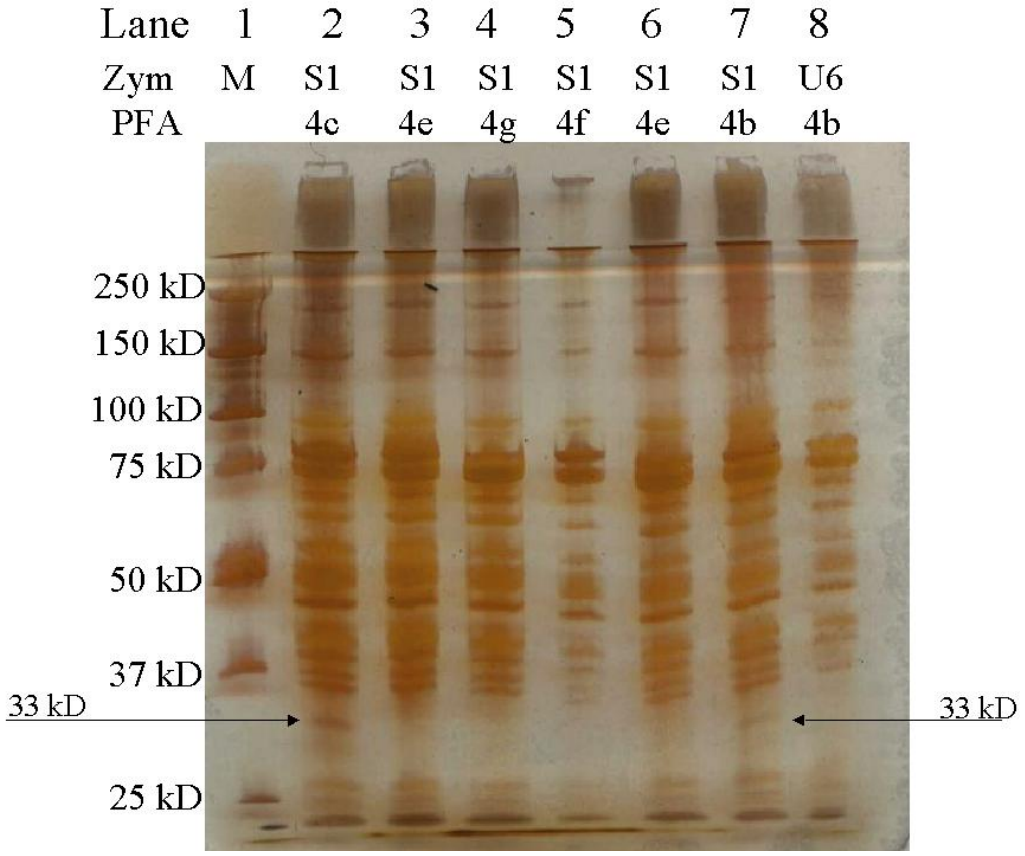


Figure 7-6. Whole cell protein profiles of molecular subtypes of isolate A198

Lane 1 = Precision protein standard; Lane 2 = 00-186-763; Lane 3 = 00-181-692; Lane 4 = 00-186-744; Lane 5 = 00-181-696; Lane 6 = 00-181-711; Lane 7 = 00-167-498; Lane 8 = 00-167-507.

A protein band at approximately 33 kD was seen in the protein patterns for PFA 4c (Figure 7-6, lane 2) and 4b (Figure 7-6, lane 7), which was not apparent in the other S1 strains or U6 strain. Overall, very little difference occurred in the whole cell protein profiles for the molecular types PFA 4b, 4c, 4e, 4f, 4g derived from strain A198 (Figure 7-6).

7.3.4.2 Results for the comparison of whole cell protein profiles of five isolates from PFA 11 clonal group

The molecular subtypes PFA 11, 11f, 11c and 11n, detected from five sheep on five farms, were similar in their protein profiles (Figure 7-7, lanes 2 to 7). Molecular subtypes of PFA 11, 11c, 11f, and 11n had identical protein bands. A minor difference was detected in the protein banding pattern for the two isolates identified as molecular type PFA 11. Isolate 00-281-95 (from Farm 150) in lane 3 had a double band at

approximately 60 kD that did not appear to be present or was not expressed at the same concentration as in isolate 00-199-824 (from Farm 142) in lane 2 (Figure 7-7).

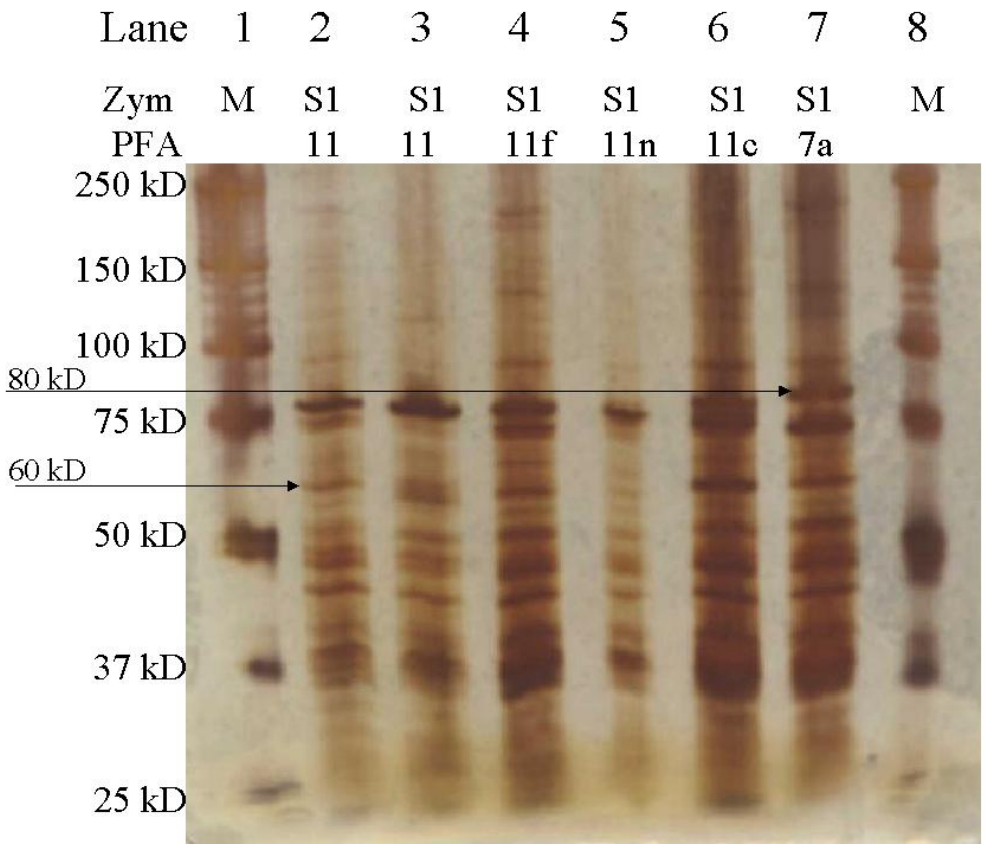


Figure 7-7. Whole cell protein profiles of isolates from clonal group PFA 11

Lane 1 = Precision protein standard (M); Lane 2 = 00-199-824; Lane 3 = 00-281-95; Lane 4 = 00-237-981 #2; Lane 5 = 00-39-109 #2; Lane 6 = 00-431-542; Lane 7 = AC2127; Lane 8 = Precision protein standard (M).

All isolates were from different farms. Control isolate AC 2127 (PFA 7a) (Figure 7-7, Lane 7) had identical bands to the isolates from clonal group PFA 11 except for a slight difference in the weights of two bands at 75 and 80 kD.

7.3.4.3 Results for the comparison of whole cell protein profiles of six isolates representing different zymogram profiles and different clonal groups

Six isolates representing three zymogram profiles and six clonal groups were different in their protein profiles. Differences in the proteins occurred between all clonal groups tested. Many protein bands were common between the clonal groups, however a number of bands were distinctly different. This difference also was noted between

isolates from different clonal groups, but of the same zymogram profile. A difference between PFA 52 (U5) and PFA 44b (S1) occurred in the region of 37 kD, although in this same region no difference was observed between PFA 24 (S1) and PFA 52 (U5). Differences at the 37 kD region occurred between the two U5 isolates (Lane 2 and Lane 5, Figure 7-8).

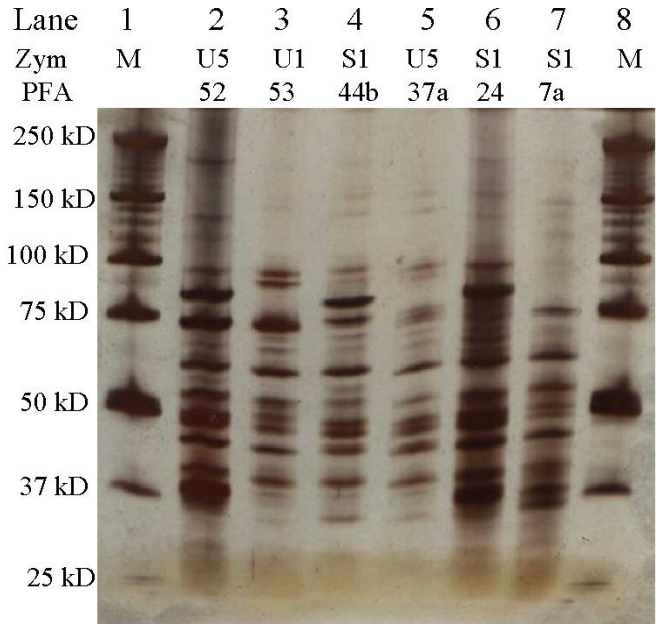


Figure 7-8. Whole cell protein profiles for isolates of different zymogram groups and different clonal groups

Lane 1 = Precision protein standard; Lane 2 = V8; Lane 3 = V23; Lane 4 = V48; Lane 5 = AC 8058; Lane 6 = V3; Lane 7 = AC2127; Lane 8 = Precision protein standard.

The region of 75-80 kD showed protein band differences between all isolates irrespective of clonal group or zymogram group. Some proteins were present in some isolates but not in others.

No whole cell proteins were identified that differentiated the virulent isolates (V3, V8 and V48) from the benign isolates (V23 and AC 8058). A protein band at approximately 80 kD was present in the virulent U5 isolate (V8) and the virulent S1 (V48) and V3, but was absent in the S1 strain AC 2127. However, this latter isolate is capable of causing a virulent lesion and has been extensively analysed in field tests in WA (Depiazzi *et al.*, 1998).

7.4 DISCUSSION

This study used PFGE to investigate the epidemiology of footrot on farms, in a single hoof, between farms, and over a prolonged time either on a farm or on farms throughout WA. In the course of this study many molecular types were identified, which could be grouped into clonal groups, and multiple molecular subtypes were found in a single hoof, on a farm or in an outbreak situation. These typing results indicated that *D. nodosus* was genetically diverse. In the current chapter the mechanisms underlying this diversity were investigated, and found to be largely due to insertions or deletions of large amounts of DNA, with some observations consistent with point mutation being a cause of the genetic diversity. The small study here to determine whether this diversity impacted on virulence, as defined by protease thermostability, indicated that the genetic change detected was random and did not appear to affect virulence. The whole cell protein profiles of isolates within the same clonal group were generally similar, whereas isolates from different clonal groups and zymogram profiles had different protein profiles.

A number of mechanisms may contribute to genetic diversity in bacteria, including: reproductive infidelity; DNA rearrangements (via transposons, insertion sequence elements, or site-specific or homologous recombination); DNA acquisition (horizontal transfer from other strains of bacteria), and normal genetic drift arising from mutations that may be selected for and accentuated by environmental pressure (Lorenz and Wackernagel, 1994; Mahillon and Chandler, 1998; Mahillon *et al.*, 1999; Prozorov, 2001). Polymorphism caused by genetic variation mechanisms can be detected using specific genomic sequencing, or by less expensive and simpler techniques such as PFGE. The PFGE method has the advantage of examining the entire genome, as all restriction bands are resolved on the gel and the sum of the molecular weights of the restriction band totals the molecular weight of the chromosomal DNA. Any genetic movement that occurs either at a restriction site (point mutation) or within a restriction fragment by the insertion or deletion of large amounts of DNA is likely to be detected. PFGE is able to detect genetic differences that may arise from natural transformation, transposons, recombination, replication errors or bacteriophage transduction. PFGE is unable to detect transfer of plasmids (van Belkum *et al.*, 2001).

The mechanism of point mutation as a means of causing genetic diversity in *D. nodosus* was observed in this study on only a few occasions. In case study one, the molecular types of two isolates detected in infected sheep on the same property were different by three restriction bands, indicating they were genetically similar. When these two fingerprints were examined it was concluded that a point mutation was likely to be responsible for the resulting genetic change between the two molecular types. A point mutation at the restriction site that had produced a 325 kb DNA fragment in molecular type PFA 24 had resulted in the loss of this restriction fragment to produce two additional restriction fragments at 125 kb and 200 kb for PFA type 24a (Figure 7-1). The observation that point mutation as a cause of genetic diversity in *D. nodosus* appeared to occur less frequently than other mechanisms is in contrast to the situation with the opportunistic pathogen, *Staphylococcus aureus*. The genetic diversity in this organism is primarily due to point mutations, as detected by multilocus sequence typing (MLST) (Feil *et al.*, 2003). Having said that, this conclusion needs to be treated with caution because point mutations may occur on a much wider scale but may not be detected with the methods used here. Unless the point mutation occurs in an *Apa*I restriction site it will not be detected by PFGE.

The most commonly noted mechanisms for the genetic diversity in *D. nodosus* were the insertion and deletion of large amounts of DNA. This was observed in strains at the farm level, at the hoof level, and in a flock of sheep artificially infected with known strains. The observation of genetic diversity occurring at the hoof level was demonstrated in case study two, where two isolates detected in the same hoof of an infected sheep had different but genetically similar molecular types. The zymogram results were difficult to interpret and the isolates were said to be a combination of a U6 and a U4 zymogram profile (M. Palmer, Australian Reference Laboratory for Ovine Footrot, Albany, unpublished data, 2003). All three PFA molecular types (PFA 39, 39a, 39c) were identical by the IRS-PCR method (IrsT 1a). A second isolate (AC 3109), from the same hoof, had a further PFA type of 39b. The difference in profiles between PFA 39, 39a, 39b and 39c was the absence or presence of a restriction fragment. PFA 39c was different to PFA 39 by the presence of a restriction band at 230 kb, which was not present in PFA 39, whereas PFA 39b was different to PFA 39 by the absence of a band at 130 kb. The difference between PFA 39 and PFA 39a was two restriction

fragments with PFA 39a having an extra band at 77kb, but the absence of a band at 82 kb. The derivation of PFA 39a is likely to be due to small amounts (approximately 5 kb) of DNA exchange, but the two molecular types PFA 39b and 39c would appear to have arisen due to large amounts of DNA being inserted or deleted from the genome or within the genome. This genetic change was not detected by the IRS-PCR method. The PFGE is likely to be more sensitive for detecting genetic change because the entire genome is examined on the gel. This was shown to be the case from the results in chapter 4. The IRS-PCR method only selects a subset of restriction fragments, those with a *Xba*I recognition site at one end, and a *Cfo*I restriction site at the other. With the PFGE method the restriction fragments are in the order of kilobases, whereas the IRS-PCR amplifies comparatively small restriction fragments of between 100 to 600 base pairs.

The observation of genetic diversity occurring at the flock level was demonstrated in case study three, which analysed molecular types of the isolates recovered from sheep artificially infected with one of three known strains. In the pulsed field profiles of *D. nodosus*, particularly those from sheep artificially infected with the Type strain (A198), there were three observations; complete loss of a restriction fragment, complete gain of a restriction fragment, or loss of a fragment and appearance of a new fragment. These same observations were seen when a transposon was infected into *Enterococcus faecalis* and the resulting strains were typed by PFGE (Thal *et al.*, 1997). The transposon had inserted into different locations on the *E. faecalis* chromosome and the PFGE typing gave rise to eight genotypes. These genotypes were different from the wild type by one to four bands, but were different from each other by up to seven bands. The transposon had inserted into the genome either once or twice and at a number of different points on the chromosome. With *D. nodosus* strain A198, six different genotypes were found from the infection trial, and these were all different to the genotype of a strain of A198 held in the culture collection. The genotypes differed from each other and from the wild type by one to five band differences. Thus, it is possible that the cause of this diversity in *D. nodosus* was the insertion and deletion of a transposon, although this needs to be verified by further studies.

The insertions and deletions of large amounts of DNA seen in the PFGE profiles for *D. nodosus* in case study three amounted to changes in the molecular weight of the entire genome by approximately 35-40 kb, which suggested insertion or deletion of DNA of this size. For example, PFA 4f appeared to derive from PFA 4b by the deletion of approximately 35 kb from the 170 kb restriction fragment in PFA 4b to give rise to a band of lower molecular weight at 135 kb (Figure 7-3, lanes 4 and 8). Similarly, PFA 4e appeared to derive from PFA 4b by the deletion of approximately 45 kb from the 120 kb restriction fragment in PFA 4b to produce a smaller fragment at 75 kb in PFA 4e (Figure 7-3, lanes 4 and 7). An insertion of approximately 40 kb into the restriction fragment at 70 kb in PFA 4b appeared to give rise to the larger fragment at 110 kb in PFA 4d (Figure 7-3, lanes 4 and 6). This 35-40 kb difference is similar in molecular weight to a reported 27 kilobase region denoted the *vrl* (virulence related locus) (Katz *et al.*, 1991). The *vrl* is present in a majority of virulent strains of *D. nodosus* and is thought to have arisen from the insertion of either a bacteriophage or plasmid through a site-specific recombination event. However, the proteins encoded by the *vrl* were not the same as those involved in virulence (Billington *et al.*, 1999).

A band shift in electrophoretic position was seen with one molecular type (PFA 4c). This type of genetic change may indicate a chromosome mutation (Warren *et al.*, 2002).

The rate of genetic change and the number of molecular subtypes that may arise from a parent or outbreak strain was indicated in the results from case study three. The molecular typing of strains re-isolated from the sheep artificially infected with the Type strain A198 provided good evidence for what might happen in an outbreak situation where there is a prolonged infection in a flock. The isolates were typed 12 to 18 months after the initial infection, therefore the time of the first appearance of molecular subtypes cannot be determined. The fact that six subtypes were identified after 12 months confirmed the results noted from field situations where prolonged infection on a farm gave rise to molecular subtypes, which eventually gave rise to a clonal group. This is a common finding for most bacteria where molecular epidemiological studies have been undertaken. Knowledge of the rate of this genetic change is important, so that the source of an outbreak can be correctly identified, or informed decisions can be made about the significance of the molecular types of isolates typed for trace-back purposes.

A rapid rate of change will produce many molecular subtypes, and the interpretation of genetic similarity to the outbreak strain may be difficult. Similarly, a slow rate of change will not produce the same number of subtypes. Two isolates may be genetically similar, but may not come from the same source strain because the change occurred over a number of years. In a flock of sheep artificially infected with strain A198, 12% of isolates examined showed genetic change. This percentage of new molecular types seen in sheep infected with the *D. nodosus* Type strain was higher than the percentage of genetic change observed in one study for the bacterium *Mycobacterium tuberculosis*. The latter study reported that 5% of patients had isolates that showed genetic change according to restriction length polymorphisms in the insertion element IS*6110* (Niemann *et al.*, 1999). The study also reported that this genetic change occurred after a period of 190 days, with little or no genetic change seen before this time. A different study on the same organism, but using isolates from the USA, indicated a higher rate of genetic change at 24% (Yeh *et al.*, 1998). It was hypothesized that this difference in genetic change rates might be due to a difference between strains.

One PFA type (PFA 4e) in case study three occurred more frequently than the other PFA types that comprised the clonal group PFA 4. No conclusions can be drawn from this without further work, but it may indicate that there is an area of the genome in *D. nodosus* that is undergoing a consistent, less random change. PFA 4e may have undergone some changes that enhanced its transmission or survival compared to the other new PFA types.

The trial involving the *D. nodosus* strains was terminated 18 months after initial infection. Future work that explores the survival of the molecular subtypes would be of interest to assist in interpretation of results for molecular epidemiology. It would also be of benefit to determine if different strains have difference rates of genetic change, and if this genetic change occurred at different time in the disease outbreak. Molecular epidemiology studies of *M. tuberculosis* involving the genetic stability and rate of change of the DNA fingerprint marker suggested that the emergence of variant strains gave rise to two populations (Warren *et al.*, 2002). One population had a higher rate of genetic change and arose prior to treatment in the phase of the disease process, which might indicate that the rate of change was influenced by active growth and adaptation of

the organism to a new environment. A second population had a slower rate of change and occurred in the latent disease phase or after treatment.

Further work in this area for *D. nodosus* should include the tracking of isolates from marked individual hoofs, and determining if the variant strains or molecular subtypes persist or in turn show further genetic change. In this study, the analysis of results was very basic, and other work suggests that involved mathematical formulae should be applied to determine a true indication of the rate of genetic change (Tanaka and Rosenberg, 2001).

Also in case study three, a U5 strain (AC 5679) that had been artificially inoculated into sheep in the same flock was examined. Five molecular subtypes (PFA 18a, 18b, 18c, 18d, 18e) that were genetically similar to the original PFA molecular type (PFA 18) were re-isolated from the artificially infected sheep. As was found with the molecular subtypes obtained for strain A198, which appeared to have arisen as a result of insertion or deletion of approximately 35-40 kb, the same molecular size seemed to be inserted or deleted in some of the subtypes obtained with strain AC 5679. In the molecular subtype PFA 18c, the 107 kb restriction band in PFA 18 and PFA 18a was absent, but was replaced by a band at 145.5 kb. The band at 145.5 kb appeared to have arisen from the insertion of approximately 35.5 kb of DNA into the band at 107 kb in PFA 18 and 18a. The molecular type PFA 18b had a very similar profile to PFA 18c, however the band at 150 kb, which was present in PFA 18 18a and PFA 18c, was slightly larger in PFA 18b at 155.5 kb. PFA 18a was similar to PFA 18 with the exception of a brightly staining band at 40 kb. The subtypes PFA 18d and 18e had a three-band difference with PFA 18 and the molecular change did not appear to be due to one event, such as the insertion or deletion of a singular fragment of DNA of 30-40 kb as in the other fingerprints. This also was found with molecular subtypes (such as PFA 4c) for strain A198, where the PFA types could not be explained by a simple insertion or deletion of 35-40 kb or DNA. It may be possible that these molecular types arose because of the insertion or deletion of DNA at multiple sites on the genome as found for the study with transposons in *E. faecalis* (Thal *et al.*, 1997). The genetic change detected by PFGE in the strains re-isolated from AC 5679 also was detected using restriction enzyme *Xba*I, and with the IRS-PCR method using PXT primer.

The second U5 strain (AC 6161) that was re-isolated from the artificially infected sheep in case study three also produced molecular subtypes by PFGE that could be attributed to insertion and deletion of DNA of approximately 35-40 kb. One of the molecular subtypes was PFA 8a, and this was different from the original type by approximately 35.5 kb. This appeared to have arisen by a deletion of 35.5 kb in fragment 155 kb in PFA 8 with a resulting fragment at 125 kb in PFA 8a (Table 7-3, Figure 7-4, Lane 20 and 21). The molecular type PFA 8b appeared to be a combination of both PFA 8 and PFA 8a as it had bands common to both subtypes. This result would need to be checked to ensure there was not a mixture of both subtypes (PFA 8a and 8b) in the culture. As large numbers of isolates derived from the initial U5 strains were not available to be typed the rate of genetic change could not be compared to the results obtained for strain A198.

Other bacteria such as the naturally transformable *Neisseria meningitidis* obtain their genetic diversity through homologous recombination, as shown by studies using MLST (Holmes *et al.*, 1999). Recombination as a possible means of inferring antigenic variation in the fimbrial gene was noted in New Zealand strains of *D. nodosus* in serogroups that were common to Australia (Zhou and Hickford, 2000a, 2000b). Strain A198 is not naturally transformable, although other strains are, and transformation and homologous recombination in these has been shown to cause antigenic variation in the fimbriae gene (Kennan *et al.*, 2003). If A198 is not naturally transformable, then the diversity seen with strain A198 in case study three by the gain and loss of restriction fragments appears to be due to some mechanism other than transformation. To fully understand the cause of the genetic diversity, many other techniques need to be applied to the study of *D. nodosus* such as whole genome sequencing to identify likely mobile genetic elements, as well as the use of DNA microarrays to compare strain-specific gene content.

The results indicate that the origin of genetic diversity in the *D. nodosus* population may be due to multiple events or to a number of different actions. In case study two where four molecular subtypes detected in the same hoof were investigated, no consistent differences in molecular weight could be found between the restriction bands that were either present or absent between molecular types. The absence or presence of

a restriction fragment could not be attributed to a single possible transposon as in the results for case study three. This would indicate that the genetic change is random, and that insertion or deletion of restriction sites is not due to a consistent genetic event or a common genetic element. The finding of molecular subtypes from a single PFA type in a single hoof indicates that the genetic change is occurring in the hoof lesion as the infection progresses. Whatever the mechanism of genetic diversity, it is taking place at one location. In this instance the genetic change is not occurring due to disease transmission to a new animal, or a new ecological site (hoof). The data obtained from case study four, section 5.3.4, showed that multiple molecular types and zymogram types could be isolated from the hoof at the same time, or at different times. This suggests that the close proximity of different strains in one infection site creates the possibility for horizontal genetic exchange between strains. However, whether this has taken place is difficult to determine from the fingerprinting data, as although a 35-40 kb difference was seen between some fingerprints, this was not a consistent finding for all subtypes either between or within strains.

The observed genetic diversity was investigated to determine if it had an impact or effect on virulence, as defined by protease thermostability. According to the methods used in this study, the U6 strains, and in some cases the U5 strains, were genetically identical to S strains isolated on the same property (chapter 6). Thus it was hypothesized that U6 or U5 strains evolve predominantly from the S strains that are present on the same property. In case study three in the present chapter the U6 zymogram types that were detected in the flock of sheep that had been artificially infected with strain A198 (S1) were the same molecular type as the most common molecular type (PFA 4b) observed for strain A198. The strains were genetically identical when tested by PFGE using both *Apa*I and *Xba*I, and by IRS-PCR using primer PXT. These results were the same as the findings reported in chapter 6. It has been shown that isolates that have restriction bands of the same molecular weight or that migrate an equal distance on the PFGE gel may not always be genetically homologous (Davis *et al.*, 2003). However, when two isolates give the same results by a number of methods then it increases the likelihood that they are genetically related or identical. The U6 strains were not only genetically identical by PFGE using two restriction enzymes, but also were identical by IRS-PCR.

The only observed difference between U6 strains and an S1 strain isolated on the same property, or the U6 and S strains from case study three, was in protease thermostability. Strain A198 produced thermostable protease in the gelatin-gel test, whereas the U6 strains produced heat labile protease. If the consistent insertion and deletion of DNA of approximately 35-40 kb in size is caused by a transposon, it does not explain the results that showed that the U6 strains were genetically identical but phenotypically different to the parent strain. Transposons that are inserted into or adjacent to a gene can alter the expression of that gene (Finnegan, 1989). If a transposon is responsible for one of the mechanisms generating genetic diversity in *D. nodosus*, it does not account for the loss of protease thermostability in some strains, as insertion into the gene would most likely produce a different DNA fingerprint. Therefore, if the genetic diversity is due to a transposon it does not appear to have an effect on virulence as determined by protease thermostability.

It has previously been suggested that benign strains may evolve from virulent strains of *D. nodosus* by the loss of the *vrl* region (which is found in virulent strains) and the gain of the *b* region, which is found in benign and avirulent strains (Liu and Yong, 1995). However, the gene products for the *b* region are not known, and work on the proteins encoded by the *vrl* region showed that they are not associated with proteins known to be involved in virulence (Billington *et al.*, 1999).

It is possible that changes in protease thermostability are due to a conformational change in the protein and is not a genetic abnormality, or is not one that can be detected by the typing methods used here. Previous work has indicated that the difference between a protease (V2) from a virulent isolate and a protease (B2) from a benign isolate was due to a single amino acid change. It was hypothesised that the protease genes may have diverged from a common ancestral gene (Riffkin *et al.*, 1995). Other research suggests that the isoenzyme bands arise from three or four closely-related genes that code for protease (Moses *et al.*, 1989).

Some bacteria have strains that are particularly virulent, which can be identified by a phenotypic or genetic characteristic. These virulent characteristics have been associated with particular clonal groups as identified by molecular typing techniques. For example, molecular typing identified two clusters that represented two major

virulent clones in *Streptococcus pneumoniae* (Overweg *et al.*, 1999). The results of PFGE typing identified a 162 kilobase pair fragment that represented a marker for the identification of strains that were a high risk of causing meningitis in neonates (Rolland *et al.*, 1999). The results from molecular typing of *D. nodosus* found that clonal groups were predominated by either protease thermostable strains or protease heat-labile strains (chapter 6). Therefore, the results were analysed to determine if virulence as defined by protease thermostability could be related to particular clones or molecular types. The data obtained from case study four provided a base-line examination of the whole cell proteins obtained from isolates that were the same clonal type, or different clonal types.

In case study four, differences in whole cell protein fingerprints between strains correlated with differences in clonal groups. Five isolates from the same clonal group (clonal group PFA 11) had either the same or very similar whole cell protein profiles, which emphasises the similarity of the strains in the same clonal group. In comparison, the difference in whole cell protein profiles from six strains of different molecular clonal groups was quite marked. This data provides background information to assist in the interpretation of the whole cell protein profiles found with the S1 and U6 variants. The whole cell protein profile of a U6 strain was the same as that of an S1 detected in sheep on the same farm. This strengthens the hypothesis that these isolates are the same, apart from their ability to produce thermostable protease. This in turn strengthens that hypothesis that the isolates are genetically identical, and therefore the loss of the thermostability of the protease is most likely to be a result of a post-translational change in the protein. It would have been useful to use more discriminatory means of examining protein profiles, such as two-dimensional gel electrophoresis. This was attempted, but there was insufficient time available to fully optimise the method so as to obtain meaningful results.

Isoenzyme forms can also arise from post-translational modification of a protein from a single gene. Although a great deal of research has been done in the area of sequencing regions on the genome of *D. nodosus* that are associated with virulence, it has not been possible to definitively differentiate virulent from benign isolates, and further work needs to be done. The database of S1 and U6 strains that have arisen from the infection trial in sheep will enable further studies such as subtractive hybridisation

(Winstanley, 2002), or DNA microarray analysis to be done to elucidate differences in the genome that contribute to virulence.

In conclusion, the results gained from the PFGE data suggest that a number of different mechanisms may be contributing to the genetic variation seen in *D. nodosus*. Point mutations occur, but the main factor contributing to the generation of genetic diversity is insertion and deletion of large amounts of DNA. In many examples, the insertion and deletion of DNA is a consistent molecular weight, but there are other cases where there is no consistent molecular size. From this data it would appear that these elements do not have an effect on virulence.

Further work needs to be done to fully elucidate the mechanisms in *D. nodosus* that contribute to genomic diversity and virulence. An understanding of the mechanisms that lead to the genetic diversity in *D. nodosus* will assist in overall knowledge of the organism's pathogenesis. This in turn will lead to improved strategies for control and eradication of the disease.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8

8. General Discussion

8.1 INTRODUCTION

The main aims of this thesis were to develop and apply DNA based typing techniques (PFGE and IRS-PCR) to study the molecular epidemiology of *Dichelobacter nodosus*. Investigation of a large number of strains led to new knowledge about the number and diversity of molecular types of the bacterium in Western Australia (WA), with a comparison made to a smaller number of isolates from three other Australian States. These results identified molecular clonal groups that were common between Australian States, and identified three main clonal groups that had been present on WA farms for a number of years. In turn it revealed that the organism is genetically diverse, and this diversity was observed at the level of the hoof, the farm, between farms, and within the State of WA. A small study to identify reasons for this diversity led to the conclusion that the diversity may be due to a number of mechanisms, with the prime mechanism being the insertion and deletion of large amounts of DNA. The mechanism that gives rise to this diversity does not appear to affect virulence of the organism as identified by protease thermostability, but it was found that protease thermostable strains capable of causing a virulent lesion in the hoof may give rise to protease heat labile strains that are benign.

8.2 MAJOR FINDINGS IN THE THESIS

8.2.1 Molecular epidemiology and genetic diversity of *Dichelobacter nodosus*

The DNA typing of a large collection of isolates of *D. nodosus* led to a greater understanding of the molecular epidemiology of the organism. This knowledge now can be applied to tracing the source of infection outbreaks, and controlling the disease. The organism was found to be extremely diverse, with genetic changes occurring frequently in the isolates infecting sheep on a farm. Based on the genetic diversity of the isolates detected on a farm, the farms fell into one of three categories. One category, consisting of approximately one third of farms, had genetically identical isolates, indicating that no detectable genetic change had occurred in the isolates on the farm. Some of these farms

had sheep infected with the same molecular type over a number of years. In these cases molecular change was not related to length of time of the infection. A second category, containing approximately 20% of farms, had isolates that were genetically similar or clonal, leading to the conclusion that isolates on these farms were undergoing constant genetic change. The remaining 50% of farms were in the third category, where isolates on these farms were genetically variable, and probably reflected the introduction of infected sheep brought onto these farms from different sources. Some farms investigated in this study were known to buy and sell sheep from a number of farms or saleyards throughout WA. This situation has consequences for trace-back and trace-forward studies when investigating an infection outbreak, because it means that a number of isolates need to be tested from a farm to establish all possible molecular types on that farm, and subsequent detection on trace farms. This is discussed further in 8.3.

The eradication program in WA is nearing completion, with only 59 agribusinesses (77 farms) in quarantine. The result of this work has led to a complete molecular type record of all farms infected with virulent footrot. Any trace-back that involves one of these farms that harbour multiple molecular types of *D. nodosus* will require a greater number of isolates being typed from each farm because of this genetic diversity. Such a complete record of the molecular types of the organism has not been established previously.

Multiple molecular types were found in a single hoof, and these types were found to be either genetically similar or substantially different. Different zymogram types also were found in a single hoof. These findings agree with research reported from New Zealand, where multiple molecular types were found in a single hoof using PCR primers that amplified the fimbrial genes (Zhou and Hickford, 2000).

The use of two molecular typing techniques enabled a better understanding of the genetic similarity between any two isolates. PFGE is more sensitive at detecting genetic change compared to the IRS-PCR method. Genetic shifts may occur on different parts of the genome, and may be detected by one method, but not another. Two isolates classed as genetically similar by one method, may be classed as genetically identical by another method, and thus are more closely related than are two isolates identified as genetically similar by both methods. This can assist with interpretation of data gathered

in the course of a disease outbreak, particularly if some time has elapsed between the initial disease outbreak and the collection of isolates from epidemiologically related farms.

The primary results from this work were the identification of different molecular types, and the recognition of considerable genetic diversity amongst *D. nodosus* strains. Diversity within *D. nodosus* has been reported previously. Molecular typing using PFGE showed that *D. nodosus* isolated from infected sheep on farms in Malaysia were diverse, with 12 molecular types reported from 15 isolates (Zakaria *et al*., 1998). Considerable antigenic variation exists within the organism (Claxton, 1989), and diversity exists in the variable region of the fimbrial gene (*fim*A) as shown by serological information (Elleman, 1988), and by gene sequencing (Mattick *et al*., 1999). In some isolates, diversity was found in the outer membrane proteins (Moses *et al*., 1995). A study using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) based on *omp*I gene framents restricted with *Hpa*II also found genetic diversity in this region. It was hypothesised that this genetic variation was due to intra- or intergenomic events such as recombination (Allworth, 1995). The results obtained in this current study contributed to the knowledge of genetic diversity in *D. nodosus* by analysing a large number of isolates by two molecular methods, and by quantifying this diversity. The genetic diversity was high by both methods when analysed by the Simpson diversity index, by a simple diversity ratio, and by the use of dendrograms. The PFGE method was more sensitive for detecting genetic change than the IRS-PCR, but both techniques are recommended to accurately type isolates, particularly when trying to establish whether an epidemiological relationship exists between two isolates.

The typing of *D. nodosus* was carried out on a large database of isolates predominantly from WA, but also included a selection of isolates from three other States in Australia. Such work has not been undertaken previously. The results confirmed what had been suspected, and that is, that some molecular types are common between States, which is likely to indicate that *D. nodosus* has been spread from State to State with the sale and purchase of healthy carrier sheep.

The typing results, particularly from the PFGE results, indicated that constant genetic change occurring in the chromosome led to the generation of clonal groups of

genetically similar isolates. The results indicated that three main clonal groups exist in WA and have done so for over 20 years. These clonal groups also were found in isolates tested from three other Australian States.

Generally, the main clonal groups in WA were predominated by isolates corresponding to either protease thermostable strains (clonal groups PFA 7 and 11), or protease heat labile strains (clonal group PFA 9).

8.2.2 Investigation into the mechanism leading to genetic diversity

Interpreting molecular epidemiological data is reliant on understanding the stability and evolution of the genome of an organism. The rate of change may be different depending on the stage of the disease (early infection), or during and after treatment. A greater understanding of the mechanisms that lead to genetic diversity, and the rate of genetic change, enables informed decisions to be made regarding the interpretation of DNA fingerprints of strains in the eradication program.

The major mechanism that leads to the extensive genetic diversity in *D. nodosus* appears to be the insertion and deletion of large amounts of DNA, with a number of other mechanisms, such as point mutations, also contributing to this diversity. The study on genetic diversity using the Type strain of *D. nodosus*, A198, that had been artificially inoculated into a flock of sheep, indicated that at least six molecular subtypes can be obtained from a single strain throughout the course of an infection. Such an intensive study of the genetic variation of one strain during an infection has not been undertaken previously for *D. nodosus*. Three other strains were artificially inoculated into sheep, but the molecular types obtained from these strains were not studied in detail. The results of such a study may give an indication as to whether or not some strains are more likely to undergo genetic variation.

Genetic diversity in *D. nodosus* has been reported in New Zealand where studies using the fimbrial gene found considerable diversity in the sequence of the variable region of the gene, which was postulated to be a result of recombination (Zhou and Hickford, 2000). Those results and the results from the present study indicate that the hoof is an ecological site where genetic exchange can take place. Further work needs to be undertaken to determine if there is an increased rate for genetic change at the time of

transmission of the infection, or at times of treatment (footbathing) of the disease, or if mixed infections contribute to an increased rate of genetic change. The influence of the environment also needs to be investigated to determine if there is a selective pressure that impacts on genetic diversity, and if some strains have a greater survival rate under different environmental conditions. Such findings may indicate if stress factors acting on the organism help drive genetic diversity. In other diseases the effect of prolonged infection, and time of treatment in regard to the stage of the disease are both factors that are known to affect the rate of genetic diversity (Niemann *et al*., 1999; Warren *et al*., 2002). Genes that are under strong selective pressure, such as the outer membrane proteins, often show great genetic variation, due mainly to partial recombination rather than to point mutation (Feil *et al*., 1995; Feil and Enright, 2004).

8.2.3 Virulence of the organism and relationship to molecular types

The disease has a spectrum of virulence that is graded according to the severity of the lesion in the hoof. Ever since the disease was first recognised in France in the late 1700s, controversy has surrounded the definition of virulence, and the identification of those isolates capable of causing a virulent lesion. The disease is seasonal, and it is now recognised and proved through research that environmental factors have an effect on the appearance of lesion severity, thus complicating the issue of what constitutes virulence and how it is detected.

In the late 1970s the recognition that virulent isolates were capable of producing a heat stable protease led to the development of the gelatin-gel test (Depiazzi and Richards, 1979) that is now used to differentiate virulent from benign isolates (Palmer, 1993). The issue of the detection of virulent isolates has been complicated by the discovery in WA of a small subgroup of isolates within the U5 zymogram profile that do not produce heat stable protease, yet cause virulent lesions. A new finding in this thesis was that isolates from the U5 zymogram group were genetically diverse when tested using PFGE and IRS-PCR methods, and have not arisen from clonal expansion of a recent common ancestor. The major finding from this work in regard to the U5 strains is that the subgroup capable of causing virulent lesions tended to be of the same genetic type as a protease thermostable (S1) isolate found on the same property. This finding that U5 strains are more related to an S1 strain than they are to each other suggests that

there may be some other as yet unrecognised virulence factors in *D. nodosus* strains, apart from protease thermostability.

Over the course of a disease outbreak atypical strains can arise. Overall, these strains (U6 and T strains) constitute a very small percentage of the population in WA. More information on the origin and behaviour of these strains is needed to determine how these isolates behave, and how they might impact on the success of the eradication program. A major finding in this thesis was the discovery that these isolates appear to have derived from a "conventional" thermostable protease producing (S1) strain detected on the same property. As was found for the U5 group of isolates, the U6 group were genetically diverse, but most were of the same molecular type as an S strain found on the same property. All T strains that were tested appeared to be derived from an S strain on the same property, and may represent an "intermediate" isoenzyme form that occurs between the thermostable protease (S1) strain and the loss of thermostability (U6). Both isolates produced the same genetic fingerprint using the molecular techniques applied in this thesis. The conclusion was made that the loss of protease thermostability may be due to a conformation change in the protein, rather than a genetic change. This is not a definitive conclusion, and it still needs to be determined if the conformation change in the protein is a result of a genetic mutation that is not detected using PFGE and IRS-PCR. Sequencing of the corresponding genes would provide this data. The mechanism(s) causing the genetic diversity that is seen amongst isolates of *D. nodosus* does not appear necessarily to affect virulence, as determined by the techniques used here.

8.2.4 Summary of results obtained in this thesis

- *D. nodosus* is genetically diverse, with a relatively rapid rate of genetic change. On some farms the isolates are more genetically stable than on other farms.
- Due to the diversity, more than four isolates per property should be typed to accurately determine all molecular types that may be present on a farm.
- Three main clonal groups exist in WA, with a predominance of S strains occurring in clonal groups PFA 7 and PFA 11, and U strains occurring predominantly in clonal group PFA 9.
- Multiple molecular types were found in individual hooves.

- Isolates within zymogram groups were genetically diverse, with U6, T and U5 strains more genetically related to S strains from the same property than they were to other isolates from the same zymogram group.
- Isolates from the same clonal group had the same or very similar whole cell protein profiles, whereas isolates from different clonal groups had different whole cell protein profiles.

8.3 APPLICATION OF THE RESULTS OBTAINED IN THIS THESIS

In WA, the eradication program is nearing a successful conclusion with 77 properties, representing 59 agribusinesses, in footrot quarantine as of March 2004. It is forecast that the complete eradication of virulent strains of *D. nodosus* will be achieved in the near future. The success of the eradication program is dependent on rapid tracing of an outbreak of infection. Sensitive molecular typing techniques enable this to be achieved, along with good records of sheep sales and purchases, good farm management, and good advice from trained staff from the WA Department of Agriculture. The DNA fingerprinting of all *D. nodosus* isolates detected on farms in quarantine is a focus of the WA Department of Agriculture. This is being achieved using the techniques of PFGE and IRS-PCR that were developed, optimised and applied to a collection of isolates used in this thesis. The information and knowledge gained about *D. nodosus* in the course of this work has enabled the correct interpretation to be applied to the results obtained from molecular typing. The results obtained in the thesis indicate that because of the rapid rate of genetic change and the high genetic diversity index for the organism, trace-back and collection of isolates must be done within a short time frame to ensure confidence that two strains are genetically related. The high genetic diversity index detected by both techniques and a simple diversity ratio of 1:4, as detected by PFGE, has led to the recommendation that more than four isolates from each property must be typed to allow for detection of all possible genetic types that might be present on the source property, and on trace properties.

The typing of a large collection of isolates selected from a 20-year period has allowed a perspective on the molecular type of an isolate detected during an infection outbreak trace-back, and in determining if a linkage exists between two infected

properties. If isolates detected on both properties have a molecular type common in WA then identifying an absolute link between the two properties is more difficult. The use of two typing methods may assist in such cases, and is almost essential when confirming an epidemiological link.

DNA typing of isolates has been used in a number of cases in WA to help prove the source of an infection in sheep on a property. Such cases have occurred when a cluster of infected properties is detected, involving either neighbouring properties or outbreaks occurring after the sale of unknowingly infected sheep to other properties. Records are not always kept, and the sale of sheep and quick re-sale can mean that it is difficult to trace an infection outbreak. DNA typing of isolates in such cases has assisted in establishing or disproving infection linkages.

As a result of the current work, a large database of molecular types is now stored in the GelCompar II program, and the construction of dendrograms provides a good visual interpretation of molecular types on a number of properties where a possible transmission link may occur. The dendrogram visually shows those isolates that are the same, as opposed to those that are unrelated. The interpretation of dendrograms needs to be confirmed by visual examination of the photographs of the gels. The results in this study identified instances where the dendrogram constructed by the GelCompar program was unable to differentiate two fingerprints that had restriction fragments with a slight difference in molecular weight. Visual examination of the two isolates located together on the same gel was the best way to determine whether or not two isolates were identical, and this always should be done when investigating individual strains from an outbreak, or in a trace-back exercise.

The finding that *D. nodosus* is capable of genetic change, which creates considerable genetic diversity, has a consequence for research projects. In this study it was noted that the Type strain that was used to infect sheep in a field trial had a slightly different molecular type to an earlier culture stored in the collection. This means that isolates being used for research work should be DNA fingerprinted before and after a trial to ensure that the same molecular type is re-isolated at the end of the experiment, and that if genetic changes do occur that they do not impact on research results and interpretations.

A collection of isolates used in the national project CHP4 (referred to in chapter 7) was typed in this study. When the cultures were received into the laboratory, two cultures were composed of two different molecular types, indicating the presence of two strains in the same culture vial. This may have serious consequences for interpretation of results, particularly interpretation of clinical virulence in foot lesions, which formed that basis of this particular research trial. This highlights the need to type or fingerprint isolates before and after clinical research trials.

8.4 FUTURE WORK

To ensure that eradication of footrot from the State of WA is achieved and maintained, the cause of genetic diversity, and further elucidation of the cause of virulence need to be identified. In particular, the small numbers of isolates that are capable of causing a virulent lesion, yet only produce a heat labile protease need to be studied further. This study found that benign strains might evolve from virulent strains. To date there has been no anecdotal or research evidence to suggest that the reverse occurs, and that a benign isolate can become virulent. If this were to occur it would have serious consequences for the eradication program. Techniques such as proteome analysis, subtractive hybridization, whole genome sequencing of different molecular types and protease stability types, gene inactivation studies, and the use of microarrays to interrogate different strains for gene content, including gene expression on and off the hoof, all would be useful modern techniques that could be applied to future study of *D. nodosus* and ovine footrot. The development of an MLST scheme for *D. nodosus* also would be helpful and could be used to obtain a better understanding of the population structure of this important pathogen.

The collection of 735 isolates used in this thesis have been well-characterised by both phenotypic and molecular techniques, and will provide a database of isolates that can be used for future work.

CHAPTER 9

REFERENCES

9. References

Allworth, M.B. (1995) Investigations of the Eradication of Footrot. PhD Thesis, University of Sydney, Sydney, Australia.

Arbeit, R., Arthur, M., Dunn, R., Kim, C., Selander, R., and Golstein, R. (1990) Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: The application of pulsed field gel electrophoresis to molecular epidemiology. *The Journal of Infectious Diseases* 161: 230-235.

Arber, W., Naas, T., and Blot, M. (1994) Generation of genetic diversity by DNA rearrangements in resting bacteria. *FEMS Microbiology Ecology* 15: 5-13.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1990) *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York, USA.

Beveridge, W.I.B. (1935) Foot-Rot in Sheep. Preliminary note on aetiology and possibility of control. *Journal of the Council for Scientific and Industrial Research* 8: 308-315.

Beveridge, W.I.B. (1936) A study of *Spirochaeta penortha* (n. sp.) isolated from foot-rot in sheep. *Australian Journal of Experimental Biological Medical Science* 14: 307-318.

Beveridge, W.I.B. (1938) Foot-rot in sheep: a preliminary note on the probable causal agent. *Journal of the Council for Scientific and Industrial Research* 11: 1-3.

Beveridge, W.I.B. (1941) Foot-rot in sheep: a transmissible disease due to infection with *Fusiformis nodosus* (n. sp.). Studies on its cause, epidemiology and control. *Council for Scientific and Industrial Research* Bulletin No. 140: 1-56.

Beveridge, W.I.B. (1956) Foot-rot of Sheep. A modern approach to an old problem. *The Veterinary Record* 68: 963-965.

Beveridge, W.I.B. (1981) The early history of foot-rot. In *Ovine Footrot*: *Report of Workshop at University of Sydney*, Sydney, Australia, pp. 200.

Billington, S.J., Johnston, J., and Rood, J. (1996) Virulence regions and virulence factors of the ovine footrot pathogen, *Dichelobacter nodosus*. *FEMS Microbiology Letters* 145: 147-156.

Billington, S.J., Huggins, A.S., Johanesen, P.A., Crellin, P.K., Cheung, J.K., Katz, M.E., Wright, C.L., Haring, V., and Rood, J.I. (1999) Complete nucleotide sequence of the 27-kilobase virulence related locus (*vrl*) of *Dichelobacter nodosus*: evidence for extrachromosomal origin. *Infection and Immunity* 67: 1277-1286.

Blaizot, L., and Blaizot, P. (1928) *Treponema podovis* (n. sp.) agent pathogene du pietin des moutons. *Comptes Rendus des Seances de l' Academie des Sciences* 187: 911-912.

Blaizot, L., and Blaizot, P. (1929) *Treponema podovis* the pathogenic cause of foot-rot in sheep. *Translated in Veterinary Record* 9: 95-96.

Bloomfield, G.A., Whittle, G., McDonagh, M.B., Katz, M.E., and Cheetham, B.F. (1997) Analysis of sequences flanking the vap regions of *Dichelobacter nodosus*: evidence for multiple integration events, a killer system, and a new genetic element. *Microbiology* 143: 553-562.

Branger, C., Gardye, C., Galdbart, J.-O., Deschamps, C., and Lambert, N. (2003) Genetic relationship between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* strains from France and from international sources: delineation of genomic groups. *Journal of Clinical Microbiology* 41: 2946-2951.

Broad, T.E., and Skerman, T.M. (1976) Partial purification and properties of extracellular proteolytic activity of *Bacteroides nodosus*. *New Zealand Journal of Agricultural Research* 19: 317-322.

Brown, G.T. (1892). *Veterinary Journal* 35: 180 and 247. Cited by Beveridge, W.I.B. (1941)

Brueggemann, A., Griffiths, D., Meats, E., Peto, T., Crook, D., and Spratt, B. (2003) Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *Journal of Infectious Diseases* 187: 1424-1432.

Cameron, D., Khambaty, F., Wachsmuth, I., Tauxe, R., and Barrett, T. (1994) Molecular characterisation of *Vibrio cholerae* 01 strains by Pulsed-Field Gel Electrophoresis. *Journal of Clinical Microbiology* 32: 1685-1690.

Casadevall, A., and Porofski, L.-A. (1999) Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infection and Immunity* 67: 3703-3713.

Chabert, P., Flandrin, P., and Huzard, J.B. (1791) *Instructions et observations sur les maladies des animaux domestiques.* Veuve Vallat-la-Chapelle, Paris, France.

Cheetham, B., Tattersall, D., Bloomfield, G., Rood, J., and Katz, M. (1995) Identification of a gene encoding a bacteriophage-related integrase in a *vap* region of the *Dichelobacter nodosus* genome. *Gene* 162: 53-58.

Cheetham, B., Tanjung, L., and Katz, M. (2004) The role of integrated genetic elements in the virulence of *Dichelobacter nodosus*. In *The Australian Society for Microbiology* Sydney, Australia, pp. 49.

Chetwin, D.H., Whitehead, L.C., and Thorley, S.E.J. (1991) The recognition and prevalence of *Bacteroides nodosus* serotype M in Australia and New Zealand. *Australian Veterinary Journal* 68: 154-155.

Chu, G., Vollrath, D., and Davis, R.W. (1986) Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* 234: 1582-1585.

Clark, B.L., J, S.D., and Emery, D.L. (1985) The role of *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* in the aetiology of interdigital necrobacillosis in cattle. *Australian Veterinary Journal* 62: 47-49.

Claxton, P.D. (1989) Antigenic classification of *Bacteroides nodosus*. In *Footrot and foot abscess of ruminants.* Egerton, J.R., Yong, W.K. and Riffkin, G.G. (eds). CRC Press, Boca Raton, USA, pp. 155-165.

Claxton, P.D., Ribeiro, L.A., and Egerton, J.R. (1983) Classification of *Bacteroides nodosus* by agglutination tests. *Australian Veterinary Journal* 60: 331-334.

Collighan, R.J., Naylor, R.D., Martin, P.K., Cooley, B.A., Buller, N., and Woodward, M.J. (2000) A spirochete isolated from a case of severe virulent ovine foot disease is closely related to a Treponeme isolated from human periodontitis and bovine digital dermatitis. *Veterinary Microbiology* 74: 249-257.

Cooper, B.S. (1977) Differences in morphology of *Bacteroides nodosus* attributable to culture media. *New Zealand Veterinary Journal* 25: 16-20.

Cross, R.F., and Parker, C.F. (1981) Zinc sulphate foot bath for control of ovine foot rot. *Journal of the American Veterinary Medical Association* 178: 706-707.

Cruickshank, R., Duguid, J., Marmion, B., and Swain, R. (1975) *Medical Microbiology.* Churchill Livingstone, Edinburgh, Scotland.

Davis, M., Hancock, D., Besser, T., and Call, D. (2003) Evaluation of Pulsed-Field Gel Electrophoresis as a tool for determining the degree of genetic relatedness between strains of *Escherichia coli* 0157:H7. *Journal of Clinical Microbiology* 41: 1843-1849.

Day, S.E.J., Thorley, C.M., and Beesley, J.E. (1986) Serotyping of *Bacteroides nodosus*: proposal for 9 further serotypes (J-R) and a study of the antigenic complexity of *B. nodosus* pili. In *Footrot in Ruminants. Proceedings of a workshop*. Stewart, D.J., Peterson, N.M., McKern, N.M. and Emery, D.L. (eds). Melbourne, Victoria, 1985: Commonwealth Scientific and Industrial Research Organization and Australian Wool Corporation, Sydney, pp. 147-159.

de Gasparin, A.-E.-P. (1821) *Des maladies contagieuses des bêtes à laine.* Huzard, Paris, France. Cited by Beveridge, W.I.B. (1981).

Deane, H.M., and Jensen, R. (1955) The pathology of contagious foot rot in sheep. *American Journal of Veterinary Research* 16: 203-208.

Demirkan, I., Carter, S.D., Winstanley, C., Bruce, K.D., McNair, N.M., Woodside, M., and Hart, C.A. (2001) Isolation and characterisation of a novel spirochaete from severe virulent ovine foot rot. *Journal of Medical Microbiology* 50: 1061-1068.

Depiazzi, L.J., and Richards, R.B. (1979) A degrading proteinase test to distinguish benign and virulent ovine isolates of *Bacteroides nodosus*. *Australian Veterinary Journal* 55: 25-28.

Depiazzi, L.J., and Richards, R.B. (1984/85) Motility in relation to virulence of *Bacteroides nodosus*. *Veterinary Microbiology* 10: 107-116.

Depiazzi, L.J., and Rood, J.I. (1984) The thermostability of proteases from virulent and benign strains of *Bacteroides nodosus*. *Veterinary Microbiology* 9: 227-236.

Depiazzi, L.J., Henderson, J., and Penhale, W.J. (1990) Measurement of protease thermostability, twitching motility and colony size of *Bacteroides nodosus*. *Veterinary Microbiology* 22: 353-363.

Depiazzi, L.J., Richards, R.B., Henderson, J., Rood, J.I., Palmer, M.A., and Penhale, W.J. (1991) Characterisation of virulent and benign strains of *Bacteroides nodosus*. *Veterinary Microbiology* 26: 151-160.

Depiazzi, L.J., Roberts, W.D., Hawkins, C.D., Palmer, M.A., Pitman, D.R., McQuade, N.C., Jelinek, P.D., Devereaux, D.J., and Rippon, R.J. (1998) Severity and persistence of footrot in Merino sheep experimentally infected with a protease

thermostable strain of *Dichelobacter nodosus* at five sites. *Australian Veterinary Journal* 76: 32-38.

Dewhirst, F.E., Paster, B.J., Fontaine, S.L., and Rood, J.I. (1990) Transfer of *Kingella indologenes* (Snell and Lapage 1976) to the genus *Suttonella* gen. nov. as *Suttonella indologenes* comb. nov.; transfer of *Bacteroides nodosus* (Beveridge 1941) to the genus *Dichelobacter* gen. nov. as *Dichelobacter nodosus* comb. nov.; and assignment of the genera *Cardiobacterium*, *Dichelobacter*, and *Suttonella* to *Cardiobacteriaceae* fam. nov. in the gamma division of *Proteobacteria* on the basis of 16S rRNA sequence comparisons. *International Journal of Systematic Bacteriology* 40: 426-433.

Dice, L.R. (1945) Measures of the amount of ecologic association between species. *Ecology* 26: 297-302.

Duck, W., Steward, C., Banerjee, S., McGowan Jr, J., and Tenover, F. (2003) Optimisation of computer software settings improves accuracy of pulsed-field gel electrophoresis macrorestriction fragment pattern analysis. *Journal of Clinical Microbiology* 41: 3035-3042.

Egerton, J.R. (1973) Surface and somatic antigens of *Fusiformis nodosus*. *Journal of Comparative Pathology* 83: 151-159.

Egerton, J.R., and Laing, E.A. (1978/1979) Characteristics of *Bacteroides nodosus* isolated from cattle. *Veterinary Microbiology* 3: 269-279.

Egerton, J.R., and Parsonson, I.M. (1969) Benign foot-rot - a specific interdigital dermatitis of sheep associated with infection by less proteolytic strains of *Fusiformis nodosus*. *Australian Veterinary Journal* 45: 345-349.

Egerton, J.R., Roberts, D.S., and Parsonson, I.M. (1969) The aetiology and pathogenesis of ovine footrot. I. A histological study of the bacterial invasion. *Journal of Comparative Pathology* 79: 209-215.

Egerton, J.R., Yong, W.K., and Riffkin, G.G. (1989) *Footrot and Foot Abscess of Ruminants.* Boca Raton, Florida: CRC Press.

Egerton, J.R., Dhungyel, O.P., Ghimire, S.C., and Rasali, D.P. (1994) Management of footrot. In *Proceedings of the Seminar on Footrot Eradication Programme in sheep and Goats in Nepal.* Vol. 94-1 Lumle-Agricultural-Centre, pp. 13-17.

Egerton, J.R., Roberts, D.S., Graham, N.P.H., and Parsonson, I.M. (1966) Ovine interdigital dermatitis and its relationship to foot-rot and foot abscess. *Australian Veterinary Journal* 42: 440.

Eisenstein, B. (1990) New molecular techniques for microbial epidemiology and the diagnosis of infectious diseases. *The Journal of Infectious Diseases* 161: 595-602.

Elleman, T.C. (1988) Pilins of *Bacteroides nodosus*: molecular basis of serotype variation and relationships to other bacterial pilins. *Microbiological Reviews* 52: 233-247.

Emery, D.L., Stewart, D.J., and Clark, B.L. (1984) The comparative susceptibility of five breeds of sheep to foot-rot. *Australian Veterinary Journal* 61: 85-88.

Escayg, A.P., Hickford, J.G.H., and Bullock, D.W. (1997) Association between alleles of the ovine major histocompatibility complex and resistance to footrot. *Research in Veterinary Science* 63: 283-287.

Everitt, B. (1974) *Cluster analysis.* Heinemann Educational Books Ltd, Lndon, UK.

Every, D. (1979) Purification of pili from *Bacteroides nodosus* and an examination of their chemical, physical and serological properties. *Journal of General Microbiology* 115: 309-316.

Every, D. (1982) Proteinase isoenzyme patterns of *Bacteroides nodosus*: distinction between ovine virulent, ovine benign isolates and bovine isolates. *Journal of General Microbiology* 128: 809-812.

Every, D., and Skerman, T.M. (1980) Ultrastructure of the *Bacteroides nodosus* cell envelope layers and surface. *Journal of Bacteriology* 141: 845-857.

Feil, E., Cooper, J., Grundmann, H., Robinson, D., Enright, M., Berendt, T., Peacock, S., Maynard Smith, J., Murphy, M., Spratt, B., Moore, C., and Day, N. (2003) How clonal is *Staphylococcus aureus*? *Journal of Bacteriology* 185: 3307-3316.

Feil, E., and Enright, M. (2004) Analyses of clonality and the evolution of bacterial pathogens. *Current Opinion in Microbiology* 7: 308-313.

Feizabadi, M., Robertson, I., Cousins, D., and Hampson, D. (1996) Genomic analysis of *Mycobacterium bovis* and other members of the *Mycobacterium tuberculosis* complex by isoenzyme analysis and pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* 34: 1136-1142.

Finnegan, D. (1989) Eukaryotic transposable elements and genome evolution. *Trends in Genetics* 5: 103-107.

Fraser, R.D.B., MacRae, T.P., and Rogers, G.E. (1959). *Nature* 183: 592.

Frisch, J.E. (1976) The comparative incidence of foot rot in *Bos taurus* and *Bos indicus* cattle. *Australian Veterinary Journal* 52: 228-229.

Ghimire, S.C., and Egerton, J.R. (1999) PCR-RFLP of outer membrane proteins gene of *Dichelobacter nodosus*: a new tool in the epidemiology of footrot. *Epidemiology and Infection* 122: 521-528.

Ghimire, S.C., Egerton, J.R., Dhungyel, O.P., and Joshi, H.D. (1998) Identification and characterisation of serogroup M among Nepalese isolates of *Dichelobacter nodosus*, the transmitting agent of footrot in small ruminants. *Veterinary Microbiology* 62: 217-233.

Godoy, D., Randle, G., Simpson, A., Aanensen, D., Pitt, T., Kinoshita, R., and Spratt, B. (2003) Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. *Journal of Clinical Microbiology* 41: 2068-2079.

Goering, R., and Tenover, F. (1997) Epidemiological interpretation of chromosomal macro-restriction fragment patterns analysed by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* 35: 2432-2433.

Gohier, J.B. (1813-1816) Mémoires et observations sur la chirurgie et la médecine vétérinaires. In *Tome Premier*. Chez Lions, Lyon, France. Cited by Beveridge W.I.B. (1981).

Gordon, L.M., Yong, W.K., and Woodward, C.A.M. (1985) Temporal relationships and characterisation of extracellular proteases from benign and virulent strains of *Bacteroides nodosus* as detected in zymogram gels. *Research in Veterinary Science* 39: 165-172.

Gradin, J.L. (1993) Serogrouping of *Bacteroides nodosus* isolates from 62 sources in the United States. *American Journal of Veterinary Research* 54: 1069-1073.

Gradin, J., Sonn, A., Bearwood, D., Schmitz, J., and Smith, A. (1991) Electron microscopic study of *Bacteroides nodosus* pili and associated structures. *American Journal of Veterinary Research* 52: 206-211.

Graham, J.R. (1870) A Treatise on the Australian Merino. *Clarson, Massina and Co. Melbourne*. Cited by Beveridge, W. I.B. (1941).

Graham, N.P.H., and Egerton, J.R. (1968) Pathogenesis of ovine foot-rot: the role of some environmental factors. *Australian Veterinary Journal* 44: 235-240.

Green, R.S. (1985a) A method to differentiate between virulent and benign isolates of *Bacteroides nodosus* based on the thermal stability of their extracellular proteinases. *New Zealand Veterinary Journal* 33.

Green, R.S. (1985b) Characterization of certain proteinase isoenzymes produced by benign and virulent strains of *Bacteroides nodosus*. *Journal of General Microbiology* 131: 2871-2876.

Gregory, T.S. (1939) Foot-rot in sheep. *Australian Veterinary Journal* 15: 160-167.

Grimont, F., and Grimont, P. (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Annuals de l'Institute Pasteur, Microbiology* 137: 165-175.

Grundmann, H., Schneider, C., Hartung, D., Daschner, F., and Pitt, T. (1995) Discrimminatory power of three DNA-based typing techniques for *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology* 33: 528-534.

Hacker, J., Blum-Oehler, G., Mühldorfer, I., and Tschäpe, H. (1997) Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Molecular Microbiology* 23: 1089-1097.

Haring, V., Billington, S.J., Wright, C.L., Huggins, A.S., Katz, M.E., and Rood, J.I. (1995) Delineation of the virulence-related locus (*vrl*) of *Dichelobacter nodosus*. *Microbiology* 141: 2081-2089.

Harriss, S.T. (1948) Proliferative dermatitis of the legs ("strawberry foot rot") in sheep. *Journal of Comparative Pathology* 58: 314-328.

Harwood, D.G., Cattell, J.H., Lewis, C.J., and Naylor, R. (1997) Virulent foot rot in sheep. *Veterinary Record* 140: 687.

Hobbs, M., Dalrymple, B., Cox, P., Livingstone, S., Delaney, S., and Mattick, J. (1991) Organization of the fimbrial gene regions of *Bacteroides nodosus*: class I and class II strains. *Molecular Microbiology* 5: 543-560.

Holmes, E., Urwin, R., and Maiden, M. (1999) The influence of recombination on the population structure and evolution of the human pathogen *Neisseria meningitidis*. *Molecular Biology and Evolution* 16: 741-749.

Hulton, C.S.J., Higgins, C.F., and Sharp, P.M. (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. *Molecular Microbiology* 5: 825-834.

Hunter, P.R., and Gaston, M.A. (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of Clinical Microbiology* 26: 2465–2466.

Imanaka, T., Shibazaki, M., and Takagi, M. (1986) A new way of enhancing the thermostability of proteases. *Nature* 324: 695-697.

Jelinek, P.D., and Depiazzi, L.J. (2003) Failure to eradicate ovine footrot associated with *Dichelobacter nodosus* strain A198 by repeated daily footbathing in zinc sulphate with surfactant. *Australian Veterinary Journal* 81: 58-62.

Jessen, P., Kristiansen, O., and Madsen, F.O. (1993) Baquiloprim/sulfadimidine in calves and young cattle. Oral treatment of enzootic pneumonia and infectious pododermatitis. *Dansk-Veterinaertidsskrift* 76: 796-798.

Jiménez, R., Piriz, S., Martín-Palomino, P., Mateos, E.M., and Vadillo, S. (2003) Aetiology of ovine footrot in the Portuguese region of Alto Alentejo. *Journal of Veterinary Medicine B* 50: 118-120.

Katz, M.E., Howarth, P.M., Yong, W.K., Riffkin, G.G., Depiazzi, L.J., and Rood, J.I. (1991) Identification of three gene regions associated with virulence in *Dichelobacter* [*Bacteroides*] *nodosus*, the causative agent of ovine footrot. *Journal of General Microbiology* 137: 2117-2124.

Katz, M.E., Strugnell, R.A., and Rood, J.I. (1992) Molecular characterization of a genomic region associated with virulence in *Dichelobacter nodosus*. *Infection and Immunity* 60: 4586-4592.

Katz, M.E., Wright, C.L., Gartside, T., Cheetham, B., Doidge, C., Moses, E., and Rood, J.I. (1994) Genetic organization of the dupicated *vap* region of the *Dichelobacter nodosus* genome. *Journal of Bacteriology* 176: 2663-2669.

Kennan, R., Dhungyel, O., Whittington, R., Egerton, J., and Rood, J. (2001) The type IV fimbrial subunit gene (*fim*A) of *Dichelobacter nodosus* is essential for virulence, protease secretion, and natural competence. *Journal of Bacteriology* 183: 4451-4458.

Kennan, R., Dhungyel, O.P., Whittington, R.J., Egerton, J.R., and Rood, J.I. (2003) Transformation-mediated serogroup conversion of *Dichelobacter nodosus*. *Veterinary Microbiology* 92: 169-178.

Kortt, A.A., O'Donnell, I.J., Stewart, D.J., and Clark, B.L. (1982) Activities and partial purification of extracellular proteases of *Bacteroides nodosus* from virulent and benign footrot. *Australian Journal of Biological Science* 35: 481-489.

Kortt, A.A., Riffkin, M.C., Focareta, A., and Stewart, D.J. (1993) Amino acid sequence of extracellular acidic protease V5 of *Dichelobacter nodosus*, the causative organism of ovine footrot. *Biochemistry and Molecular Biology International* 29: 989-998.

Kortt, A.A., Burns, J.E., Vaughan, J.A., and Stewart, D.J. (1994a) Purification of the extracellular acidic proteases of *Dichelobacter nodosus*. *Biochemistry and Molecular Biology International* 34: 1157-1166.

Kortt, A.A., Caldwell, J.B., Lilley, G.G., Edwards, R., Vaughan, J., and Stewart, D.J. (1994b) Characterization of a basic serine proteinase (pI 9.5) secreted by virulent strains of *Dichelobacter nodosus* and identification of a distinct, but closely related, proteinase secreted by benign strains. *Biochemical Journal* 299: 521-525.

La Fontaine, S., and Rood, J. (1997) Physical and genetic map of the chromosome of *Dichelobacter nodosus* strain A198. *Gene* 184: 291-298.

Laing, E.A., and Egerton, J.R. (1978) The occurrence, prevalence and transmission of *Bacteroides nodosus* infection in cattle. *Research in Veterinary Science* 24: 300-304.

Lee, S., Alexander, B., and McGowan, B. (1983) Purification, characterisation, and serological characteristics of *Bacteroides nodosus* pili and use of a purified pili vaccine in sheep. *American Journal of Veterinary Research* 44: 1676-1681.

Lilley, G.G., Stewart, D.J., and Kortt, A.A. (1992) Amino acid and DNA sequences of an extracellular basic protease of *Dichelobacter nodosus* show that it is a member of the subtilisin family of proteases. *European Journal of Biochemistry* 210: 13-21.

Lilley, G.G., Riffkin, M.C., Stewart, D.J., and Kortt, A.A. (1995) Nucleotide and deduced protein sequence of the extracellular, serine basic protease gene (bprB) from *Dichelobacter nodosus* strain 305: comparison with the basic protease gene (bprV) from virulent strain 198. *Biochemistry and Molecular Biology International* 36: 101-111.

Lin, J.-J., Kuo, J., and Ma, J. (1996) PCR-based DNA fingerprinting technique: AFLP for molecular typing of bacteria. *Nucleic Acids Research* 24: 3649-3650.

Liu, D., Roycroft, C., Samuel, J., and Webber, J. (1995) Relationship between clinical manifestations of footrot and specific DNA products of *Dichelobacter nodosus* amplified through PCR. *Research in Veterinary Science* 59: 102-105.

Liu, D., and Yong, W.K. (1995) Molecular basis for the virulence of *Dichelobacter nodosus*. *Trends in Microbiology* 3: 474-475.

Lorenz, M., and Wackernagel, W. (1994) Bacterial gene transfer by natural genetic transformation in the environment. *Microbiological Reviews* 58: 563-602.

Lymberry, A., Hampson, D., Hopkins, R., Combs, B., and Mhoma, J. (1990) Multilocus enzyme electrophoresis for identification and typing of *Treponema hyodysenteriae* and related spirochaetes. *Veterinary Microbiology* 22: 89-99.

Mahillon, J., and Chandler, M. (1998) Insertion Sequences. *Microbiology Molecular Biology Reviews* 62: 725-774.

Mahillon, J., Léonard, C., and Chandler, M. (1999) IS elements as constituents of bacterial genomes. *Research in Microbiology* 150: 675-687.

Maiden, M., Bygraves, J., Feil, E., Morelli, G., Russell, J., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D., Feavers, I., Achtman, M., and Spratt, B. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of National Academy of Science USA* 17: 3140-3145.

Malecki, J.C., and Coffey, L. (1987) Treatment of ovine virulent footrot with zinc sulphate/sodium lauryl sulphate footbathing. *Australian Veterinary Journal* 64: 301-304.

Marsh, H., and Tunnicliff, E.A. (1934) Experimental studies of Foot-rot in sheep. *Montana Experimental Station Bulletin* 285: 1-29.

Marshall, D.J., Walker, R.I., Cullis, B.R., and Luff, M.F. (1991) The effect of footrot on body weight and wool growth of sheep. *Australian Veterinary Journal* 68: 45-49.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., and Hakenbeck, R. (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research* 20: 3479-3483.

Mattick, J., Anderson, B., Cox, P., Dalrymple, B., Billis, M., Hobbs, M., and Egerton, J.R. (1991) Gene sequences and comparison of the fimbrial subunits representative of *Bacteroides nodosus* serotypes A to I: class I and class II strains. *Molecular Microbiology* 5: 561-573.

Matushek, M.G., Bonten, M.J.M., and Hayden, M.K. (1996) Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* 34: 2598-2600.

Mazurek, G.H., Reddy, V., Marston, B.J., Haas, W.H., and Crawford, J.T. (1996) DNA Fingerprinting by Infrequent-Restriction-Site Amplification. *Journal of Clinical Microbiology* 34: 2386-2390.

Mazurier, S., van de Giessen, A., Heuvelman, K., and Wernars, K. (1992) RAPD analysis of Campylobacter isolates: DNA fingerprinting without the need to purify DNA. *Letters in Applied Microbiology* 14: 260-262.

McKern, N.M., O'Donnell, I.J., Inglis, A.S., Stewart, D.J., and Clark, B.L. (1983) Amino acid sequence of pilin from *Bacteroides nodosus* (strain 198), the causative organism of ovine footrot. *Federation of European Biochemical Societies Letters* 164: 149-153.

McKern, N.M., Elleman, T.C., and Hoyne, P.A. (1989) Molecular basis of antigenic variation in pili (fimbriae) of *Bacteroides nodosus* - application to footrot vaccines. In *Footrot and foot abscess of ruminants. 1989*. Egerton, J.R., Yong, W. and Riffkin, G. (eds). CRC Press, Boca Raton, USA, pp. 219-234.

Meats, E., Feil, E., Stringer, S., Cody, A., Goldstein, R., Kroll, J., Popovic, T., and Spratt, B. (2003) Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. *Journal of Clinical Microbiology* 41: 1623-1636.

Mohler, J., and Washburn, H. (1905) *Foot-rot of sheep: its nature, cause and treatment.* Government Printing Office, Washington, USA.

Monaco, A.P. (1995) *Pulsed Field Gel Electrophoresis: A practical approach.* The Practical Approach Series, IRL Press, Oxford, UK.

Monis, P.T., and Andrews, R.H. (1998) Molecular Epidemiology: assumptions and limitations of commonly applied methods. *International Journal for Parasitology* 28: 981-987.

Moses, E., Rood, J., Yong, W., and Riffkin, G. (1989) Molecular analysis of one of multiple protease-encoding genes from the prototype virulent strain of *Bacteroides nodosus*. *Gene* 77: 219-228.

Moses, E.K., Good, R.T., Sinistaj, M., Billington, S.J., Langford, C.J., and Rood, J.I. (1995) A multiple site-specific DNA-inversion model for the control of Omp1 phase and antigenic variation in *Dichelobacter nodosus*. *Molecular Microbiology* 17: 183-196.

Muralidharan, K., and Wakeland, E. (1993) Concentration of primer and template quality affects products in random-amplified polymorphic DNA PCR. *BioTechniques* 14: 362-364.

Murnane, D. (1933) Footrot in Sheep. *Journal of the Council for Scientific and Industrial Research* 6: 252-259.

Naylor, R., Martin, P., Jones, J., and Burnell, M. (1998) Isolation of spirochaetes from an incident of severe virulent ovine footrot. *Veterinary Record* 143: 690-691.

Niemann, S., Richter, E., and Rusch-Gerdes, S. (1999) Stability of *Mycobacterium tuberculosis* IS6110 restriction fragment length polymorphism patterns and spoligotypes determined by analyzing serial isolates from patients with drug-resistant tuberculosis. *Journal of Clinical Microbiology* 37: 409-412.

Olive, D.M., and Bean, P. (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical Microbiology* 37: 1661-1669.

Oliver, J.D. (1999) The viable but non-culturable state and cellular resuscitation. In *8th International Symposium on Microbial Ecology*. Bell, C.R., Brylinsky, M. and Johnson-Green, P. (eds.), Atlantic Canadian Society for Microbial Ecology, Halifax, Canada.

Olson, M.E., Gard, S., and Morck, D.W. (1998) Serological classification and virulence determination of *Dichelobacter nodosus* isolated from Alberta and British Columbia sheep. *Canadian Journal of Veterinary Research* 62: 33-37.

Ørskov, F., and Ørskov, I. (1983) From the National Institutes of Health. Summary of a workshop on the clone concept in the epidemiology, taxonomy, and evolution of the enterobacteriaceae and other bacteria. *The Journal of Infectious Diseases* 148: 346-357.

Outteridge, P.M., Stewart, D.J., Skerman, T.M., Dufty, J.H., Egerton, J.R., Ferrier, G., and Marshall, D.J. (1989) A positive association between resistance to ovine footrot and particular lymphocyte antigen types. *Australian Veterinary Journal* 66: 175-179.

Overweg, K., Hermans, P., Trzcinski, K., Sluijter, M., de Groot, R., and Hryniewicz, W. (1999) Multidrug-resistant *Streptococcus pneumoniae* in Poland: identification of emerging clones. *Journal of Clinical Microbiology* 37: 1739-1745.

Palmer, M.A. (1993) A gelatin test to detect activity and stability of proteases produced by *Dichelobacter* (*Bacteroides*) *nodosus*. *Veterinary Microbiology* 36: 113-122.

Parsonson, I.M., Egerton, J.R., and Roberts, D.S. (1967) Ovine interdigital dermatitis. *Journal of Comparative Pathology* 77: 309-313.

Piriz, S., Cuenca, R., Valle, J., and Vadillo, S. (1990) Isolation and identification of anaerobic bacteria from ovine foot rot in Spain. *Research in Veterinary Science* 49: 245-247.

Píriz, S., Hurtado, M.A., Valle, J., Mateos, E.M., Martin-Palomino, P., and Vadillo, S. (1996) Bacteriological study of footrot in pigs: a preliminary note. *The Veterinary Record* 139: 17-19.

Pitman, D.R., Palmer, M.A., and Depiazzi, L.J. (1994) The laboratory culture of *Dichelobacter nodosus* in a footrot eradication program. *Australian Veterinary Journal* 71: 109-112.

Popoff, M.R. (1991) Causal agent of foot rot: description, pathogenicity, vaccination. *Revue-de-Medecine-Veterinaire* 142: 453-462.

Prozorov, A. (2001) Recombinational rearrangements in bacterial genome and bacterial adaptation to the environment. *Microbiology* 70.

Richards, R.B., Depiazzi, L.J., Edwards, J.R., and Wilkinson, F.C. (1980) Isolation and characterisation of *Bacteroides nodosus* from foot lesions of cattle in Western Australia. *Australian Veterinary Journal* 56: 517-521.

Riffard, S., Presti, F., Vandenesch, F., Forey, F., Reyrolle, M., and Etienne, J. (1998) Comparative analysis of infrequent-restriction-site PCR and pulsed field gel electrophoresis for epidemiological typing of *Legionella pneumophila* serogroup 1 strains. *Journal of Clinical Microbiology* 36: 161-167.

Riffkin, M.C., Focareta, A., Edwards, R., Stewart, D.J., and Kortt, A.A. (1993) Cloning, sequencing and expression of the gene (*apr*V5) encoding extracellular serine acidic protease V5 from *Dichelobacter nododus*. *Gene* 137: 259-264.

Riffkin, M.C., Wang, L., Kortt, A.A., Stewart, D.J., and Wang, L.F. (1995) A single amino-acid change between the antigenically different extracellular serine proteases V2 and B2 from *Dichelobacter nodosus*. *Gene* 167: 279-283.

Rinderknecht, H., Geokas, M.C., Silverman, P., and Haverback, B.J. (1968) A new ultrasensitive method for the determination of proteolytic activity. *Clinica chim Acta* 21: 197-203.

Rivera, I., Chowdhury, M., Huq, A., Jacobs, D., Martins, M., and Colwell, R. (1995) Enterobacterial repetitive intergenic consensus sequences and the PCR to generate fingerprints of genomic DNAs from *Vibrio cholerae* O1, O139, and non-O1 strains. *Applied and Environmental Microbiology* 61: 2898-2904.

Roberts, D.S., Graham, N.P.H., Egerton, J.R., and Parsonson, I.M. (1968) Infective bulbar necrosis (heel abscess) of sheep, a mixed infection with *Fusiformis necrophorus* and *Corynebacterium pyogenes*. *Journal of Comparative Pathology* 78: 1-8.

Robertson, W.A.N., (ed) (1932) *Milestones in the pastoral age of Australia.* Australian and New Zealand Association for Advancement of Science, Sydney, Australia.

Rolland, K., Marois, C., Siquier, V., Cattier, B., and Quentin, R. (1999) Genetic features of *Streptococcus agalactiae* strains causing severe neonatal infections, as revealed by pulsed-field gel electrophoresis and *hyl*B gene analysis. *Journal of Clinical Microbiology* 37: 1892-1898.

Rollins, D.M., and Colwell, R.R. (1986) Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Applied and Environmental Microbiology* 52: 531-538.

Rood, J.I., Howarth, P.A., Haring, V., Billington, S.J., Yong, W.K., Liu, D., Palmer, M.A., Pitman, D.R., Links, I., Stewart, D.J., and Vaughan, J.A. (1996) Comparison of gene probe and conventional methods for the differentiation of ovine foot rot isolates of *Dichelobacter nodosus*. *Veterinary Microbiology* 52: 127-141.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular cloning. A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, pp. E.10.

Saulnier, P., Bourneix, C., Prévost, G., and Andremont, A. (1993) Random amplified polymorphic DNA assay is less discriminant than pulsed-field gel electrophoresis for typing strains of methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology* 31: 982-985.

Scherba, G., Wiemers, J., Siegel, A., Jin, L., Austin, C., Bowman, L., Redeford, B., Johnston, N., and Weigel, R. (1999) Application of a quantitative algorithm to restriction endonuclease analysis of Aujeszky's disease (pseudorabies) virus from a geographically localized outbreak. *Journal of Veterinary Diagnostic Investigation* 11: 423-431.

Schlichting, C., Branger, C., Fournier, J.-M., Witte, W., Boutonnier, A., Wolz, C., Goullet, P., and Döring, G. (1993) Typing of *Staphylococcus aureus* by pulsed-field gel electrophoresis, zymotyping, capsular typing, and phage typing: resolution of clonal relationships. *Journal of Clinical Microbiology* 31: 227-232.

Schwartz, D.C., Saffran, W., Welsh, J., Haas, R., Goldenberg, M., and Cantor, C.R. (1983) New techniques for purifying large DNAs and studying their properties and packaging. *Cold Spring Harbor Symposium on Quantitative Biology* 47: 189-195.

Schwartzkoff, C.L., Egerton, J.R., Stewart, D.J., Lehrbach, P.R., Elleman, T.C., and Hoyne, P.A. (1993) The effects of antigenic competition on the efficacy of multivalent footrot vaccines. *Australian Veterinary Journal* 70: 123-126.

Selander, R., Caugant, D., Ochman, H., Musser, J., Gilmour, M., and Whittam, T. (1986) Methods of multilocus enzyme electrophoresis for bacterial population genetics and sysematics. *Applied and Environmental Microbiology* 51: 873-884.

Short, J.A., Thorley, C.M., and Walker, P.D. (1976) An electron microscope study of *Bacteroides nodosus*: ultrastructure of organisms from primary isolates and different colony types. *Journal of Applied Bacteriology* 40: 311-315.

Simpson, E.H. (1949) Measurement of diversity. *Nature* 163: 688.

Skerman, T.M. (1975) Determination of some in vitro growth requirements of *Bacteroides nodosus*. *Journal of General Microbiology* 87: 107-119.

Skov, M., Pedersen, K., and Larsen, J. (1995a) Comparison of pulsed field gel electrophoresis, ribotyping, and plasmid profiling for typing of *Vibrio anguillarum* serovar 01. *Applied and Environmental Microbiology* 61: 1540-1545.

Skov, M., Pedersen, K., and Larsen, J. (1995b) Comparison of pulsed-field gel electrophoresis, ribotyping, and plasmid profiling for typing of *Vibrio anguillarum* serovar 01. *Applied and Environmental Microbiology* 61: 1540-1545.

Sneath, P.H.A., and Snokal, R.R. (1973) Numerical Taxonomy: the principles and practice of numerical classification. W.H. Freeman and Company, San Francisco, USA, pp. 230-234.

Southern, E., and Elder, J. (1995) Theories of gel electrophoresis of high molecular weight DNA. In *Pulsed Field Gel Electrophoresis: A Practical Approach*. Monaco, A. (ed). IRL Press, Oxford, UK.

Stewart, D.J. (1978) The role of various antigenic factors of *Bacteroides nodosus* in eliciting protection against foot rot in vaccinated sheep. *Research in Veterinary Science* 24: 14-19.

Stewart, D.J. (1979) The role of elastase in the differentiation of *Bacteroides nodosus* infections in sheep and cattle. *Research in Veterinary Science* 27: 99-105.

Stewart, D.J., and Claxton, P.D. (1993) Ovine footrot: clinical diagnosis and bacteriology. In *Australian Standard Diagnostic Techniques for Animal Diseases*. Corner, L.A. and Bagust, T.J. (eds). CSIRO for the Standing Committee on Agriculture and Resource Management, East Melbourne, Australia, pp. 1-27.

Strom, M.S., and Lory, S. (1993) Structure-function and biogenesis of type IV pili. *Annual Review of Microbiology* 47: 565-596.

Tanaka, M.M., and Rosenberg, N.A. (2001) Optimal estimation of transposition rates of insertion sequences for molecular epidemiology. *Statistics in Medicine* 20: 2409-2420.

Tenover, F., Arbeit, R., Goering, R., Mickelsen, P., Murray, B., Persing, D., and Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* 33: 2233-2239.

Thal, L., Silverman, J., Donabedian, S., and Zervos, M. (1997) The effect of Tn*916* insertions on contour-clamped homogenous electrophoresis patterns of *Enterococcus faecalis*. *Journal of Clinical Microbiology* 35: 969-972.

Thomas, J.H. (1962a) The differential diagnosis of footrot in sheep. *Australian Veterinary Journal* 38: 159-163.

Thomas, J.H. (1962b) The bacteriology and histopathology of footrot in sheep. *Australian Journal of Agricultural Research* 13: 725-732.

Thomas, J.H. (1964a) Proteolytic enzymes produced in liquid media by *Fusiformis nodosus*. *Australian Journal of Agriculture Research* 15: 417-426.

Thomas, J.H. (1964b) The pathogenesis of footrot in sheep with reference to proteases of *Fusiformis nodosus*. *Australian Journal of Agriculture Research* 15: 1001-1016.

Thorley, C.M. (1976) A simplified method for the isolation of *Bacteroides nodosus* from ovine foot rot and studies on its colony morphology and serology. *Journal of Applied Bacteriology* 40: 301-309.

Thorley, C.M., and Day, S.E.J. (1986) Serotyping survey of 1926 strains of *Bacteroides nodosus* isolated from sheep and cattle in Great Britain and western Europe. In *Footrot in Ruminants. Proceedings of a workshop*. Stewart, D.J., Peterson, N.M., McKern, N.M. and Emery, D.L. (eds). Melbourne, Victoria, 1985: Commonwealth Scientific and Industrial Research Organization and Australian Wool Corporation, Sydney, pp. 135-142.

Toussaint, E.R., and Cornelisse, J.L. (1971) The specific contagious inflammation of the interdigital skin of cattle. *Veterinary Medical Review* 2-3: 223.

Trott, D., Masters, A., Carson, J., Ellis, T., and Hampson, D. (1995) Genetic analysis of *Dermatophilus* spp. using multilocus enzyme electrophoresis. *Zentralblatt für Bakteriologie* 282: 24-34.

Tyler, K., Wang, G., Tyler, S., and Johnson, W. (1997) Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. *Journal of Clinical Microbiology* 35: 339-346.

Urban, D.L. (2004) Multivariate Analysis. Cluster Analysis. Introduction. http://www.env.duke.edu/landscape/classes/env358/mv_cluster.pdf

Valsangiacomo, C., Baggi, F., Gaia, V., Balmelli, T., Peduzzi, R., and Piffaretti, J.-C. (1995) Use of amplified fragment length polymorphism in molecular typing of *Legionella pneumophila* and application to epidemiological studies. *Journal of Clinical Microbiology* 33: 1716-1719.

van Belkum, A., Sluijuter, M., de Groot, R., Verbrugh, H., and Hermans, P. (1996) Novel BOX repeat PCR assay for high-resolution typing of *Streptococcus pneumoniae* strains. *Journal of Clinical Microbiology* 34: 1176-1179.

van Belkum, A., Struelens, M., de Visser, A., Verbrugh, H., and Tibayrenc, M. (2001) Role of genomic typing in taxonomy, evolutionary genetics, and microbial epidemiology. *Clinical Microbiology Reviews* 14: 547-560.

Versalovic, J., Koeuth, T., and Lupski, J. (1991) Distribution of repetitive DNA sequences in eubacteria and applications to fingerprinting of bacterial genomes. *Nucleic Acids Research* 19: 6823-6831.

Walker, P.D., Short, J., Thomson, R.O., and Roberts, D.S. (1973) The fine structure of *Fusiformis nodosus* with special reference to the location of antigens associated with immunogenicity. *Journal of General Microbiology* 77: 351-361.

Walker, R.L., Read, D.H., Loretz, K.J., and Nordhausen, R.W. (1995) Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. *Veterinary Microbiology* 47: 343-355.

Wani, S.A., Samanta, I., Bhat, M.A., and Buchh, A.S. (2004) Molecular detection and characterisation of *Dichelobacter nodosus* in ovine footrot in India. *Molecular and Cellular Probes* 18: 289-291.

Warren, R.M., van der Spuy, G.D., Richardson, M., Beyers, N., Borgdorff, M.W., Behr, M.A., and van Helden, P.D. (2002) Calculation of the stability of the IS6110 banding pattern in patients with persistent *Mycobacterium tuberculosis* disease. *Journal of Clinical Microbiology* 40: 1705-1708.

Welsh, J., and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* 18: 7213-7218.

Whittington, R.J., and Nicholls, P.J. (1995a) Grading the lesions of ovine footrot. *Research in Veterinary Science* 58: 26-34.

Whittington, R.J., and Nicholls, P.J. (1995b) Effects of the severity and duration of lesions on the primary and anamnestic humoral responses of sheep to *Dichelobacter nodosus* and observations of natural resistance to footrot. *Research in Veterinary Science* 59: 128-135.

Whittle, G., Bloomfield, G.A., Katz, M.E., and Cheetham, B.F. (1999) The site-specific integration of genetic elements may modulate thermostable protease production, a virulence factor in *Dichelobacter nodosus*, the causative agent of ovine footrot. *Microbiology* 145: 2845-2855.

Whittle, G., Katz, M.E., Clayton, E.H., and Cheetham, B.F. (2000) Identification and characterization of a native *Dichelobacter nodosus* plasmid, pDN1. *Plasmid* 43: 230-234.

Williams, J., Kubelik, A., Livak, K., Rafalski, J., and Tingey, S. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18: 6531-6535.

Winstanley, C. (2002) Spot the difference: applications of subtractive hybridisation to the study of bacterial pathogens. *Journal of Medical Microbiology* 51: 459-467.

Wood, D. (1995) PFGE in the study of a bacterial pathogen (*Haemophilus influenzae*). In *Pulsed Field Gel Electrophoresis: A Practical Approach*. Monaco, A. (ed). IRL Press, Oxford, UK, pp. 159-176.

Yeh, R.W., de Leon, A.P., Agasino, C.B., Hahn, J.A., Daley, C.L., Hopewell, P.C., and Small, P.M. (1998) Stability of *Mycobacterium tuberculosis* DNA genotypes. *Journal of Infectious Diseases* 177: 1107-1111.

Youatt, W. (1837) *Sheep: Their Breeds, Management and Diseases.* Baldwin and Cradock, London, UK, pp. 523. Cited by Beveridge, W.I.B. (1981).

Zakaria, Z., Radu, S., Sheikhomar, A.R., Mutalib, A.R., Joseph, P.G., and Rusul, G. (1998) Molecular analysis of *Dichelobacter nodosus* isolated from footrot in sheep in Malaysia. *Veterinary Microbiology* 62: 243-250.

Zhou, H., and Hickford, J. (2000a) Novel fimbrial subunit genes of *Dichelobacter nodosus*: recombination in vivo or in vitro? *Veterinary Microbiology* 76: 163-174.

Zhou, H., and Hickford, J.G.H. (2000b) Extensive diversity in New Zealand *Dichelobacter nodosus* strains from infected sheep and goats. *Veterinary Microbiology* 71: 113-123.

Zhou, H., Hickford, J., and Armstrong, K. (2001) Rapid and accurate typing of *Dichelobacter nodosus* using PCR amplification and reverse dot-blot hybridisation. *Veterinary Microbiology* 80: 149-162.

APPENDICES

APPENDICES

Appendix A Reagents, equipment and protocols

Appendix A.1 List of reagents

Reagent	Catalogue No.	Supplier
Agar	12177	Calbiochem
Agarose (Metaphor)	50180	Scott Scientific
*Apa*I 10U/ul, 5000 units	R6361	Promega
*Apa*I (80 units/ul)	R4364	Promega
Aquarium filter wool		Aquarium suppliers
Bacto Casitone	0259-17-9	Difco
*Bgl*II	E1021Y	Amersham International
Bromophenol Blue	161-0404	BioRad
Boric Acid	194810	ICN
*Cfo*I (*Hha*I) (10 units/ul)	R6241	Promega
dATP	U1201	Promega
dCTP	U1221	Promega
dGTP	U1211	Promega
dTTP	U1231	Promega
DNA lambda ladder	170-3635	BiocRad
*Eco*RV		Boehringer Mannheim
EDTA, 1 Kg, ICN	800683	Australian Biosearch
EDTA disodium salt	RT 195173	ICN
Film Polaroid High Speed film, 4 x 5 Instant	Type 57	Polaroid Australia
Film (High Speed)	Polaroid T57	PR Agencies
Lab Lemco powder	LP 029B	Oxoid
Loading Buffer (Supplied with molecular weight marker		Geneworks
Lysozyme	100831	ICN
Microtubes EZPP 500 uL	223-9503	Australian Biosearch
Mitsubishi Gas Company (MGC) Gas Pak	MGC10-01	Blackaby Diagnostics
*Mlu*I	MluI-1	Biotech International

List of reagents continued

Reagent	Catalogue No.	Supplier
Molecular weight marker, PFGE	170-3635	Australian Biosearch
Molecular weight marker 100 bp	27-4001-01	Promega
Molecular weight marker (100 ng/μl) DNA 100 base pair	DMW-1002	Geneworks
N-Lauroyl Sarcosine	L9150	Sigma
*Not*I	1014 706	Boehringer Mannheim
Nunc cryotubes	3-66656	Medos
Operon primers	Kit A	Operon Technologies, USA
Pipette – Gilson P1000	H-23602	John Morris Scientific
Pipette- Gilson Pipetteman P200	H 23601	John Morris Scientific
Pipette – Graduated 25 ml, BRRU 27079	391318,	Selby
Pipette Tips MLA pipette 5-200uL	223-9025	Australian Biosearch
Pipette tips BR-37	223-9037	Australian Biosearch
Primers		Biotech International
Plug mould for PFGE	170-3713	BioRad
PMSF	195381	ICN
Polyacrylamide Phast gel homogenous 12.5 %	17-0623-01	Pharmacia
Protein Precision Standard	161-0362	BioRad
Proteinase K, 100 mg	V3021	Promega
Rnase A, 100 mg, ICN	101076	Australian Biosearch
SDS Buffer strips	17-0516-01	Pharmacia
*Sfi*I	1288 016	Roche Boehringer Mannheim
Silver stain kit (Phast)	17-1150-01	Pharmacia
*Sma*I	SmaI	Roche Boehringer Mannheim
Starch gel (30 g Sigma starch gel and 5 g BDH starch)		Sigma (30 gm) and BDH (5 gm)
T4 DNA Ligase (1unit/ul)	716359	Roche Boehringer Manheim
Taq (*Tth* Plus DNA polymerase) 250 units	BIL 986-101	Biotech International
Taq enzyme (Amplitaq)	N808-0172	Applied Biosystems
Tips for 1000 ul Gilson Pipetteman	152 146	Medos

List of reagents continued

Tips Trace 1 ml	1312-1	Australian Biosearch
Tris Base, 1 Kg, ICN	103133	Australian Biosearch
Tubes 5 ml. Trace	1781-1	Australian Biosearch
Tris base	103133	ICN
*Xba*I restriction enzyme (80 units/ul)	R4184	Promega

Appendix A.2 List of Equipment

Equipment	Details	Supplier
Anaerobe Pack	MGC10-01	Blackaby Diagnostics
Anaerogen gas pak	ANO 25A	Oxoid
Anaerobic indicator	BRO 55B	Oxoid
Anaerobic indicator	96118	Blackaby Diagnostics
Anaerobic jar	96127	Blackaby Diagnostics
Centrifuge (bench top)	Heraeus Biofuge *pico* #3325B	Lab Supplies
Centrifuge	Centra with a SG 34 rotor	
CHEF DR III (PFGE)	170-3695	Bio-Rad
Dark ground illumination	Olympus BH microscope	Olympus
Electrophoresis tank for IRS-PCR = wide mini-sub cell GT Electrophoresis cell with gel caster	170-4469	Bio-Rad
GelCompar II software	version 3.1	Applied Maths, Kortrijk, Belgium
Glass beads	106 μm diameter	Sigma Chemical Co
McFarland opacity tubes		Difco
SDS-PAGE		Phast system, Pharmacia
Sonicator	Labsonic	B. Braun, Melsungen, Germany
PowerPac 300	165-5051	Bio-Rad
Thermocycler	FTS 320	Corbett

List of equipment, continued

Equipment	Details	Supplier
Thoma counting chamber (Hawskley Ltd, London).	1x400 mm^2	Weber Scientific International, 40 Udney Park Rd, Teddington Middlesex, TW11, (GB, England)

Appendix A.3 Formulae for media and buffers

The catalogue numbers and suppliers of all reagents and chemicals are listed in Appendix A.1.

Appendix A.3.1 Media for *D. nodosus*

Modified Stuart's Transport Medium

Stuarts transport medium is modified by the addition of agar to a concentration of 0.6%. This makes it slightly more anaerobic and therefore more suitable for the transport of hoof material from suspect cases of footrot. The media is prepared from the modified recipe from Oxoid and extra agar added to increase the agar concentration to 3 g/500 ml. Oxoid's Stuarts transport media contains 2.5 g/500 ml. Required for *D. nodosus* isolation.

Reagents	Amount
Oxoid Stuarts Transport Medium (Modified)	8.0 gm
Agar (Calbiochem)	0.5 gm
Distilled Water	500 ml

Combine powders into distilled water and boil to dissolve the agar. Dispense to completely fill Bijou's (approx 7 ml). The bottles are tightly capped and autoclaved at 121°C for 15 min. When cooled the bottles are mixed by inversion and stored at 4°C.

Basic TAS agar

Reagents	Amount
Difco Casitone	6.0 gm
Oxoid Lab Lemco Powder	2.0 gm
Difco Proteose Peptone No 3	2.0 gm
Difco Yeast Extract	0.8 gm
L-Arginine HCl	2.0 gm
L-Cysteine HCl	0.2 gm
DL-Serine	0.6 gm
Calbiochem Agar (see below)	6.0 gm
Oxoid Haemoglobin Powder	1.0 gm
Distilled Water	400 ml

Dissolve all powders, except haemoglobin and agar, in 300 ml water. Add haemoglobin to 50 mls of warm distilled water, and add to other ingredients and adjust to pH 7.8. Adjust volume to 400 ml and add agar. Autoclave at 10lbs/20mins. Cool to 50°C in waterbath and pour thick plates in 90 mm Petri dishes.

Appendix 3.2 Buffers for PFGE

Wash Buffer

Reagents	Amount
1M NaCl	58.44 gm
10 mM Tris Base (pH 8.0)	1.21 gm
10 mM EDTA(di-sodium salt)	3.72 gm
Milli Q water	1000 ml

Suspend Tris in 900 ml of water and adjust the pH to 8.0 while stirring. When the Tris has dissolved and the pH remains at pH 8.0 add the other reagents and stir to dissolve. Adjust the pH to 8.0 again if necessary. Make up to the final volume of 1000 ml with Milli Q water. Autoclave at 121°C for 15 minutes. Store at 4°C.

Lysis buffer

Reagents	Amount
1M NaCl	29.22 gm
10 mM Tris (pH 8.0)	0.605 gm
100 mM EDTA (di-sodium salt)	18.60 gm
0.5% Sarkosyl (= N-Lauroylsarcosine)	2.5 gm
0.2% Sodium deoxycholate	1.0 gm

lysozyme	1 mg/ml	see below
Rnase	2 μg/ml	see below

The Tris is suspended in 400 ml of water and adjusted to pH to 8.0 while stirring. When the Tris is dissolved, the EDTA is added and warmed in a 50^{o}C waterbath for 30 minutes. The pH is again adjusted while stirring. When the EDTA has dissolved the NaCl, sarkosyl and deoxycholate are added and stirred to dissolve. The pH is checked again before the volume is made up to the final volume of 1000 ml with MilliQ. The buffer is stored at 4^{o}C.

Lysozyme Stock Solution

Make a stock solution of Lysozyme of 50 mg/ml. Aliquot 400 μl amounts into microfuge tubes. Store at –20^{o}C.

Rnase Stock Solution

Make a stock solution of Rnase of 10 mg/ml. Aliquot 100 μl amounts into microfuge tubes and store at –20^{o}C.

For Use: Note. Only make up the final buffer on the day of use. For the preparation of a 100 ml volume of lysis buffer add 2 ml of lysozyme stock solution to 100 ml lysis buffer stock. Add 20 μl of Rnase stock solution. Mix well and filter through a low protein binding Sartorius Ministart filter into a sterile McCartney bottle.

It was also found that the lysis buffer could be stored frozen for up to one month without loss of activity.

ESP Buffer

Reagents	**Amount**
0.5 M EDTA (di-sodium salt)	93.06 gm
1% sarkosyl	5.0 gm
MilliQ water	500 ml
Proteinase K	100 ug/ml.

The EDTA was suspended in 400 ml of water and warmed in a 50^{o}C waterbath for 30 minutes stirring occasionally. The pH was adjusted to 8.0 while stirring until the

EDTA dissolved completely. The sarkosyl was added and stirred to dissolve. The volume was made up to the final volume with MilliQ. The buffer was stored at 4^{o}C.

For Use (100 ml): Prepare on the day to be used. Measure 100 ml of ESP buffer stock. Add 10mg Proteinase K. Mix well and filter through a low protein binding Sartorius Ministart filter.

It was also found that the ESP buffer containing the proteinase K could be stored frozen for up to one month without loss of activity.

TBE Buffer 10x Stock Solution

Reagents	**Amount**
89 mM Tris base	108 g
89 mM boric acid	55 g
0.5 M EDTA (Disodium salt pH 8.0)	40 ml
Milli Q Water	1,000 ml

Make stock solution of 0.5M EDTA by adding 37.22 g of EDTA disodium salt (MW 372.23) to approximate volume of Milli Q water. Stir and warm in waterbath to dissolve and adjust pH to 8.0. Make up to 200 ml. Add 40 ml of this stock solution Tris and Boric acid in a 1000 mL conical flask with approx 900 ml of Milli Q water. Make up to 1,000 ml and autoclave at 120°C for 15 min.

For use dilute 1:20 to obtain working solution of 0.5 M TBE.

TE Buffer

Reagents	**Amount**
10 mM Tris [pH 8.0]	1.21 g
1 mM EDTA (di-sodium salt)	0.37 g
MilliQ water	1000 ml.

The Tris was suspended in 900 ml water and the pH adjusted to 8.0 while stirring. When the Tris was dissolved the EDTA was added and stirred to dissolve. The pH was re-checked. The final volume was made up to 1000 ml with MilliQ water and autoclaved at 121°C for 15 minutes. The buffer was stored at room temperature, but once opened was kept at 4°C.

PMSF

Reagents

0.1M phenylmethylsulfonyl fluoride	34.84 g
iso-propyl alcohol	2.0 ml

The PMSF powder is added to the alcohol in a 5 ml glass bijou and stirred to dissolve. It was stored –20^{o}C.

Appendix 3.3 Media, buffers and protocols for MLEE

TC6	per 2 litres final volume
Tris (preferable Sigma 7-9)	133.25 g
citric acid (monohydrate)	79 g
pH 6; store in refrigerator	

TC8	per 2 litres final volume
Tris	208 g
Citric acid	79 g
pH 8; store in refrigerator	

TEB	per 2 litres final volume
Tris	218 g
Boric acid	61.8 g
Disodium EDTA	14.9 g
Store at room temperature	

SP7	per 2 litres final volume
NaH_2PO_4	60.8 g
Na_2HPO_4	86.6.g
pH 7; store at room temperature	

LiOH gel buffer	per 2 litres final volume
Tris	54.5 g
Citric acid	15.1 g
Electrode stock buffer	200 ml
store in refrigerator	

LiOH electrode buffer	per 2 litres final volume
LiOH (hazardous)	12.6 g
Boric acid	118.9 g
store in refrigerator	

Poulik gel buffer	per 1 litre final volume
Tris	92.1 g
Citric acid	10.5 g
store in refrigerator	

Poulik electrode buffer	per 2 litre final volume
Boric acid	185.5 g
NaOH	24 g
store at room temperature	

Tris maleate buffer	per 2 litre final volume
Tris	121 g
Maleic acid	116 g
EDTA (disodium)	37.2 g
$MgCl_2$	20.0 g
NaOH	50.0 g
Adjust pH to 7.4 with NaOH	

MLEE running conditions

Buffer	Gel dilution buffer:water (mls)	Tank dilution buffer:water (mls)	running current (mA)	running voltage (V)	running time (hours)
TM	5:250	1:4	25 (constant)	55-65	24
SP7	5:250	1:4	30 (constant)	85-100	16
Poulik	25:230	1;4	20-30	120 (constant)	18
LiOH	27:225	1:4	20-30	120 (constant)	18

Preparation of starch gel (11.4%) for MLEE

Starch (Sigma starch)	30 gm
Starch (BDH)	5 gm
Buffer (variable depending on buffer system used)	250 ml

The starch and buffer was placed into a round bottomed flask and mixed well. It was heated over a bunsen burner and continually swirled to prevent the starch sticking to the base of the flask. The fluid become less viscous and was ready when the mixture was liquid and “cleared” slightly. It was degassed in a vacuum for 30 seconds, or until it had come to the full boil. The gel was poured in the gel tray, covered and left to cool at room temperature for 1 hour. The gel was chilled at 4°C for 1-4 hours, and then stored in a plastic bag to prevent loss of moisture. It could be prepared a day ahead.

Appendix 3.4 Molecular biology procedures used in this thesis

IRS-PCR

Based on Mazurek *et al*. 1996.

Schedule for Infrequent Restriction Site PCR Method

Extract **DNA** from Agarose Plugs	
Reconstitute **Primers**	15 min
Prepare **Adaptors** AX and AH	1 hour
Set up **Restriction Digest**	1 hour
Ligation [use thermocycler]	1 hour
Inactivate at 65^{o}C for 20 min [use thermocycler]	20 min
Re-Digest	15 min
PCR	overnight
Run gel (4%)	2.5 hr (150 v)
Stain gel	45 min
Photograph gel	15 min

Reconstituting Primers and Oligonucleotides

Primers and oligonucleotides were reconstituted in TE buffer. The tubes were spun briefly to collect the DNA in bottom of tube before the appropriate volume of buffer was added. The manufacturer recommended that primers be prepared in amounts greater than 10 μmolar and stored at –80°C. Stability of reconstituted material was said to be >6 months.

Micromolar concentrations of primer = pmoles/μl. Thus 20 pmoles of primer in 100 μl PCR mixture = 20 micromolar (20 μM). Example: If primer is supplied at 29 nanomoles. This = 29,000 picomoles. If 290 μl of water is added this gives 100 picomoles. For working solution a dilution of 1:5 was prepared to give 20 picomoles.

Preparation of dinucleotide mixture for use in the PCR

Stocks of dNTPs are received as 100 mM. The final concentration in the PCR was either 100 μM or 200 μM depending on optimisation standards. Each dNTP twas prepared to 10 mM by diluting 1:10. A working solution was prepared by adding equal volumes of each dNTP plus an equal volume of water, that is a 1:5 dilution for each

dNTP. Either 1.25 µl or 2.5 µl was used in the PCR reaction volume of 25 µl to obtain a final concentration of 100 µM or 200 µM respectively of each dNTP.

It was found that the concentration of dNTP needed to be re-optimised when new primers were used for the first time, as there appeared to be a difference between primer batches. The optimum final concentration in the PCR mix varied from 100 µM to 200 µM depending on the batch of primers.

Protocols when preparing PCR master mix and adding DNA

The PCR Master Mix was prepared in a dedicated Biological Cabinet using dedicated pipettes. This assisted in prevention of amplification of non-specific products, cross-contamination from other DNA sources, and false positives due to contamination from amplified product. Pipettes were UV sterilized after use.

20 µl of PCR Master Mix was aliquoted into individual 0.6 ml microfuge tubes. 2 µl of Restriction/Ligation mix was added to each PCR mixture tube. This step was performed in a separate biological safety cabinet to the one used to prepare the PCR master mix, so as to prevent cross-contamination and the risk of erroneous results.

Preparation of gel for IRS-PCR

Metaphor agarose was used as this had the equivalent sieving and pore size as polyacrylamide and the same results were achieved as PAGE, chapter 3. A 4% gel was prepared using 3.6 g in 90 ml of 0.5M TBE buffer. For this type of agarose the powder had to be added to cold buffer while continually stirring to prevent the formation of lumps. The agar was melted in a microwave using short times at high power. When melted, the agarose was de-gassed by placing in an anaerobe jar and applying a slight vacuum – enough to cause boiling.

The gel was cooled to 55°C and poured into a Bio-Rad gel pouring apparatus, and a 30-well comb was inserted before the agarose set. The apparatus was placed at 4°C to allow quick setting of the gel.

Gel electrophoresis for IRS-PCR

Ice-cold 0.5 M TBE buffer was added to the electrophoresis tank to a level that covered the gel to a depth of 1 cm. The gel was prepared and set at 4°C for 20 minutes. Each well on the gel was loaded with 5 μl of PCR product plus 5 μl of Loading buffer. The gel was electrophoresed at 150 volts for 2.5 hr. Ice was packed tightly around the edges, in the space underneath the tank, and on top of the electrophoresis tank to keep the gel and buffer cool. This was important because the high voltage through the thick gel generated a great deal of heat and this caused distortion of the restriction bands if the gel became too hot.

Preparation of agarose gel for PFGE

A 10X stock solution of TBE buffer was diluted to 0.5M (1:20 dilution) in MilliQ water (100 μl of 10X stock in 1.9L water).

The 1% agarose gel was prepared in 0.5M TBE buffer (1.3 g of Chromosomal Grade agarose (BioRad) to 130 ml of 0.5 M TBE buffer). The agarose was melted in a microwave and poured into the gel bed with the comb inserted. The gel was set on the bench for 1 hour before being refrigerated for ½ to 1 hour before loading the plugs.

DNA extraction using the CTAB method (Sambrook, 1989)

This method of extracting DNA from bacterial cells was used for the RAPD method.

Cultures of *D. nodosus* were grown on TASH MM plates, in an anaerobic atmosphere. The cells were scraped from the plates and washed twice in TE buffer. To the bacterial pellet (rice-grain in size) is added 567 μl of TE buffer, 30 μl of 10% SDS and 3 μl of Proteinase K (20 mg/ml). The cell mixture was vortexed gently until smooth and then incubated at 37°C for one hour. 100 μl of 5 M NaCl and 80 μl CTAB reagent was added to the mixture and incubated at 65°C for 10 minutes. 600 μl of chloroform/isoamyl alcohol (24:1) was added and the mixture vortexed gently for 20 seconds. The solution was centrifuged at 10,000 rpm (10,000 g) for 5 minutes. The upper aqueous layer was removed to a fresh microfuge tube and a further 600 μl of chloroform/isoamyl alcohol (24:1) was added. The solution was centrifuged at 10,000

rpm (10,000 g) for 5 minutes, and the upper aqueous layer removed to a fresh microfuge tube. 400 µl ice-cold isopropanol was added and the tube rocked gently to precipitate the DNA. The DNA was pelleted by centrifugation at 5,000 g (6,000 rpm) for 5 minutes. The precipitate was washed with 400 µl of 70 % alcohol, twice using centrifuge at 6,500 rpm for 5 minutes each time. A final wash was carried out with 95% alcohol, and then the pellet air-dried for 20 minutes at 37°C. The DNA was reconstituted with 50 µl of ultra pure water and stored at 4°C.

Reagents for CTAB method

CTAB/NaCl Solution

NaCl	4.1 gm
Distilled water	80 ml
CTAB (hexadecyltrimthyl ammonium bromide)	10 gm

Dissolve NaCl in water, slowly add CTAB and heat to 65°C. Adjust final volume to 100 ml.

Sodium Dodecyl Sulphate (10%)

SDS	100 gm
Distilled water	900 ml

Heat to 68°C to dissolve. Adjust pH to 7.2 using HCl and adjust volume to 1 litre.

5M NaCl

NaCl	29.22 gm
Distilled water	100 ml.

Appendix B Bacterial isolates and their results for PFGE and IRS-PCR typing

Appendix B.1 Results of DNA fingerprinting of isolates

The following tables present the results of molecular typing using PFGE and IRS-PCR for isolates tested from Western Australia (Table B-1), from South Australia (Table B-2), from New South Wales (Table B-3) and from Victoria (Table B-4).

Table B-1. Isolate identification and results of PFGE and IRS-PCR typing for isolates used from WA

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
1	7756	97-281	01212	S	S1	24a		2			3	
1	7757	97-281	01218	S	S1	24		2				
1		01-506	674	S	S1	7a+		13				
1		01-506	673	U	U6	7		9				
1	9099	01-478	12573	S	S1	7		13				
1	6909	95-0505	776	S	S1	24a		2				
1	3596	91-1586	99	S	S1	59		13a				
1	3816	92-0143	787	S	S1	24		60				
1	4306	92-1302	512	S	S1	24c		2e				
1	4284	92-1286	470	S	S1	24d		2e				
1	3817	92-0144	789	S	S1	24		2				
1	3818	92-0144	792	S	S1	24		2				
1	4278	92-1276	447	S	S1	24		2				
1	4279	92-1276	449	S	S1			17				
1	4280	92-1276	452	S	S1	24a		2e				
1	4281	92-1276	453	S	S1	24		2				
1	6906	95-0505	771	S	S1	24		2				
2	7741	97-204	00801	S	S1	7L		2e				
3	2449	90-1255		S	S1	7	1a	13	8			
4		97-340	01478	U	U4	49		1d				
4		97-340	01480	U	U4	48		1b	10			
4		97-340	01482	U	U4	48a		1d			1	
4		97-340	481	U	U4	48		1b			2	
4		97-340	479	U	U1	48						
5	3264	91-1156	1141	U	U5	7k		13g	1		4	
5	8058	98-481	5317#2	U	U5	37a		1d				
5	8056	98-481	5313	S	S1	44		10				
5	8057	98-481	5314	S	S1	44		10				
5		98-481	5315	U	U1	19h		1d				
6	5928	94-0452	10354	U	U1	7c		5a				
6		97-180	00624	U	U5	9d		5				
6		97-180	00626	U	U1	9d		5				
6		97-180	00628	U	U5	9o		5				
6		97-432	01930	U	U5	9d		5	4			
6		97-432	936	U	U5	9d		5a	4a			
6		97-432	933-1	U	U5	9d		5a	4			
7		96-0022	14972	U	U1	12a						
7	7155	96-0022	14973	U	U1	12a						
7		96-0022	14976	U	U1	12a						
7		96-0022	14977	U	U1	12a		5b				
7		96-0022	14978	U	U1	12c		1				
7	7170	96-0022	14979	U	U1	12c						
7	7157	96-0022	14980	U	U1	12e		1				

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
7		96-0022	14981	U	U1	12b		1				
7	7158	96-0022	14982	U	U1	12c						
7		96-0024	14985	U	U1	12a						
7	7161	96-0024	14987	U	U1	12d		1				
7		96-0024	14988	U	U1	12a						
7	7162	96-0024	14989	U	U1	12a		5b				
7		96-0024	14991	U	U1	12a						
7	7163	96-0024	14992	U	U1	12a						
7	7160	96-0024	14986	U	U1	12a						
7		96-0024	14990	U	U1	12a		5b				
8	7748	97-235	01000	S	S1	11d		28				
9		97-175	00599	U	U1	9n		21				
9		97-175	00602	S	S1	19g		13a				
10	915	85-		U	U2	9a		1g				
11	5944	94-0471	10412	U	U5	12		5	5	1		
11	6361	94-0869	11637	U	U5	12		5	5	1		
12		97-100	18560	S	S1	29		13a		7		
12		97-100	18561	S	S1	29		13a		8		
13	6095	94-0648		S	S1	11a		2				
14	2505	90-1246		S	S1	7	1a	13	8			
15	6697	95-0297	12989	S	S1	11		2				
15	6698	95-0297	12990	S	S1	7c						
15	6701	95-0297	12998	S	S1	11a		2				
16	5615	94-0191	9509	S	S3	46a		29c				
17	3008	91-0492		S	S1	7	1	13	8			
17	3009	91-0492		S	S	7	1	13	8			
17	7742	97-219	00919	S	S1	7		13	8			
17	7743	97-231	00976	S	S1	7						
17	7744	97-231	00979	S	S1	7		13	8			
17	7749	97-239	01015	S	S1	7						
17	6041	94-0587	689	S	S1	7c		13	8			
17	2378	90-1113	138	S	S1	7		13	8			
17	3619	91-1620	187	S	S1	7		14	8a			
17	3624	91-1620	196	S	S1	7c		13	8			
17	5041	93-0986	386	S	S1	7		13	8			
17	5042	93-0986	389	S	S1	7	1					
17	5289	93-1258	273	S	S1	7		13	8			
17	5290	93-1258	276	S	S1	7						
17	6041	94-0587	689	S	S1	7c		13	8			
17	6043	94-0587	692	S	S1	7c						
17	6058	94-0598	729	S	S1	7c		13	8			
17	6770	95-0394	393	S	S1	7c		13	8			
17	6925	95-0476	651	S	S1	7c		13	8			
17	2447	90-1254		S	S	7	1a	13	8			
19	7740	97-214	00876	S	S1	11a		28				
20	6971	95-0582	14071	U	U5	11a		2				
20	6167	94-0618	10786	S	S1	11a		2				
20		97-060	18114	U	U5	11a						
20		97-060	18115	U	U5	11a	10	2				
20	3357	91-1306		U	U1	11a	10	2	1			
20	4369	92-1360	4686	U	U1	11a		2	1			
20	6065	94-0618	10784	U	U1	9a		1	3			
20	6622	95-0251	12810	U	U1	26b		1d				
20	6623	95-0251	12811	U	U1	9a		1	3			
20	6624	95-0251	12812	U	U1	9a	9	1	3			
21	6324	94-0867	632	U	U1	19		1d		1		
22	7758	97-292	01242	S	S1	26		21				
23		97-102	18622	S	S	29		13e				
24		97-386	01680	U	U1	9e		1	11a			
25		97-192	00700	U	U1	38		13a				
25		97-192	00701	U	U1	37		23				
25		97-192	00702	U	U1	37		23				
25	7185	96-0037	15067	S	S1	67	18	7				
26	5545	94-0106	9245	U	U4	69		1				

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
26		00-320	238	S	S1	7		13				
26		00-499	774	S	S1	78		3a				
26		00-431	542	S	S1	11c		13c				
26		00-499	777	S	S1	11n		14b				
26		00-492	746	S	S1	77		2				
26		00-492	748	S	S2	77		2				
26		00-492	745	S	S1	7N		13h				
26		01-226	746	S	S1	7b		28				
26	9057	01-365	12193	S	S1	82						
26	9056	01-365	12191	S	S1	82		28				
26		01-480	12576	S	S1	21a		16				
26		01-480	12577	S	S1	21a		16				
26		01-481	12580	S	S1	78b						
26		01-510	683	S	S1	82		28a				
26		02-368	849	S	S1	82		28				
26		02-512	380	S	S1	7		14				
27	2459	90-1261		S	S1	7	1a	13	8			
28	7153	96-0012	14937	U	U2	27		29c				
28		96-0019	14958	U	U2	9d		5b				
28		96-0019	14959	U	U2	9d		5b				
29	2512	90-1304		S	S1	7		13	8			
30	2458	90-1260		S	S1	7	1	13	8			
31	4123	92-0970	3807	S	S3			15a				
32	6040	94-0585	10682	U	U1	14b		1g				
32	2444	90-1248		S	S1	7	1	13	8			
33	2839	91-0153	739	S	S1	7		13				
34	7381	96-0136	15621	S	S1	11h		13e				
34	7760	97-304	01286	S	S1	11c		13a		10		
34	7761	97-316	01372	S	S1	11c		13a				
34		96-0136	15626	S	S1	11h		13e				
35	3798	92-0133	2757	S	S1	59	17	13a				
36	6482	95-0139	12275	U	U1	9		1				
37		97-170	00573	S	S1	7f		14				
37		97-170	00576	S	S1	7f		14				
37		97-170	00579	S	S1	7h		28c				
37		97-209	00825	S	S1	7f						
37	7739	97-209	00829	S	S1	1		29d				
37	7738	97-209	00826	S	S1	7i		28c				
37	7750	97-241	01038	S	S1	7f						
38		97-210	00841	S	S1	9		1				
38		97-210	00854	U	U1	12a		5b				
38		97-210	00857	U	U1	12a		5b				
38		97-210	00861	U	U1	12a		5b				
38		97-210	00864	U	U1	12a		5b				
38		97-210	00842	S	S1	9		1				
38		96-0181	16025	S	S1	11c		13				
38		96-0181	16026	S	S1	11c						
39		96-0135	15613	U	U1	9a		1				
39		96-0135	15615	S	S1	11c		13				
39		96-0135	15616	S	S1	11h		13a				
39	7380	96-0135	15617	S	S1	11h		13a				
39	7377	96-0135	15610	S	S1	11h		13a				
40	7419	96-0159	15858	U	U1	9		1				
40		96-0159	15870	U	U1	19d		1a				
40		96-0159	15859	U	U1	9a		1				
40		96-0159	15860	U	U1	9a		1				
40		96-0159	15868	U	U1	12a		5b				
40	7423	96-0159	15871	U	U1	19d		1a				
41	7783	97-373	635	S	S1	11e		13c	8			
40		96-0159	15867	U	U1	9b		1				
40		96-0159	15875	U	U1			23				
41		97-373	638-1	S	S1	7		13h				
41		97-373	638-2	S	S1	19g		29c				
42		96-0067	15151	U	U1	9b		1d				

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
42	7203	96-0067	15152	U	U1	9		1d				
42	7204	96-0067	15154	U	U1	9b		1d				
42	7204	96-0067	15153	U	U1	9						
43	5469	93-1440	8888	U	U1	33		1				
44		97-352	01530-1	U	U2	9d		5				
44		97-352	01532	U	U2	41		5c				
44		97-352	01530-3	U	U2	41		5c	20			
44		97-352	533#2	U	U2	92						
45	2508	90-1292		S	S1	7		13	8			
46		97-108	18696	S	S	14						
46	7671	97-108	18697	S	S	14a		14				
46		97-108	18698	S	S	14						
46	7672	97-108	18699	S	S	14		14				
46	4223	92-1187		S	S1	11	10	2				
48		97-405	01766	U	U3	19c		1c	1			
48		97-405	01769	U	U3	9		1c				
48		97-405	765	U	U1	19d		1d	11			
49		97-328	01419A	U	U1	9d		25		1		
49		97-328	01419B	S	S1	11e		2				
49		97-328	01424	S	S1	11e		2		2		
49	7775	97-328	01425	S	S1	11e						
50	7767	97-309	01328	S	S1	11a		2				
50		02-019	792	S	S1	11a						
51	3107	91-0923	00011	U	U6/U4	39	23	1a	1			
51	3107	91-0923	00011[B]	U	U6/U4	39a	23	1a				
51	3109	91-0923	00012	U	U1	39b	24	1a				
51	3107	91-0923	11c	U	U6/U4	39c	23	1a				
52	7677	97-128	00098B	U	U1	29		1				
52	7677	97-128	0098C	U	U1	29a		1				
52		02-029	834	U	U5	9o		1c				
53	7351	96-0130	15514	U	U	9e		1d				
53	7330	96-0120	15414	U	U1	9d						
53		96-0028	15021	U	U1	8c		13a				
53		96-0028	15023	U	U1	9		1				
53		96-0028	15026	U	U1	9		1				
53		96-0028	15029	U	U1	9i		1				
53		96-0028	15030	U	U1	9d		1				
53		96-0028	15032	U	U1	9i		1				
53	7172	96-0027	15011	U	U1			1				
54	7774	97-348	516	S	S1	26c		27	16			
54	7759	97-294	01255	S	S1	26a		21/1				
55		97-383	01673	U	U1	9k		1d				
55		97-383	1675	U	U1	17						
55		97-383	1668	U	U1	17		27				
55		97-383	1670	U	U1	17						
56	2441	90-1244		S	S	7	1	13	8			
57	6251	94-0757	00015	U	U6	11e	6	3a	3			
57	7117	95-0734	00025	S	S1	11e	5	2	2b			
58	7159	96-0023	14984	U	U1	12c		1d				
59	2647	90-1529	707	U	S1	9a		13				
59	8660	00-393	448	E	U1	19d		1d	18			
60		96-0034	15054	U	U1	9i		1d				
60		96-0034	15055	U	U1	9i		1d				
60		96-0034	15056	U	U1	9i		1d				
60		96-0034	15049	U	U1	9i		1d				
60		96-0034	15051	U	U1	9i		1d				
60	7179	96-0034	15052	U	U1	9i		1d				
60		96-0034	15053	U	U1	9i		1d				
61	7755	97-266	01158	S	S1	7h		28c				
62	2469	90-1220		S	S1	7		13	8			
63	7165	96-0025	14996	U	U6	9e	4	1				
63	5627	94-0219	00028	S	S1	20	2	3a	3			
63	6016	94-0566	10635	S	S1	20	2	3a	3			
63	6033	94-0566	10632	S	S1	20a	2	2	2a			

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
63	6034	94-0566	10634	S	S1	20	2	3a	3			
63	6325	94-0870	643	S	S1	20	2	3a				
63		96-0025	14993	U	U1	9a	3	21				
63	7164	96-0025	14994	U	U1	9a	3	21				
63		96-0025	14995	U	U6	9e		1a				
63		96-0025	14998	U	U1			1				
64	7825	98-030	02177	U	U5	8		29b	19			
64	6161	94-0704	11022	U	U5	8		6	19a	5		
64		98-467	205#2	U	U5	8	28	29				
65	2452	90-1257		S	S	7	1a	13	8			
66	7208	96-0055	15108	S	S1	11c		2				
66	7209	96-0055	15109	S	S1	11e		28				
67	7776	97-341	01488	U	T	11L		13	12			
67		97-341	01491	U	T	11L						
68	5679	94-		U	U5	18	11	1g	5	3		
69	1572	88-1179		S	S1	7	1	13a		6		
70		76-656		U	U1	9c		1				
71	2407	90-1166		S	S1	6		2				
72	6612	95-0240	00021	U	U6	11e	7	14	2a			
72	6613	95-0240	00022	U	U6	11e	7					
73	5113	93-1061		S	S1	11	12a	2				
74	7754	97-263	01148	S	S1	11		28				
74		97-327	01411	U	U1	14c		1		1		
74		97-327	01416	U	U1	9b		1d				
74	3089	91-0827	605	U	U3	22		1				
75	4953	93-0868		S	S1	87	10a	2				
76	6502	95-0164	00018	U	U6	11e	7	14	2a			
77	857	85-614		S	S1	11	12	2				
78		97-342	492	S	S1	6c		17a	14			
79		97-184	00640	S	S1	7b						
79		97-184	00641	S	S1	7b		13d				
79		97-184	00645	S	S	7b		13d				
80	211	78-594		S	S1	3		13a				
81	7786	97-400	01735	S	S1	11e		14				
81		97-400	01736	S	S1	11e		14b				
81	7787	97-400	01737	S	S1	11e		14b	9			
81		97-400	01738	S	S1	11a		14b				
81		97-400	01739	S	S1	11a		14b				
82		95-0572	14026	S	S	11f						
82		95-0572	14030	S	S	11e						
82	6967	95-0572	14032	S	S	11e						
82		95-0572	14033	S	S	11f		28				
82		95-0572	14034	S	S	11e						
82		95-0572	14035	S	S	11e						
82		95-0572	33 A1	S	S1	11e		28				
82		95-0572	33 B5	S	S1	11g		28				
82		95-0572	33 B8	S	S1	11i		28				
83	5260	93-1205		S	S1	11	12b	2				
84		97-174	00596	U	U	9p		32				
84		97-174	00597	U	U1	9f		32a				
85	8060	98-483	5326	U	U5	8b		29c				
85	8061	98-483	5328	U	U5	8b		29b				
86		97-190	00691	U	U4	36		23				
86		97-190	00692	U	U4	36		23				
86		97-190	00694	U	U1	9m		1				
86		97-190	00696	U	U1	9m		1				
87		96-1168	15902	U	U1	12a		5a				
87		96-1168	15906	U	U1	12a		5d				
88	2443	90-1247		S	S	7	1	13	8			
89	2588	90-1396		S	S1	7	1	13	8			
90	2434	90-1236		S	S	60	19	15	9			
91		97-064	18122	U	U1	9b		1d				
92	6386	94-0906		S	S1	11a	15a	2				
93	7777	97-345	01503	S	S1	7b		17	15			

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
94	6170	94-0693	10965	S	S1	7		13	8			
94	6483	95-0132	12266	S	S1	7		13				
95	4307	92-1305	4518	U	U5	9r		30		1		
96		97-300	01268	S	S1	11k		17		1		
97	6412	95-0022	00016[B]	U	U1	9	9	1a	4			
97	6476	95-0126	00017	U	U6	9c	8	1				
98	7676	97-118	18849	S	S2	19a		18a				
98	7676	97-118	18849[B]	E	S2U4	19b		18		9		
98	7678	97-129	00103#1	U	U4	19		18				
98	7678	97-129	00103#5	U	U4	19		18				
98	7679	97-129	00104#3	S	S2	19a		18				
98	7890	98-179	3570	S	S1	11		20				
98	7891	98-179	3571	S	S1	11		20				
98	7892	98-179	3572	S	S1	11		20				
98		98-179	3573	S	S1	11		20				
98		98-179	3574	S	S1	11		20				
98	7679	97-143	5Ai	E	U4	19b		18				
98	7679	97-143	6Eii	S	S2	19e		19				
98	7679	97-143	5Bii	E	U4	19e		19				
99	7367	96-0130	15513	U	U1	9e		1d				
99	7350	96-0130	15511	U	U1	9e						
99		00-269	067	S	S1	19		57				
100		00-505	788	U	T	21d		2d				
100		00-505	789	U	T	21		2d				
100	4821	93-0585	631	U	U6	21		?				
100	4288	92-1287	00325	S	S1	21		2				
100	4289	92-1287	00326	S	S1	21		2				
100	4290	92-1304	00327	S	S1	21		2				
100	4291	92-1304	00328	S	S1	21						
100	6013	94-0558	00329	U	U1	25						
100	6903	95-0503	00331	U	U1	25a		22				
100	6904	95-0503	00332	U	U1	9c		1d				
100	7938	98-341	4725	U	T	21		2d				
100	9095	01-466	12524	S	T	21e		2d				
100		01-516	707	E	S1	21						
100	9152	02-026	823	S	S1	21a		2d				
100		02-048	896	U	U5	21a		2d				
100		01-516	706	U	T	21e		2d				
100	9098	01-475	12556	S	S1	21a						
100	9094	01-466	12523	U	T	21a		2d				
100		01-416	12382	S	S1	21a		2d				
100		01-421	12405	S	S1	21		2d				
100		01-323	041	S	S1	21a						
100		01-323	040	U	T	21a						
100	9254	02-164	311	S	S1	21		2d				
100	9255	02-164	313	S	S1	21a						
100		01-416	12437	S	S1	90		2d				
100		01-516	708	S	S1	21						
101	7782	97-369	625	S	S1	9d	21	11a	17			
101	7781	97-369	623	U	U6	9d-1	9	11				
101		97-369	626#1ii	S	S1	61	20	29a	7			
101		97-369	626#1	S	S1	9d		12				
102		97-111	18772	U	U2	9a		1d				
103		96-0129	15505	S	S1	15		13				
103		96-0129	15507	S	S1	15		13				
103		96-0129	15508	S	S1	15		13				
104		97-333	01441	U	U4	7e		1e				
104		97-333	01442	S	S1	11c		13c				
104		97-333	01443	S	S1	7a-1		13				
105	2127	90-675		S	S1	7a		2	8			
106	968	85-842		S	S1	11	12	2				
107	830	85-540		S	S1	11	12	2				
108	5404	93-1358	643	U	U4	9i		30				
109		95-0595	14167	S	S1	11b		3a				

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
109		95-0595	14168	S	S1	11b						
110	2503	90-1219		S	S1	7	1a	13	8			
111		97-159	00461	S	S1	28		14				
111		97-159	00463	S	S1	28		14				
112	7022	95-0634	00024	U	U6	11e	7	14	2a			
112	7136	96-0002	00334	S	S1	11e	7	14				
112	7138	96-0002	00336-896	U	U1	11e		14				
112	6344	94-0883	00338	S	S1	11e		14				
112	6345	94-0883	00339	S	S1	11e		14				
112	5336	93-1305	00341	S	S1	11e	7	14				
112	6346	97-145	340	S	S1	11e	ng	3				
113		97-057	18085	S	S1	11e						
113		97-331	01433	S	S1	11b						
113	7763	97-321	394	S	S2	11b		24				
114		97-055	18079	U	U1	19		1	4a	1		
114		97-055	18081	U	U1	19		1	4a			
115		97-106	18679	U	U5	9e		1d	4			
115	7340	96-0125	15487	S	S	3a		13a	6			
115	7341	96-0125	15488	S	S	3		13a	7			
115	7343	96-0125	15490	S	S	7		13a				
115	7369	96-0133	15578	U	T	7		13a				
116		96-0147	15741	S	S1	11c		13h				
117	6718	95-0297	13035	S	S1	28		2				
117	6720	95-0297	13037	S	S1	30		10				
118	305			U	U1	9d		54				
118	A198	95-0112	12222	S	S1	4a		26				
118	6468	95-0112	12225	S	S3	68		29d				
119	6488	95-0146	12291	S	S1	7g		6a				
120	7701	97-133	182	S	S1	9d		1d				
120	7702	97-133	183	S	S1	9d		1d				
121	6207	94-0739	11156	U	U1	85		5				
122		01-450	12476	S	S1	87		13a				
122	6047	94-0593	10715	U	U1	9b		1d				
122	6048	94-0593	10716	U	U1	83		1d				
123		97-134	185	S	S1	7f		13c				
123		97-134	187	S	S1	7f		13c				
123		01-464	12517	S	S1	78b		13g				
123		01-464	12520	S	S1	78b		13g				
123		00-527	865	S	S1	78b		13g				
123		00-529	870	S	S1	7f		13g				
124		01-456	495	S	S1	7f		28a				
125		00-434	547#1	S	S1	43a		29c				
125		00-434	546	S	S1	43a		10				
126	6171	94-0710	11045	U	U1	9d		1a				
127	1685	89-428		S	S1	66	2	29c				
128	6059	94-0603	10744	S	S1	84		13				
129	5136	93-1073	7624	U	U6	7	22	2				
129		97-176	00604	U	U1	70a						
129		97-176	00606	U	U1	70						
129		97-119	18852	U	U1	34						
129	6326	94-0872	646	U	U3	9a						
129		98-169	03541	U	U3	9		1				
129		97-169	541	U	U3	9		1				
130		01-436	12435	S	S2	11		2				
131	4851	93-0683	6743	S	S1	59a	17	6b				
132		00-438	561	S	S1	19x		29b				
132		00-438	562	S	S1	7a-2		14d				
132		00-438	563	S	S1	7a		14c				
133	5659	94-		U	U3	9e		13g				
134	2406	90-1165		S	S1	7	1a	13	8			
135	8797	01-032	11069	S	S1	9u		27a				
136	6205	94-0730	11126	U	U3	9b		1e				
137		00-144	314	U	U5	8b						
137		00-143	310	U	U5	8b						

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
137		00-143	308	U	U5	8b						
137		00-143	306	U	U5	8b	29	29d				
138	8536	01-034	11077	U	U1	64a						
138	8537	00-209	889	U	U5	12		5				
138	8536	00-209	888	U	U5	64a		1d				
138		00-194	819	S	S1	17a		1d				
138		00-468	669	U	U5	12		5				
138		00-468	666	U	U5	12e						
139	8231	00-189	065	U	U5	9s		21				
139		00-180	684	U	U1	9s		21				
140		00-199	825	S	S1	11		28				
140		00-199	824	S	S1	11		28				
141		01-037	102	S	S1	77b		28a				
142	8796	01-023	11044	S	S1	37(9T)		27a				
142	8540	00-237	981 #1	S	S1	71		8				
142	8541	00-237	981 #2	S	S1	11f		14				
142	8542	00-237	982	S	S1	11k		17				
143	8539	00-230	967	S	S1	11c		13				
144	8543	00-244	995	S	S1	72		2				
144	8544	00-244	996	S	S1	ng		58				
145		00-256	032	S	S1	37		10				
145	8547	00-256	031	S	S1	37b		29a				
145		00-256	030	S	S1	19d		14b				
145		00-256	033	S	S1	37		29a				
146		00-391	442	S	S1	79a						
146		00-391	440	S	S1	14 (23)		14				
146		00-257	037	S	S1	79a		10				
146		00-257	036	S	S1	79a		10				
146		00-464	654	S	S1	79		10				
146		00-464	652	S	S1	76		2e				
146		00-464	655	S	S1	79a		10				
147		00-258	038	S	S1	11n		14				
147		00-258	039	S	S1	11n		14				
147		00-258	040	S	S1	11		14				
148	8655	00-282	097	S	S2	7a		28				
148		01-372	12225	S	S2	11?		2				
148		01-372	12226	S	S2	11?						
149	8558	00-238	983	S	S1	7f		50b				
150	8563	00-281	094	S	S1	11						
150	8561	00-281	092	S	S1	11						
150	8565	00-281	096	S	S1	11		28				
150	8564	00-281	095	S	S1	11						
150	8562	00-281	093	S	S1	11						
150		01-006	962	S	S1	11		28				
150		01-006	964	S	S1	11		28				
150	8559	00-281	090	S	S1	11		28				
150	8560	00-281	091	S	S1	11		28				
150a		00-304	202	S	S1	87		13a				
150a		00-337	295	S	S1	73		13g				
150a		00-304	201	S	S1	87						
150a		00-304	199	S	S1	87						
151	8572	00-287	135	U	U5	12		5				
152	8574	00-292	147	S	S1	75		14b				
152	8573	00-292	146	U	U1	74		1d				
152	9005	01-240	777	S	S1	11h		2a				
152	9014	01-253	824	S	S1	11e		2				
152		01-220	731	S	S1	11?						
153	8586	01-034	11079	U	U5	8a		29b				
154		00-295	165	S	S1	9u		1d				
154	8587	00-295	160	S	S1	9u						
154		00-295	161	S	S1	11N						
154		00-295	162	S	S1	11N		14				
154		00-295	164	S	S1	11N		2				
154		00-295	163	S	S1	9u		1d				

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
154		00-322	251	S	S2	88						
154		00-322	252	S	S2	88		1				
155		00-306	209	S	S1	7L		2e				
155		00-306	210	S	S1	7L						
156		00-307	212	U	U1	19e		29c				
156		00-307	211	S	S1	7a		28a				
157		01-452	12481	S	S1	21		2d				
157	9055	01-362	12181	U	U5	8e		29				
158	9199	02-096	058	S	S1	88						
158		01-461	513	S	S1	88		1e				
159		00-498	773	S	S1	11n		2				
159		00-377	407	S	S1	9c		1d				
160		00-324	255	S	S1	37(9T)						
160		00-324	256	S	S1	37(9T)		15a				
160		00-324	257	S	S1	37(9T)						
160		00-324	258	S	S1	37(9T)						
160		00-324	259	S	S1	37(9T)						
161	9092	01-458	12500	S	S1	14b		14				
161		01-458	12501	S	S1	14a		14d				
161		01-458	12497	S	S1	14		14				
162		00-332	283	S	S1	11h-b		28a				
162		00-340	312	S	S1	11h-b						
162		00-340	311	S	S1	11h-b						
162		00-340	310	S	S1	11h-b						
162		00-340	309	S	S1	11h-b						
162		00-340	307	S	S1	11h-b						
162		00-339	304	S	S1	11h-b						
162		00-340	313	S	S1	11h-b		28a				
162		00-339	302	S	S1	11h-b						
162		00-339	305	S	S1	11K?						
163		00-300	190	U	U5	8a		29a				
164		01-041	117	S	S1	77b		14d				
164		01-041	116	S	S1	7a		14c				
165		00-473	683	S	S1	7a		13h				
165		00-473	684	S	S1	7a		13h				
166		01-042	120	S	S1	89d		13				
166		01-042	119	S	S1	89c		13				
166		01-325	046	S	S1	89		13a				
166		01-325	048	S	S1	89a		13a				
166		01-325	047	S	S1	89						
167		01-423	12411	S	S2	11c		2				
168		01-168	610	S	S1	13		13f				
169		01-449	12470	S	S1	7a		13g				
170		00-450	607	S	S2	19d		15a				
170		00-450	609	S	S2	19d		15b				
170		00-450	610	S	S1	12		21				
170	8777	01-009	974	S	S1	12e?		1b				
170		01-009	976 #1	S	S1	19d?		15a				
170		01-009	977	S	S2	19d?		15a				
171		00-439	565	S	S1	9m		21				
171		00-439	566	S	S1	9m		21				
171		00-435	549	S	S1	82		2				
171		00-435	550	S	S1	82a		2				
171		01-015	005	S	S1	82		2				
171		01-015	006	S	S1	82		2				
171		01-015	007	S	S1	9a		1d				
171		01-015	008	S	S1	7i		2				
171		01-015	009	S	S1	83		13g				
172		01-038	104	S	S1	9b		21				
172		01-049	154	S	S1	7f		2				
172		01-050	155	S	S1	7f		2e				
172		01-049	153	S	S1	7f		2				
172	8809	01-046	144	S	S1	11N		2				
172	8803	01-039	110	S	S1	11N		14				

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
172		01-050	157	S	S1	7f		2e				
172	8802	01-039	109 #2	S	S1	11N						
172	8802	01-039	109 #1	S	S1	11N		2e				
172		01-049	152	S	S1	7f		2e				
172		01-050	156	S	S1	9L+		21				
172		01-038	103	S	S1	9a		21a				
173		00-544	921	S	S1	44		28a				
173		00-544	920	S	S1	44		28a				
173		00-467	664	S	S1	11h-a		28a				
173	9129	01-515	697	S	S1	11a		28a				
173	9341	02-435	77	S	S1	11h		13a				
174	8870	01-064	189	U	U5	37		5				
174	8869	01-064	190	U	U*	37		5				
175		00-471	677	S	S1	7a		13h				
175		00-471	678	S	S1	7		13h				
175		00-471	676	S	S1	7a		13g				
176		00-528	868	S	S1	11n+						
176		00-528	866	S	S1	11n+		13g				
176		00-531	876	S	S1	11h-b		28				
176		02-549	517	S	S2	21a		2				
176		02-549	518b	S	S1	7a		13a				
176		02-031	838	S	S1	77b		28a				
176		02-031	836	S	S2	77b		28a				
176		02-031	837	S	S1	77b		28a				
176		01-250	810	S	S1	77a		28a				
176		01-250	813	S	S1	77a		28a				
177		01-012	992	T	T	11c		13g				
177		01-012	994	S	S1	11c		13g				
177		01-012	993	S	S1	11c		13g				
177		01-012	990	S	S1	11c		13g				
177		01-012	991	T	T	11c		13g				
177		02-020	795	S	S1	11c						
177	9144	02-020	839	S	S1	11c						
178		01-311	008	S	S1	7c		13c				
178		01-311	009	S	S1	7c		13g				
179		00-443	576	S	S1	11c?		14				
180		00-405	474 #1	S	S1	7a		2				
180		01-448	12467	S	S1	82b						
180		01-448	12465	S	S1	82b		17				
180		00-410	493	S	S1	7a						
180		02-077	999	S	S1	82b						
181		01-014	004	S	S1	7		13a				
181		02-108	089	S	S2	7		14				
182		00-491	742	S	S1	72		2				
182		00-491	740	S	S1	72a		2				
183		00-380	413	S	S1	73		13				
183		00-380	414	S	S1	73						
184	8674	00-436	554	S	S1	7f-a						
184		00-422	515	S	S1	7c		13c				
184		00-441	569	S	S1	7m		28				
184		00-513	820	S	S1	7c		13g				
184		00-513	823	S	S1	7c		13g				
184		00-513	821	S	S1	7c		13g				
184		00-513	822	S	S1	7c		13g				
184		00-441	568	S	S1	7c		28				
184		00-371	383	S	S1	7f						
184		00-513	819	S	S1	7c		13g				
185		00-421	514	S	S1	7c		28				
185	8672	00-421	513	U	U1	19d		1d				
186		01-052	160	S	S1	11k		17				
186		02-025	820	S	S1	11						
187		00-385	424	S	S1	7h		28				
188		00-521	849	S	S1	11a		13g				
188		00-521	852	S	S1	11c		13g				

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
189		00-413	500	S	S1	19d		1d				
190		00-496	763	S	S1	77a		14d				
191		01-051	159	S	S2	7f		2				
192		00-489	734	S	S1	76		2				
192		00-489	735	S	S1	76		2				
192		00-489	736	S	S1	76a		2				
192		00-489	737	S	S1	76b		2				
193		00-509	800	S	S1	86		29c				
193		01-055	178	S	S1	86a		29c				
193		01-055	173	S	S1	86						
193		01-055	174	S	S1	86						
193		01-055	175	S	S1	86						
194		00-404	470	S	S1	86		2				
195		00-444	584#1	S	S1	24c		60				
195		00-444	587	S	S1	81		28a				
195		00-444	586	S	S1	24		28				
195		00-444	578	S	S1	81		28				
195		00-444	585#1	S	S1	24		2				
195		00-444	588	S	S1	81		28a				
195		00-444	582	S	S1	24c		60				
195		00-444	581	S	S1	21b		24				
195		00-444	583	S	S1	21c		28a.				
195		00-444	580	S	S1	21		28				
195		00-444	585#2	S	S1	24d		60				
195	8695	00-444	579#2	S	S1	9		27				
195		00-444	584#2	S	S1	81		28a				
195		00-444	579#1	S	S1	24b		17				
196		00-343	318	U	U1	19e		1d				
196		00-347	326	U	UI	19d		1d				
196		00-347	325	U	U1	19d		1d				
197		00-457	627	S	S1	80						
197		00-457	629	S	S1	80		4				
198		01-459	12502	S	S1	11		28				
199	9054	01-361	12177	U	U5	8e		29b				
200	9096	01-467	12529	S	S1	11K		2				
201	8924	01-092	419 #2	S	S1	1		10				
201	8926	01-092	420 #2	S	S1	1						
202		01-451	12479	S	S1	7a		13h				
203	9048	01-355	12130	S	S1	82		17				
203	9051	01-355	12134	S	S1	82		17				
203	9050	01-355	12132	S	S1	11K		17				
204	8927	01-095	425	S	S1	11c		14				
205	9193	02-089	035	S	S1	7		2				
205		01-399	12332	S	S1	11e		2				
205		00-487	731	S	S1	11n		2				
205		02-089	037	S	S1	7						
205	9194	02-089	038	S	S1	7						
205	9195	02-089	039	S	S1	7L		2				
206		01-517	712	S	S1	7f		13g				
207	9208	02-114	108	S	S1	82		17				
207	9207	02-114	105	S	S1	82		17				
207	9206	02-114	104	S	S1	82		17				
207	9205	02-114	103	S	S1	82		17				
207		02-055	928	S	S1	7f						
207		02-075	996	S	S1	82						
207		02-075	992	U	U6	7i		2				
207		02-075	993	S	S1	7e						
208		01-210	709	S	S1	73		13				
208		01-210	710	S	S1	73						
208		01-210	718	S	S1	73		13				
208		01-210	716	S	S1	73						
208		01-210	715	S	S1	73						
208		01-210	714	S	S1	73						
208		01-210	713	S	S1	73						

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
208		01-210	711	S	S1	73						
208		02-016	784	S	S1	7		13g				
208		02-016	780	S	S1	7						
209	8974	01-184	635	S	S1	7		14				
209	8975	01-184	636	S	S1	7						
210		01-222	737	S	S1	11						
210		01-222	738	S	S1	11K		17				
210		02-032	842	S	S1	11						
211	9017	01-281	901	S	S1	9a		27				
212		00-427	527	S	S1	78a		2				
213		01-279	897	S	S1	7c		28a				
214		01-334	089	S	S1	14		14				
215	9012	01-265	856	S	S1	7		13h				
216		01-409	12367	S	S1	1		10				
217		02-035	849	S	S1	73		13				
218		00-336	292	U	U5	8d		29a				
219	9021	01-294	939	S	S1	7m		29a				
220		02-037	861	S	S1	11e		28				
221		01-192	655	U	U5	8d						
221		01-192	657	U	U5	8d		29a				
222		01-315	015	S	S1	83		10				
222		01-315	017	S	S1	7a						
222		01-315	019	S	S1	7a		2				
223		02-036	852	S	S1	7a						
224		02-073	989	S	S1	11c		13				
225		98-067	02394	U	U5	11a		1d	4			
225	7836	98-067	02393	U	U5	11a		1d	4			
226	7821	98-009	02122	S	S1	7h		28c	8			
226	7822	98-009	02124	S	?S4	8a		10	13			
227		00-514	825	S	S1	7a		13g				
227		00-514	824	S	S1	7a		13g				
228		00-246	998	S	S1	37		10				
229	8570	00-283	101	S	S1	73		13				
229	8569	00-283	100	S	S1	73		13				
229	8668	00-283	099	S	S1	73						
229	8567	00-283	098	S	S1	73		13				
230		00-303	198	U	U5	8c		29a				
231	9243	02-136	221	S	S1	91						
231	9240	02-136	214	S	S1	91		29				
231	9241	02-136	215	S	S1	91		29a				
231	9242	02-136	219	S	S1	91		29a				
232		02-140	234	S		11c		28				
232	9246	02-140	235	S	S1	11c		28				
232	9244	02-140	236	S	S1	91		29a				
233	9247	02-146	250	S	S1	83d		13c				
233	9248	02-146	254	S	S1	83b		13c				
233	9249	02-146	255	U	U6	83c		13c				
233		02-146	257	S	S1	83c		13c				
233		02-146	258	S	S1	83a		13c				
233	9250	02-151	274	S	S1	11L		13c				
233	9252	02-151	277	S	S1	11N		13c				
233	9253	02-151	278	S	S1	11N		13c				
234		02-575	618	S	S1	7e		13c				
235	8005	95-0662-1	V8	U	U5+	52		28				
236	8007	95-0558-2B	V10	S	S1	42		33				
237	8017	95-0296-1	V20	S	S1+	6b		20				
238	8004	96-0009-1	V7	U	U1	4		35				
239	8015 A	95-0296-1	V18 d	U	U5	9a		13g				
240	8072	95-0588-1	V3	S	S1+	24		2				
241	8002	96-0063-4A	V5	S	S1	3b		13				
242	8015 B	95-0296-1	V18ii m	S	S1	52		50				
251		02-146		S	S1	9L		1c				
253		02-146		S	S1	9L		1c				
300	4834	93-0612	6667	U	U5	23		29d	2	2		
301	5637	94-0229	9640	U	U6	31		60				

Table B-1 (continued)

Farm ID	AC No.	Case No.	Isolate	GGel	Zym	PFA	PFX	IrsT	IrsG	IrsC	IrsA	IrsX
302		00-530	873	U	U5	1a		29b				
302		00-530	874	U	U5	12c		1d				

AC = Albany culture number; Farm ID = farm identification code number; Case No. = laboratory case number; Isolate No. = isolate identification number; GGel = gelatin-gel result; Zym = zymogram pattern number; PFA = molecular type using PFGE and enzyme *Apa*I; PFX = molecular type using PFGE and enzyme *Xba*I; IrsT = molecular type using IRS-PCR and primer PXT; IrsG = molecular type using IRS-PCR and primer PXG; IrsC = molecular type using IRS-PCR and primer PXC; V = National Culture Collection number; S = thermostable protease; U = thermolabile (unstable) protease. A blank indicates that the isolate was not tested by that method.

Table B-2. Results of molecular typing of isolates from farms in SA

Farm ID	Year Isolated	Case No.	Isolate No.	GGel	Zym	PFA	IrsT
31	1992	AC 4123		S	S3	NT	15a
300	1993	AC 4834		U	U5	23	29d
301	1994	AC 5637		U	U6	31	60
276	1993	93-1123	4A	U	U1	43	52
277	1993	93-11229	4	S	S1	44b	46a
278	1993	93-10127	6A	S	S1+	57	2b
279	1993	93-10083	1	U	U5	9d	29e
280	1994	94-100999	3B	U	U1	46d	29d
281	1994	94-11164	1	S	S1+	11a	2
282	1993	93-11047	2B	S	S1+	63	3b
283	1993	93-1191	2	S	S1+	9e	1b
284	1993	93-11969	2A	S	T	54	46a
285	1993	93-10543	3A	S	S2	16	2
286	1993	93-10737	2	S	S1+	1b	65
287	1994	94-10998	1	U	U1	7d	13a
288	1994	94-00132	2	U	U5	18	1d
289	1993	93-11697	5	S	S1	1c	53
290	1994	94-7991	1	S	S1+	7	13
291	1994	94-9799	1A	U	U1	9b	5c
297	1993	93-9669	5	S	S1+	6a	46
299	1993	93-9669	5	S	S1+	6a	46

AC = Albany culture number; GGel = gelatin-gel method; ID = identification number; IrsT = molecular type using primer PXT and IRS-PCR; No. = number; PFA = molecular type using *Apa*I enzyme and PFGE; NT = not tested; S = thermostable protease; SA = South Australia; U = unstable protease; Zym = zymogram profile number.

Table B-3. Results of molecular typing of isolates from farms in NSW

Farm ID	Year Isolated	Case No.	Isolate	GGel	Zym	PFA	IrsT
86	1997	97-190	694	U	U1	9m	1
86	1997	97-190	696	U	U1	9m	1
86	1997	97-190	691	U	U4	36	23
86	1997	97-190	692	U	U4	36	23
264		830		S	S1+	10	2a
265	1995	95-3567	4A	U	U1	9a	1
266	1995	95-4321	1A	U	U1	9e	13
267	1995	95-4187	4a	U	U1	53	40
268	1995	95-5203	1A	U	U1	50	34
269	1995	95-5156	2A	S	S1+	11a	2
270	1995	95-4401	2A	U	U1	6b	29d
271	1995	95-4589	5A	S	S1+	46c	39
272	1995	95-3394	1A	S	S1+	3e	36
273	1995	95-4244	2A	U	U1	46	13g
274	1995	95-4010	5A	U	U1	7L	1b
275		829		S	S1+	6d	48

GGel = gelatin-gel method; ID = identification number; IrsT = molecular type using primer PXT and IRS-PCR; No. = number; NSW = New South Wales; PFA = molecular type using *Apa*I enzyme and PFGE; S = thermostable protease; U = unstable protease; Zym = zymogram profile number.

Table B-4. Results of molecular typing of isolates from farms in Victoria

Farm ID	Year Isolated	Case No.	Isolate	GGel	Zym	PFA	IrsT
118	1995	C305		U	U1	9d	54
118	1995	95-0112	A198	S	S1	4a	26
118	1995	95-0112-225	6468	S	S3	68	29d
243	1993	93-6558	3	S	S1+	13	13
244	1993	93-6206	3A	S	S1+	31	2c
245	1994	94-6057	4A	E	S3	9q	1a
246	1994	94-6115	3A	S	S1+	19f	1a
247	1996	96-0183	1	S	S1+	3c	3a
248	1995	95-0181	4A	U	U1	3d	14b
249	1993	93-6559	6A	U	U1	44a	5a
250	1993	93-6782	3A	E	S3	1d	39
251	1994	94-6475	1	E	S3	45	14b
252	1994	94-6132	1A	S	S1+	7	2
253	1993	93-6216	2B	E	S3	47	1d
254	1993	93-6885	4	S	S1+	55	3
255	1993	93-6728	1a	U	U1	9d	25
256	1993	93-6713	6A	S	S1+	64	61
257	1993	93-6603	1A	E	S3	38	39
258	1993	93-6239	1	E	S3	11	2
259	1993	93-6024	1A	E	S3	38	39
260	1993	93-6163	1	E	S3	46b	39
261	1993	93-5969	1A	S	S1+	11	2
262	1994	94-5526	1	S	S1+	65	13d
263			721	S	S1+	7L	18b

GGel = gelatin-gel method; ID = identification number; IrsT = molecular type using primer PXT and IRS-PCR; No. = number; PFA = molecular type using *Apa*I enzyme and PFGE; S = thermostable protease; U = unstable protease; Zym = zymogram profile number.