Research Article

Characterization of Footrot Bacteria *Dichelobacter nodosus* Using PCR Amplification and DNA Sequence Analysis

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Abstract: *Dichelobacter nodosus* is an essential causative agent of footrot in ruminants, particularly in sheep, goats and cattle. In this study, more than 100 footrot samples were collected from 4 different farming regions in New Zealand (NZ). Selective media were chosen and isolation and routine growth conditions were optimized for NZ *D. nodosus* serotypes. Approximately 1000 primary plates were anaerobically subcultured several times and examined with Gram staining in order to detect single colonies of *D. nodosus*. Both the variable region and a part of the conserved region of fimbrial subunit gene (*fimA*) were amplified from bacterial DNA using polymerase chain reaction (PCR). On the basis of PCR and DNA sequencing, 6 new *D. nodosus* isolates were identified and characterized from NZ. These sequences of *D. nodosus fimA* gene from NZ were genetically different from previously reported strains in the GenBank (NCBI) and commercial vaccine strains.

Key Words: Footrot, *Dichelobacter nodosus*, *fimA* gene, PCR, DNA sequencing

Ayak Çürüğü Bakterisi *Dichelobacter nodosus*'un PCR ve DNA Dizi Analizi Kullanarak Karakterizasyonu

Özet: *Dichelobacter nodosus* özellikle koyun, keçi ve inekler olmak üzere tüm geviş getiren hayvanlarda ayak çürümesi (footrot) hastalığının etkenidir. Bu çalışmada Yeni Zelanda da bulunan dört farklı çiftlik bölgesinden yüzden fazla ayak çürümesi lezyon örneği alındı. Yeni Zelanda *D. nodosus* serotiplerinin izolasyonu ve rutin üreyebilmesi için uygun seçici besiyerleri kullanıldı ve ortam koşulları optimize edildi. Yaklaşık bine yakın petri kabı tek *D. nodosus* kolonisi görülünceye kadar anaerobik koşullarda üretildi ve Gram boyama ile incelendi. Bakteriyal genomik DNA ekstratları kullanılarak *fimA* geninin değişken ve sabit bölümünün bir kısmı polimeraz zincir reaksiyonu (PCR) kullanılarak amplifiye edildi. PCR ve DNA dizi analizlerinden elde edilen sonuçlara bakılarak 6 Yeni Zelanda *D. nodosus* izolatı tanımlandı ve karakterize edildi. Altı yeni *D. nodosus* DNA dizisi, ticari aşı türlerinden ve Uluslararası Gen Bankasında (NCBI) daha önce rapor edilen diğer *D. nodosus* türlerinden genetik olarak oldukça farklı bulunmuştur.

Anahtar Sözcükler: Footrot, *Dichelobacter nodosus*, *fimA* geni, PCR, DNA dizi analizi

Introduction

Footrot is a highly contagious disease of ruminants. It is an acute, sub-acute or chronic interdigital dermatitis which can exhibit a range of clinical signs depending upon such factors as the virulence of the infecting strains of *D. nodosus* (*Bacteroides nodosus*) and pasture conditions (1). Despite its worldwide presence, the disease has significant economic impact in those sheep farming countries (1). Clinical signs include loss of condition and lameness, which results from severe under running of the horn of the hoof and separation of the horn from underlying tissues (2,3). The disease is responsible for decrease in body weight and generating loss production of wool growth in affected animals and increases susceptibility to other infectious diseases (3).

Although several common soil bacteria are involved in the initiation of the infection, a multi-strain, Gram-negative, anaerobic *D. nodosus* has been shown to be the essential causal pathogen (4). The isolation and growing of *D. nodosus* is an extremely difficult and time consuming process, partially because of the fastidious nature of this strict anaerobe, but also because of the

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large number of different bacteria comprising the footrot lesion microflora (5).

Traditionally, the identification of *D. nodosus* has relied on isolation of the bacterium from footrot lesion material and subsequent microbiological and biochemical tests (6,7). More recently, molecular techniques have been applied to footrot diagnosis. PCR based on the *fimA* gene (8) can be used as a practical method for the detection, identification and serotyping of *D. nodosus* isolates.

There have been many studies in which the *fimA* genes from different isolates of *D. nodosus* were sequenced (9-11). However, there are no reports of both the isolation and identification of this bacterium and the sequencing of the *fimA* gene from NZ isolates, although Zhou and Hickford (12) have reported sequences from the *fimA* gene of uncultured NZ footrot samples.

In this study, a number of *D. nodosus* strains from NZ sheep with footrot were isolated and identified using both conventional microbiological and PCR-based molecular techniques. *FimA* sequences of new isolates were cloned and analyzed and their sequence data were submitted to the GenBank (NCBI).

These results indicated that PCR and further molecular techniques are very specific and useful tools for identification and characterization of footrot cultures. Isolation of NZ endemic footrot isolates is very important to diagnose and treat footrot. This study is also the first step to produce a serogroup-specific biotechnological vaccine for the future.

Materials and Methods

Bacterial strains and culture conditions

The *D. nodosus* reference strain no. 25549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

Footrot samples were collected from 4 farming regions in New Zealand (Temuka, Lake Coleridge, Timaru and Canterbury). The cotton ends of the swabs were cut off into 1.5 ml tubes containing 1 ml of phosphate buffered saline (PBS) [145 mM NaCl, 8.7 mM Na_2HPO_4, 1.2 mM NaH_2PO_4, pH 8.0) and 20 mM Na_2EDTA] and into 5 ml bottles containing Thorley's transport medium (13). Samples were also rapidly transferred for processing in the laboratory.

All primary isolates were cultured for 3 to 4 days on Hoof agar (14), and Eugon agar (BBL, Microbiology Systems, MD, USA). Additional agar (2%-4%), yeast extract (2%) and defibrinated sheep blood (5%) (Invitrogen Corporation, San Diego, CA, USA) were added to enhance certain features of the *D. nodosus* colonies. Streaked plates were incubated in anaerobic jars (BBL) at 37 $^{\circ}$C in 10% H_2, 10% CO_2, 80% N_2 and the reaction being catalyzed with aluminum-palladium pellets. The preliminary cultures of *D. nodosus* were examined and checked for purity and confirmed by microscopic examination of Gram-stained smears.

DNA extractions

DNA from swab samples was extracted using a rapid boiling method (15). Genomic DNA from NZ *D. nodosus* strains were isolated from either Eugon agar or Eugon broth cultures by the modified method described by Anderson et al. (16) and Liu et al. (17). Selected single colonies of *D. nodosus* cells were either scraped from agar plates into 2 ml of PBS or grown in 100 ml of TAS (Tripticase-Arginine-Serine) or Eugon broth. Cell pellets were obtained by centrifugation at 13,000 ×g for 10 min. The cells were rewashed in 10 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and resuspended in 4 ml of SET (Sodium-EDTA-Tris) solution (75 mM NaCl, 25 mM EDTA and 20 mM Tris, pH 7.5). Lysozyme (Roche) was added to a final concentration of 4 mg/ml and the cells were incubated on ice for a minimum of 20 min or maximum overnight. Ten percent SDS and 200 mg/ml of proteinase K were added and the tubes were inverted gently and incubated at 55 $^{\circ}$C for at least 2 h with occasional inversion. Subsequently, 1.2 ml of 5 M NaCl and 4 ml of phenol/chloroform (1:1) were added and the contents mixed. The tubes were shaken for 50 s to lyse the bacterial cells. The lysates were then centrifuged at 13,000 ×g for 2 min and the aqueous phase containing the DNA was collected. The crude lysates were extracted with equal volumes of phenol: chloroform (50:50) (twice), phenol: chloroform: isoamyl alcohol (25:24:1), and chloroform: isoamyl alcohol (24:1). The genomic DNA was then recovered by ethanol precipitation and resuspended in 50 to 200 ml of TE buffer.

PCR primers and amplification of the *fimA* gene

Two primer sets were designed to isolate the *fimA* gene from different serogroups and serotypes of *D. nodosus* (Figure 1). The primer binding regions for *D.*

Primer name	Sequence (5' $\longrightarrow$ 3')	Binding region (5' $\longrightarrow$ 3')	Length (b)	% G+C	Tm (^{o}C)
Forward					
U1	ATCCCTGCATACAACGACTACAT	83-105	23	43	48
U2	GCTATTCCACAATACCAAAACTACAT	80-105	26	34	48
Reverse					
D1	ACTCAAGAGAGAGGCTTTTAAGTAAG	427-451	26	38	50
D2	AGAGAGGCTTTCACATTTAAGAGC	398-422	24	41	49
D3	GTACCGAAGTACACCTTTGATTG	423-445	23	43	48

Figure 1. Primers used to amplify the *fimA* gene of *D. nodosus.*

nodosus fimA genes are also shown in Figure 1. Primers were synthesised by GIBCO BRL (Life Technologies, Inc., Rockville, MD, USA).

DNA from bacterial culture was amplified in a 20 ml reaction containing 0.25 mM of each primer, 200 mM of each dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 1 ml of extracted DNA, 1 unit of *Taq* DNA polymerase (Qiagen, Clifton Hill, Victoria, Australia), and 1×PCR buffer (containing 1.5 mM $MgCl_2$). DNA from swab samples was amplified using the same procedure as above, but with the addition of BSA (200 ng/ml) (Sigma Chemical Company, St Louis, MO, USA) and with a higher concentration of $MgCl_2$ (3.5 mM).

Amplifications were carried out in a GeneAmp PCR System 2400 (PE Biosystems, Foster City, CA, USA) and consisted of denaturing at 94 ºC for 2 min, followed by 30 or 35 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 62 ^{o}C for 30 s or 1 min, extension at 72 ^{o}C for 50 s, with a final extension at 72 ^{o}C for 7 min. The PCR amplimers were performed by electrophoresis 1% (w/v) agarose (Seakem¨ LE, FMC, Rockland, Maine, USA) and visualized over a UV transilluminator at 254 nm (UVP White/UV TMW20, San Gabriel, CA, USA).

Cloning of the *fimA* gene

Amplimers (~440 bp) were cloned using the pGEM¨-T Easy Vector System (Promega Corporation, Madison, WI, USA) and then ligated. If primer-dimers or non-specific PCR products were present, the amplimer was isolated and purified using a Qiagen QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). A 2 ml ligation mixture

was used to transform competent *E. coli* cells (Invitrogen™, One Shot™ INVaF'). Several white colonies were picked for each transformation and incubated overnight in Terrific broth (GIBCO BRL, Life Technologies) at 37 ^{o}C in a 225 rpm shaking incubator.

Plasmid isolation and DNA sequence analysis

Plasmid DNA was recovered from bacteria using a Quantum Prep$^{®}$ Plasmid Miniprep Kit (BioRad Laboratories, Hercules, CA). DNA concentration was adjusted to 200 ng/ml.

DNA was sequenced at the Waikato DNA Sequencing Facility, University of Waikato, NZ. Sequencing was performed using M13 forward and reverse primers. To minimize the impact of PCR or sequencing errors, at least 2 independent PCR amplifications from the same colony were used. DNA sequence alignments were performed using DNAMAN™ version 4.0 (Lynnon BioSoft, Canada).

Results

Footrot was seen most frequently from late autumn to early spring in NZ. The interdigital skin of infected feet was usually swollen, very smelly and often covered with a moist film of gray to cream colored necrotic material. Inflammation was usually confined to the posterior half of the interdigital region. Sometimes there was erosion of the adjacent horn, often accompanied by separation. Footrot samples were taken from the deeper part of the lesions that had moisture and smell. Gram-stained slides were examined from approximately 500 footrot samples (Figure 2).
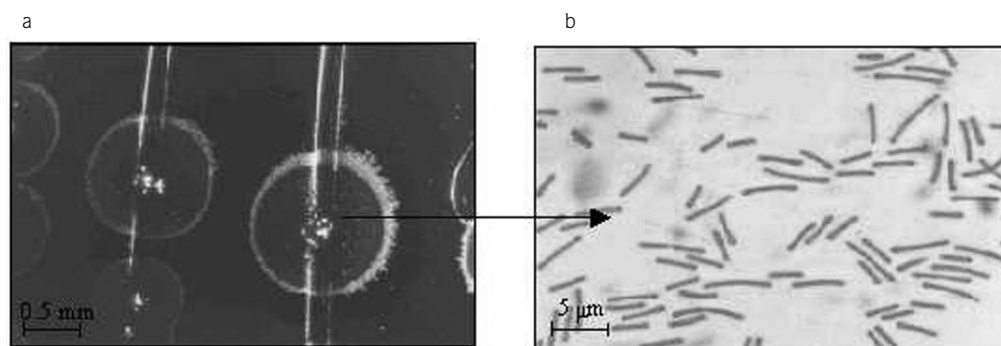
Figure 2. Morphology of *D. nodosus* isolate. a) Stereomicroscope colony morphology of *D. nodosus* from a culture grown on Eugon agar for 5 days (Bar = 0.5 mm). b) Light microscope morphology of *D. nodosus* cells stained by Gram's method (Bar = 5 mm).

It was found that successful isolation of *D. nodosus* from infected hoof tissue could only be achieved after the rapid transportation of field samples to the laboratory. The isolation from field samples was inhibited by the numerous contaminating organisms from the soil environment and excessive moisture in anaerobic jars. Therefore, the dilution of samples in PBS or 0.25 M sucrose before culturing, and adding extra desiccant such as dry silicon particles to the jars was necessary for the first inoculation of *D. nodosus* colonies, especially with heavily infected footrot samples.

Repeated subculturing on solid medium was invariably required to isolate single *D. nodosus* colonies. Colonies appeared as flat concentric zones with a finely granulated surface texture. They were colorless on the agar surface and varied in size from 0.5 to 3 mm in diameter with irregular margins. Colonies were hardly visible to the naked eye after 3 days of incubation.

DNA extracts from swab samples and cultured *D. nodosus* isolates were subjected to PCR amplification using specific primers. An amplimer of ~ 440 bp was obtained from each swab samples and cultured *D. nodosus* extract. The amplimers for each sample were compared with positive (*D. nodosus* A198) and negative controls (*P. aeruginosa*). Typical results are shown in Figures 3 and 4.

DNA sequencing results observed that 6 strains were different from previously published NZ strains. These 6 new *fimA* sequences from NZ isolates were submitted to GenBank (NCBI) and the following accession numbers recorded: A-NZ$_2$ (AY835827), B$_1$-NZ$_1$, C-NZ$_3$ (AY835829), E-NZ$_2$ (AY835831), E-NZ$_3$ (AY835832), F-NZ$_2$(AY835835).

The predicted amino acid sequences of *fimA* from new strains were aligned with other published amino acid sequences of *fimA* obtained from the GenBank (NCBI) (Figure 5). It was revealed that there were several sequence differences between the NZ and Australian strains. These sequence differences were confirmed by duplicating the PCR amplification, cloning and sequencing.
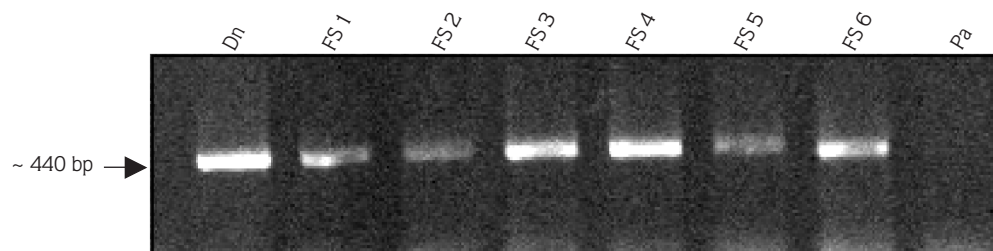

Figure 3. PCR amplimers of *D. nodosus* from field sample. Lane 1 is positive control *D. nodosus* A198 (Dn), Lanes 2 to 7 are from different field samples and Lane 8 is negative control *P. aeroginosa* (Pa). The size of PCR products was ~ 440 bp.
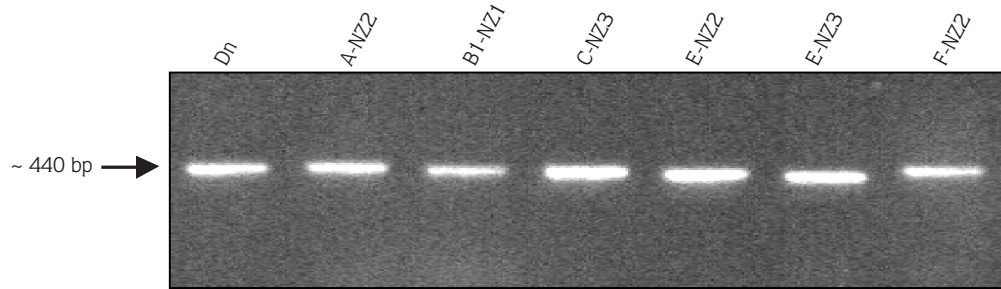
Figure 4. PCR amplimers of cultured *D. nodosus* isolates. Lane 1 is a positive control *D. nodosus* A198 (Dn), and Lanes 2 to 6 are from different isolates from NZ. The size of PCR products was ~ 440 bp.

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A1-VCS1001  DYIARSQAAEGLTLADGLKVRISDHLESGECKGDANPASGSLGNDDKGKYALATIDGDYNKDAKTADEKNGCKVVITYGQGTAGEKISKLIVGKKLVLDQ  100
A-SPAHL     ------------------------------------------------------------------------------------------------    100
A-NZ1       ----------------------------e---a-v-------------s----d----n--d--------n-------------------------    100
A-NZ2       ---------.---------------k---------------------p-----------------------------------------f-------   99

B1-AC293    DYIARSQAAEGVSLADGLKVRIAENLQDGECKGPDADPQSGVVGNEDKGKYGLAKIEGDYDASKTEAGDPNGCKVEITYGQGTAGDKISKLITGKKLVLD   100
B1-SPAHL    -------------------------------------------k-t---a--e-d-t------a---------n-------a--------------    100
B-NZ1       ------------------------------g-----------a------------d--a---------------eg-------------    100
B-NZ2       ------------------------------------------a------------d--a---------------eg-------------    100
B1-NZ1      -------------------------------------------------n--------------------------    100

C2-VCS1617  DYIARTQVSEGVSLADGLKIRIADNLQDGDCVTKGDSSTGEVGNEDKGKYALATILGTPAQNLSELKAEDPNGCQVKIEYGKGTSGGSVSALINNTELVL   100
C1-SPAHL    -------------------------------t----a---------------e----a--------ek---l------------------    100
C-NZ1       -------------------------------t----a---------------e----a--------ek---l------------------    100
C-NZ2       ------------------------------------------------------------------------------------n-----------    100
C-NZ3       ----------------------------------a-----------------e-----------ek--wl-----------n-----------    100

E1-VCS1137  DYIARSQAAEGLTLADGLKIRIADHLENGSCTEDANAGAGEKGNEDKGKYALAVIEGDYAQNATDLKPEDKNGCKVVITYGQGTAGSKISKLIDTKVLEL   100
E2-VCS1114  -------------------------------------------------q-t------e-g-t---d--n---------t-t----k----a------------   100
E-SPAHL     -------------------------------k----------------------------------------------------------    100
E-NZ1       -------------------------------k----------------------------------------------------------    100
E-NZ2       ..------------------------------------..--------q-t------e-g-t---d--n---------t-t-a--k----p------------   96
E-NZ3       ..------g----------------------------..--------q-t------e-g-t---d--n---------t-t----k----p------------   96

F1-VCS1017  DYIARSQAAEGLTLADGLKIRIADHLENGSCMETANAGAGEKGNQDIGKYGLAEISGDYDESKTDAKDENGCKVTITYGQGTAGEKVSKLIKGKTLILLQ   100
F-SPAHL     -----------------------------------------------------------------------------------------------h-    100
F-NZ1       -----------------------------------------------------------------------------------------------h-    100
F-NZ2       -.--------------.--------------------------------------------------s---i----------------------h-    98
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Figure 5. Comparison of the predicted amino acid sequences of *D. nodosus fimA* sequences from NZ strains and other published sequences. Identical regions of sequences are shown with dashes and sequence variations are indicated with lower case letters.

Discussion

This paper describes the isolation, culturing, identification, and cloning of *D. nodosus* isolates from footrot samples from NZ.

In our observation it was not possible to grow *D. nodosus* on plates, if the footrot samples on swabs had remained in transport media, or on the laboratory bench longer than 2-3 h. However, Skerman (6) reported that fully grown *D. nodosus* cultures would tolerate limited exposure to air and that plates may be left on the bench for up to about 12 h without significant viability being lost.

D. nodosus grown on Hoof agar plates was particularly slow-growing and very difficult to visualize, due to the colorless nature of the colonies. This observation is consistent with that of Egerton and Parsonson (18). Thus, Hoof agar was not the best medium for initial inoculations. Eugon agar was however easy to prepare and the color derived from added defibrinated blood allowed easier visualization of the colonies. A higher concentration of agar was used (4% and 5%), which enhanced development of *D. nodosus* colonies from the first inoculation, as well as suppressing the spreading growth of some contaminants. There was no obvious relationship between apparent virulence

assessed at collection, colony type and the degree of fimbriation in cultured *D. nodosus* isolates.

Traditionally, the isolation and definitive identification of *D. nodosus* from footrot microflora is difficult and requires Gram stains and other laboratory tests such as the elastase and the gelatin tests (19). The results presented in this study indicate that *fimA*-specific PCR can be used to quickly and easily detect *D. nodosus* in DNA obtained from footrot lesions, or plates.

On the basis of DNA sequencing, 6 new *D. nodosus* strains (serogroups A, B, C, E, and F) belonging to 8 different serogroups were detected in this research. Isolates from serogroups D, G, H and I were not found in this study. Kingsley et al. (20) reported that serogroup D was the second most common serogroup in New Zealand. This study found that serogroup E was the most common and was present in around 30% of the samples taken from the 4 New Zealand farms. According to our findings the second most common serogroup was serogroup B and the third was serogroup A. These findings are consistent with Chetwin et al. (21), who supported the finding that serogroup E occurs in around 20% of New Zealand flocks. Coincidentally, serogroup E was the most common pathogenic serogroup in Nepal, with a sheep

industry that originated from imported rams from New Zealand (22,23), while serogroup E is relatively uncommon in Australia (24), the United Kingdom (20,25) and the United States of America (26). The 6 new *D. nodosus* strains from New Zealand were genetically different from previously reported strains. These differences could have been the result of errors introduced by *Taq* polymerase upon amplification. However, sequencing from independent PCR amplifications from the same colony confirmed the DNA sequences, suggesting that this difference reflects variation in the gene, rather than amplification or sequencing errors.

Acknowledgments

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