



UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERISATION OF *DICHELOBACTER NODOSUS* FROM FOOTROT INFECTED SHEEP IN MALAYSIA

ZUNITA BTE ZAKARIA

FPV 1998 10

**ISOLATION AND CHARACTERISATION OF *DICHELOBACTER
NODOSUS* FROM FOOTROT INFECTED SHEEP IN MALAYSIA**

By

ZUNITA BTE ZAKARIA

**Thesis Submitted In Fulfilment of the Requirements for the
Degree of Master of Science In the Faculty of
Veterinary Medicine and Animal Science
Universiti Putra Malaysia**

March 1998



Specially dedicated to Abah and Mak.....



ACKNOWLEDGEMENTS

This thesis was an ambitious project from the start and would never have been completed without the skills and talents of many people. Though only my name appears on the cover, much credit and my heartfelt thanks are owed to the following people:

Especially to my supervisor, **Professor Dato' Dr. Sheikh Omar Abdul Rahman** and the members of the supervisory committee, **Dr. Abdul Rahim Mutalib**, **Dr. Son Radu** and **Dr. P.G. Joseph** for their encouragement, invaluable advice and suggestions, guidance, patience and kindness.

To the **Department of Veterinary Services** which kindly provided samples for this project. To **Professor J.R. Egerton** of the University of Sydney and the **Crawford Fund** for their help in enabling me to attend a training course on footrot disease in Australia.

To my family and husband, **Hasnal**, who I owe the most for their patience, understanding and support through this long and demanding project.



Finally to all the staffs and academicians of the Faculty of Veterinary Medicine and Animal Science and my friends for their help and support during the course of the project.



TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
LIST OF PLATES.....	x
ABSTRACT.....	xi
ABSTRAK.....	xiii
CHAPTER	
1 GENERAL INTRODUCTION.....	1
2 LITERATURE REVIEW.....	4
Introduction.....	4
Footrot Disease.....	5
Historical Background.....	5
Pathogenesis.....	5
Clinical Diagnosis.....	8
Laboratory Diagnosis of Footrot.....	9
Treatment and Control.....	10
Etiology of Ovine Footrot.....	14
Taxonomic Status of <i>Dichelobacter nodosus</i>	14
Morphological and Bacteriological Characteristics of <i>Dichelobacter nodosus</i>	15
Molecular Characteristics.....	16
Antigens of <i>Dichelobacter nodosus</i>	19
Virulence of <i>Dichelobacter nodosus</i>	22
Virulence Determination of <i>Dichelobacter nodosus</i>	25
Sensitivity to Antimicrobial Agents.....	26
3 ISOLATION AND IDENTIFICATION.....	29
Introduction.....	29
Materials and Methods.....	31
Study Area.....	31
Sampling Procedure.....	31
Direct Smear Identification.....	32
Preparation of Culture Media	32
Isolation.....	34
Storage and Maintenance of Cultures.....	34



	Polymerase Chain Reaction (PCR) Amplification of 16S rRNA.....	36
	Serology.....	38
	Results.....	41
	Direct Smear Examination.....	41
	Clinical Observation.....	41
	Isolation.....	43
	Serogrouping and Serotyping.....	47
	Species Confirmation.....	47
	Discussion.....	50
4	ANTIBIOTIC SUSCEPTIBILITY TEST.....	55
	Introduction.....	55
	Materials and Methods.....	56
	Antibiotics.....	56
	Susceptibility Testing.....	56
	Interpretation of MIC Reading.....	58
	Results.....	60
	Discussion.....	63
5	VIRULENCE ASSESSMENT.....	68
	Introduction.....	68
	Materials and Methods.....	70
	Bacterial Isolates.....	70
	Gelatin Gel Test.....	70
	Elastase Test.....	72
	Results.....	73
	Gelatin Gel Test.....	73
	Elastase Test.....	75
	Discussion.....	76
6	MOLECULAR CHARACTERISATION.....	82
	Introduction.....	82
	Materials and Methods.....	83
	Preparation of Chromosomal DNA for Pulsed Field Gel Electrophoresis (PFGE) Analysis.....	83
	Restriction Endonuclease Digestion and Pulsed Field Gel Electrophoresis (PFGE) Analysis.....	85
	Size Markers for Pulsed Field Gels.....	
	Restriction Endonucleases.....	87
	Interpretation of Pulsed Field Gels.....	88
	Small Scale Isolation of Plasmid DNA.....	89
	Results.....	91
	Discussion.....	100
7	GENERAL DISCUSSION AND CONCLUSIONS.....	107

REFERENCES	113
APPENDIX	123
A Definition of Scoring System.....	123
B Gram Stain - Kopeloff's Modification.....	125
C Solutions.....	127
VITAE	128



LIST OF TABLES

Table		Page
1	Biochemical properties of <i>Dichelobacter nodosus</i>	17
2	Tube agglutination reactions between <i>Dichelobacter nodosus</i> and antisera prepared in rabbits.....	21
3	Agglutination titre reaction between <i>Dichelobacter nodosus</i> antigen and serotype antisera.....	48
4	NCCLS-approved breakpoints for antimicrobial agents.....	59
5	Sensitivity of 12 <i>D. nodosus</i> isolates to nine antibiotics.....	61
6	MIC _{50%} and MIC _{90%} values of nine antibiotics against 12 <i>D. nodosus</i> isolates.....	62
7	Comparative examination of 12 <i>D. nodosus</i> strains using elastase and gelatin gel tests.....	74
8	Criteria for interpreting pulsed field gel electrophoresis patterns.....	89
9	F values of each PFGE pattern for 12 <i>D. nodosus</i> isolates.....	94
10	Comparison of 12 <i>D. nodosus</i> isolates patterns in pulsed field gel electrophoresis.....	103



LIST OF FIGURES

Figure		Page
1	A schematic representative of <i>ApaI</i> -pulsed field gel electrophoresis fingerprints.....	93
2	A schematic representative of <i>SfiI</i> - pulsed field gel electrophoresis fingerprints.....	97
3	A schematic representative of <i>SmaI</i> -pulsed field gel electrophoresis fingerprints.....	99



LIST OF PLATES

Plates	Page	
1	A Gram-stained smear from a footrot lesion showing a bacilli resembling <i>D. nodosus</i> seen as a large Gram-negative rod with swollen ends.....	42
2	Gram-stained smears from footrot lesion showing bacteria resembling <i>D. nodosus</i> seen among other contaminating bacteria.....	42
3	A smear from a footrot lesion showing a long, slender Gram-negative rod with tapering ends resembling <i>Fusobacterium necrophorum</i>	44
4	Necrotising lesion of the interdigital skin which involved part of the soft horn of the axial wall of the digit.....	44
5	A 2% HA plate showing colony morphology of <i>D. nodosus</i> with a mucoid central zone, transparent mid zone and smooth edges.....	45
6	A 2% HA plate showing colony morphology of <i>D. nodosus</i> with a beaded central zone, transparent mid zone and fimbriate edges.....	45
7	A 2% HA plate showing swarming growth of flat colonies of <i>D. nodosus</i>	46
8	Agarose gel electrophoresis of <i>D. nodosus</i> genomic DNA amplification products by using the Ac and C primer combination.....	49
9	Agarose gel electrophoresis of <i>D. nodosus</i> genomic DNA amplification products using the Ac and C primer combination.....	49
10	<i>Apa</i> I-pulsed field gel electrophoresis of <i>D. nodosus</i> genomic DNA.....	92
11	<i>Sfi</i> I-pulsed field gel electrophoresis of <i>D. nodosus</i> genomic DNA.....	96
12	<i>Sma</i> I-pulsed field gel electrophoresis of <i>D. nodosus</i> genomic DNA.....	98



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirements for the degree of Master of Science

**ISOLATION AND CHARACTERISATION OF *DICHELOBACTER*
NODOSUS FROM FOOTROT INFECTED SHEEP IN MALAYSIA**

BY

ZUNITA ZAKARIA

MARCH 1998

Chairman: Professor Dato' Dr. Sheikh Omar Abdul Rahman

Faculty: Veterinary Medicine and Animal Science

Twelve *Dichelobacter nodosus* were isolated from 12 sheep with footrot with lesion score 2. The isolates were studied and the results analysed. Diagnosis was done successfully by Gram-stain method while polymerase chain reaction (PCR) method with species specific primers, A and Ac were employed for species confirmation. All 12 isolates reacted positively in the PCR method by producing a single product of approximately 780 basepairs. All isolates, although obtained from distant locations, were from serogroup B (10 isolates were B2 serotype, 2 isolates were B1 serotype). The E-test method was used to determine the minimum inhibition concentration (MIC) values of nine



antimicrobial agents against all 12 isolates. Penicillin G proved to be the most effective antibiotic with MIC_{90%} of 0.023 µg/ml. Two standard conventional methods, the elastase and gelatin gel tests, were used in assessing the virulence of the isolates. Generally, the isolates exhibited variation in the laboratory characteristics although they had been isolated from similar lesion score. Some of the isolates which appeared to have the capability of causing virulent footrot *in-vitro*, failed to show clinical signs of virulent form of footrot. This was probably due to the frequent topical regimen adhered to and result of the vaccination programme by the farm management. All isolates were found not to contain plasmid by standard plasmid extracting method. This indicates that the genes coding for virulence of the isolates were not plasmid-mediated. Molecular typing of the isolates was successfully carried out by pulsed field gel electrophoresis (PFGE) analysis. Significant patterns were generated by three GC-rich enzymes (*ApaI*, *SfiI* and *SmaI*) discriminating the isolates into eight genome types. Isolates from the same flock were also shown to possess variation in their PFGE profiles.

These results demonstrate the diversity of *D. nodosus* strains infecting sheep in Malaysia and also indicated that the isolates were from diverse sources.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra
Malaysia sebagai memenuhi keperluan ijazah Master Sains

PEMENCILAN DAN PENCIRIAN *DICHELOBACTER NODOSUS*
DARI BEBIRI BURUK KUKU DI MALAYSIA

OLEH

ZUNITA ZAKARIA

MARCH 1998

Pengerusi: Professor Dato' Dr. Sheikh Omar Abdul Rahman

Fakulti: Kedoktoran Veterinar dan Sains Peternakan

Dua belas pencilan *Dichelobacter nodosus* telah dipencilkan dari bebiri yang menghadapi buruk kuku dengan lesi skor 2. Pencilan tersebut dikaji dan dianalisa. Diagnosa telah berjaya dilakukan melalui kaedah pewarnaan Gram manakala kaedah reaksi polimerasi rantai (PCR) menggunakan primer spesifik, A dan Ac digunakan untuk konfirmasi spesis. Kesemua pencilan menunjukkan reaksi positif dalam kaedah PCR di mana semua pencilan menghasilkan satu produk 780 pasangan bes. Walaupun semua pencilan adalah dari tempat berbeza, mereka semua didapati adalah didalam satu kumpulan sero B (10 pencilan adalah serotip B2, 2 pencilan adalah serotip B1). Kaedah



E-test telah digunakan untuk menentukan nilai konsentrasi inhibisi minima (MIC). Sembilan agen antimikrobia telah diuji menentang 12 pencilan *D. nodosus*. Terbukti bahawa Penicillin G adalah antibiotik yang paling efektif dengan MIC_{90%} 0.023 µg/ml. Dua kaedah konvensional iaitu ujian elastase dan gelatin gel digunakan dalam penentuan kadar virulen setiap pencilan. Umumnya, walaupun semua pencilan diasingkan dari lesi skor yang sama, terdapat variasi di dalam ciri makmal. Seseengah pencilan yang mempunyai keupayaan untuk menghasilkan penyakit buruk kuku yang virulen didapati gagal untuk menunjukkan ciri-ciri klinikal bagi buruk kuku virulen berbuat demikian. Keputusan ini mungkin adalah hasil dari pemberian ubatan yang berterusan dan juga program vaksinasi yang telah dijalankan oleh pihak ladang. Tiada plasmid dijumpai di dalam semua 12 pencilan, oleh itu gen-gen yang mengkod kevirulenan bukan terletak di atas plasmid. Pecirian molekular pencilan telah dilangsungkan melalui kaedah analisis elektroforesis pulsed field (PFGE). Corak yang signifikan telah dihasilkan oleh tiga jenis enzim yang kaya dengan bes GC iaitu (*ApaI*, *SfiI* dan *SmaI*). Semua 12 pencilan berjaya di bahagikan kepada lapan jenis genom. Terdapat juga pencilan yang berasal dari tempat yang sama menunjukkan variasi dalam profail corak PFGE.

Keputusan ini menunjukkan kepelbagaian strain *D. nodosus* di Malaysia dan berpunca dari tempat yang pelbagai.

CHAPTER 1

GENERAL INTRODUCTION

Footrot is a contagious disease of ruminants, particularly sheep and goats although cattle and deer may also be affected. It is present worldwide and has a significant economic impact in sheep farming countries with temperate climate and moderate to high rainfall, such as Australia and New Zealand (Stewart, 1989). Footrot is responsible for a 10% production loss in body weight and wool growth, and for an increased cost of treatment and control (Stewart *et al.*, 1984; Marshall *et al.*, 1991; Glynn, 1993). Although several common soil bacteria are involved in the initiation of infection, a Gram-negative, obligate anaerobe, *Dichelobacter nodosus* (formerly *Bacteroides nodosus*) has been shown to be the essential causative agent (Dewhirst *et al.*, 1990).

Footrot varies in its clinical severity depending on the climatic conditions and the virulence of the invading *D. nodosus* strain. *D. nodosus* isolates are classified as virulent, intermediate or benign, although the spectrum of disease manifested is commonly described as a continuum ranging from virulent to benign footrot.



The laboratory diagnosis of ovine footrot currently has depended upon the isolation and identification of *D. nodosus* from footrot lesion material (Skerman, 1989; Pitman *et al.*, 1994). Conventional tests to assess the virulence of the isolate include the elastase test, the gelatin gel test and other assays based on differences in the properties of its extracellular proteases (Stewart, 1979; Kortt *et al.*, 1983; Skerman, 1989; Depiazzi *et al.*, 1991). More recently, molecular techniques have been applied as a useful diagnostic tool for footrot. Polymerase chain reaction (PCR) methods, based on the amplification of the 16S rRNA sequences have been developed and used for the identification of *D. nodosus* isolates (La Fontaine *et al.*, 1993).

There are several options for the control and treatment of footrot. These options include footbathing with antiseptic solutions, parenteral antibiotic therapy and vaccination. However, despite recent advances in the prevention and treatment measures, footrot remains one of the most economically important endemic diseases affecting the sheep industry.

The first case of footrot in Malaysia was detected in early 1994 at the Institut Haiwan Kluang (IHK) farm (Yii, 1995) and the disease is now known to be present in other farms as well. To date, there is only one report on the epidemiology and pathology of ovine footrot in this country (Yii, 1995). Further information on the etiological agent of the

disease is vital to understand the situation in Malaysia enabling formulation of suitable measures to control and if possible to eradicate footrot.

The objectives of this study were:

- (1) to isolate *D. nodosus* from clinical cases of footrot in sheep kept in farms in Malaysia and,
- (2) to identify and characterise *D. nodosus* isolates obtained in Malaysia.

To achieve the above objectives the following experiments were carried out.

- (i) clinical and laboratory diagnostic tests to determine and confirm the occurrence of footrot disease in the farms,
- (ii) virulence assessment tests to determine the capabilities of *D. nodosus* in producing different degrees of footrot,
- (iii) plasmid analysis and pulsed field gel electrophoresis (PFGE) analysis to determine the molecular characteristics of each of the isolates,
- (iv) antimicrobial sensitivity tests to determine the susceptibility of each of the isolates towards several antimicrobial agents for anaerobic bacteria.

CHAPTER 2

LITERATURE REVIEW

Introduction

Ovine footrot is a highly contagious disease characterised by inflammation of the interdigital skin and hoof matrix leading to underrunning and separation of the hoof from the epidermal tissues. The main etiological agent is *Dichelobacter nodosus* (Dewhirst *et al.*, 1990), formerly known as *Bacteroides nodosus*. The disease is present in most countries rearing commercial flocks of sheep and goats. Footrot results in debilitating lameness with marked loss of productivity and reduced market value as it affects wool production, body weight and fertility. It is an economically important disease in some major sheep producing countries world-wide such as Australia and New Zealand. Footrot has been estimated to cost the New South Wales (Australia) sheep industry A\$43 million annually due to additional cost of control and treatment (Egerton and Raadsma, 1993).



Footrot Disease

Historical Background

Footrot is one of the oldest known diseases of sheep and was first described in France by Chabert in 1791. The history of footrot dates back as early as the eighteenth century when it became endemic in Britain. Its presence in France, Germany, USA and Australia was reported in the early nineteenth century, although little was known then about the cause and methods of its spread. At that time, the most common belief was that it occurred spontaneously under wet and lush conditions (Beveridge, 1981). Bacteria comprising the footrot microflora were tested to determine the main causative agent of the disease. Finally, Beveridge (1941) found a non-sporeforming anaerobe, which he named "*Fusiformis nodosus*" (*D. nodosus*). After extensive experimental investigations, *D. nodosus* was concluded to be the primary causative agent of footrot (Marsh and Claus, 1969).

Pathogenesis

The lesions of footrot result from a combined synergistic invasion by several bacteria which individually and separately were incapable of causing footrot. The main bacteria involved in the pathogenesis of footrot are *D. nodosus* and a gastrointestinal inhabitant, *Fusobacterium*



necrophorum, while other environmental bacteria such as *Spirochaeta penortha* would only subsequently invade the primary lesions. *D. nodosus* is now considered to be the main causative agent of footrot. In its absence, footrot lesions do not develop. Experimentally, it is also the only bacteria in the footrot microflora capable of reproducing the disease when applied in pure culture to scarified feet or when injected near the skin-horn junction of the heel (Thomas, 1962). He also concluded that after the injury to the skin-horn junction, *D. nodosus* alone is capable of causing typical severe footrot with progressive separation of the horn of the hoof. *D. nodosus* lives only in diseased hooves and survives no longer than 14 days in faeces, soil or pasture. This ability to survive despite being anaerobic, is probably assisted by some common aerobic bacteria that decreases the oxygen tension in the microenvironment (Laing and Egerton, 1981).

Although *D. nodosus* and *F. necrophorum* work synergistically, mere presence of both bacteria is insufficient to produce the disease. Studies have shown that flocks of sheep where the disease is known to be present, has freedom from lameness during certain time of the year. Further studies subsequently showed that environmental factors play an important role in predisposing footrot outbreaks especially in temperate countries (White, 1991). Footrot spreads under restricted environmental conditions requiring favourable conditions of moisture and temperature. The outbreaks are usually confined to regions that

have a sufficiently high annual average rainfall. Dampness, warm weather and a susceptible host together with a source of infection, favour outbreaks of the disease. It is most common in wet, lush pastures. Wet conditions cause softening of the foot allowing easier access to the invasive bacteria. Moreover, warm conditions facilitate the growth of the bacteria. The disease process begins with the presence of predisposing conditions, whereby *F. necrophorum* will colonise the moist epidermal surface, causing scalding of the interdigital skin. This will then allow *D. nodosus* to penetrate the surface tissue and invade the epidermis of the hoof. Proteases of *D. nodosus* will liquefy the stratum granulosum and stratum spinosum, cleaving the cells in the area and separating the hoof corneum from the basal epithelium. This would give rise to the symptoms of severe lameness and pain, causing the sheep to walk on its knees when only the front feet were affected, or lying prone when all four feet had the condition.

Footrot is transmitted by direct and indirect contacts with *D. nodosus*. Infected feet carry viable *D. nodosus* in cavities, cracks and other deformities of the hooves. Under favourable conditions, the bacteria will multiply in the host and contaminate moist soil, pasture and manure where they may come in contact with other susceptible host. Macerated feet and feet affected with scald causing a moist superficial interdigital dermatitis, are highly susceptible to this pathogen. Mechanical transmission through truck tyres, boots and

others does not occur as in highly contagious viral diseases (Walker, 1988; Stewart, 1989).

Clinical Diagnosis

The initial lesion of footrot is the inflammation of the interdigital skin which may extend abaxially and cause separation or underrunning of the keratin matrix of the hoof. Footrot is accurately diagnosed by location, fetid odour and the characteristic swelling of the interdigital area and the bulbs of the heel. A careful clinical examination will clearly distinguish the disease from other allied infections e.g. foot abscesses, foot and mouth diseases and traumatic injuries. In 1971, Egerton and Roberts used a simple system for scoring the lesions of individual foot. A score of 1 or 2 is given based on the presence and severity of the interdigital skin lesion alone while a score 3 is given if in addition the horn of the hoof has an underrun. A score 4 is given if the underrunning has extended to the abaxial margin of the sole of the hoof. This system was modified by Stewart and others (1982b) in which they subdivided the score 3 into 3a, 3b and 3c according to the degree of underrunning (**Appendix A**). These methods have been adopted by most researchers although further modifications are being proposed (Whittington and Nicholls, 1995).

Laboratory Diagnosis of Footrot

Field diagnosis of footrot can be routinely confirmed by demonstrating the presence of *D. nodosus* in smears from diseased tissues (Marsh and Claus, 1969; Hungerford, 1989). The laboratory confirmation of footrot for regulatory purposes also had depended on microscopic examination of Gram-stained smears for detecting the presence of *D. nodosus* (Stewart and Claxton, 1993). The fluorescent antibody test with lyophilised FITC IgG anti-*D. nodosus* reagent has been developed as an alternative for confirmation of diagnosis. However, because the characteristic morphology of *D. nodosus* in smears is so easily recognised, the latter test is not widely used (Stewart and Claxton, 1993).

Attempts are now made to develop rapid, sensitive and specific diagnostic tests for the detection and identification of bacterial pathogens. The concept of using rRNA sequences as targets in diagnostic tests has been extended to include the use of polymerase chain reaction (PCR) methodology (Cox *et al.*, 1991, Ho *et al.*, 1991). The use of specific oligonucleotides primers makes PCR amplification of the 16S rRNA sequences a highly sensitive and specific method for the detection and identification of bacteria. La Fontaine and co-workers (1993) designed two oligonucleotides primers namely primers A and Ac which were shown to be specific for *D. nodosus*. These primers

amplify a 783 base pair segment of the 16S rRNA and thus allow the detection of *D. nodosus* in cultures or in lesion materials from footrot infected sheep without the need to culture the organism. The PCR amplification of the 16S rRNA genes has provided a highly specific and sensitive method for detecting small numbers of *D. nodosus* cells (less than 10 cells) or 1 fg of *D. nodosus* DNA template (La Fontaine *et al.*, 1993).

Treatment and Control

The control and prevention of footrot relies upon a combination of treatment, vaccination programmes and quarantine regulations (Egerton *et al.*, 1983). There are several options for treatments of this disease. At present, footbathing combined with vaccination is the most widely used method. Footbaths containing antiseptic solution have been used for many years for both preventing and treating footrot. In fact, it is more practical when dealing with a large number of sheep and it is relatively inexpensive compared to other treatments. The effectiveness of footbathing depends on the antiseptic coming in contact with the interdigital skin and the underrunning area, and killing the pathogenic bacteria i.e. exhibiting a bactericidal action. Its success is also a function of time i.e. the speed of the sheep going through the bath. It is also very dependent on good access of the antiseptic to the lesions which is improved through paring. Either 5% formalin solution