

Serotyping *Dichelobacter nodosus* with PCR-SSCP

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Abstract: In this study, we reported rapid approach for identification of *D. nodosus* serotypes that causes footrot, highly contagious diseases of the ruminants in worldwide with using Polymerase Chain Reaction-Single Strand Conformational Polymorphism (PCR-SSCP) analysis. A total of 500 swab samples from infected hoof lesion were investigated for presence of *D. nodosus* by PCR amplification of *fimA* gene. PCR-SSCP patterns revealed that each sample contained two or three different *D. nodosus* serotypes. Additionally on the basis of SSCP patterns, 8 different serogroups (A- G and I) were identified from New Zealand samples. These findings were confirmed by cloning and sequences of *fimA* gene.

Key words: *Dichelobacter nodosus*, *fimA* gene, PCR, Single Strand Conformational Polymorphism (SSCP), sequencing, sample

INTRODUCTION

D. nodosus is an anaerobe, fastidious, slow growing and gram negative bacterium (Stewart, 1989). *D. nodosus* has a number of serogroups based on its fimbrial antigens (Whittington *et al.*, 1997; Moore *et al.*, 2005). The current Australian classification systems classifies *D. nodosus* into at least 10 serogroups (A-I and M) (Claxton *et al.*, 1983; Ghimire *et al.*, 1998). Traditionally, identifying and characterization of *D. nodosus* serotypes have required sample collection from infected hooves followed by careful and rapid transport of lesion material in specialized media to the laboratory.

Following this, specific plating media are used to grow the *D. nodosus* (Stewart *et al.*, 1986). This is then followed by streak plating to obtain single colonies and subsequent analysis of both the morphology of these single colonies and antigenic analysis with serological methods which could take 3-4 weeks to verify the presence of *D. nodosus* (Hindmarsh and Fraser, 1985; Depiazzi *et al.*, 1991; Gradin *et al.*, 1993). Previously, PCR has been used in identifying bacteria based on the amplification of the *fimA* gene (John *et al.*, 1999; Dhungyel *et al.*, 2002; Myers *et al.*, 2007) and *16S rDNA* gene (Zakaria *et al.*, 1998; Wani *et al.*, 2007; Zhou *et al.*, 2010). While this gives a more definitive identification of *D. nodosus* and down to the level of individual isolates, the approach is still reliant upon the retrieval and culture of live cells, something that is both time-consuming and technically costly. Moreover, the observation that multi-strains of *D. nodosus* occur on a single hoof means that

multiple plates need to be screened to be sure that all the strains present on a footrot infected hoof are identified. The objective of present investigation is describe a rapid approach that involves the PCR-SSCP-based direct analysis of field samples collected off footrot infected hooves and without the need for live cell retrieval, culturing or classical morphological identification for serotyping *D. nodosus*.

MATERIALS AND METHODS

Sample collection: Samples were collected from footrot infected hooves using swabs to scrape the axial skin-horn junction of the hoof. The ends of the swabs were cut off into 5 mL screw-capped Bijou bottles containing Stuart's transport medium (Skerman, 1989) and transferred to the laboratory for DNA extraction. Reference *D. nodosus* 25549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cultures of 12 *D. nodosus* strains from serogroup A-M were obtained from Shearing-Plough Animal Health Ltd. (SPAHL), Upper Hutt, New Zealand.

DNA extraction and PCR amplification: DNA from field swabs and reference cultures were extracted as described by Cagatay and Hickford (2005). The *D. nodosus fimA* genes was amplified, using of mixture of primers sets (fU1, fU2, rD1-rD3) as described previously by Cagatay and Hickford (2006). Amplicons were visualized by electrophoresis in 1% agarose (BioWhittaker Molecular Applications, Rockland, ME) gels, using

1×TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM Na₂EDTA) containing 200 ng of ethidium bromide mL⁻¹.

SSCP analysis and sequence analysis: A 2 µL of each amplicon was mixed with 7 µL of loading dye (95% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) and after denaturation at 95°C for 5 min, samples were rapidly cooled on wet ice and then loaded to 16×18 cm, 8% acrylamide: bisacrylamide (49:1; Bio-Rad Laboratories, Inc., Hercules, CA, USA) gels.

Electrophoresis was performed using protean IIxi cells (Bio-Rad) at 220 V for 16-18 h at 4°C in 0.5×TBE buffer and gels were silver-stained (Bassam *et al.*, 1991). Amplicons representative of unique PCR-SSCP patterns were ligated into the Invitrogen™, One Shot™ INVαF system (Invitrogen, USA). The ligation mixture were used

to transform competent *E. coli* cells following the manufacturer's instructions. Fifteen insert positive colonies for each transformation were picked and incubated overnight in Terrific broth (Invitrogen) at 37°C in a shaking rotary incubator. Amplicons from these clones and the corresponding genomic DNA were run adjacent to each other on SSCP gels for comparison. Clones where the SSCP pattern matched those of the corresponding genomic *D. nodosus* DNA were selected for DNA sequencing. Sequencing was performed as described previously by Cagatay and Hickford (2006).

RESULTS AND DISCUSSION

About 500 potential swabs were examined with gram stain and then PCR for presence of *D. nodosus*. DNA from positive samples were typically revealed an amplicon of approximately 440 bp of the *fim4* gene (Fig. 1a-c). A 112

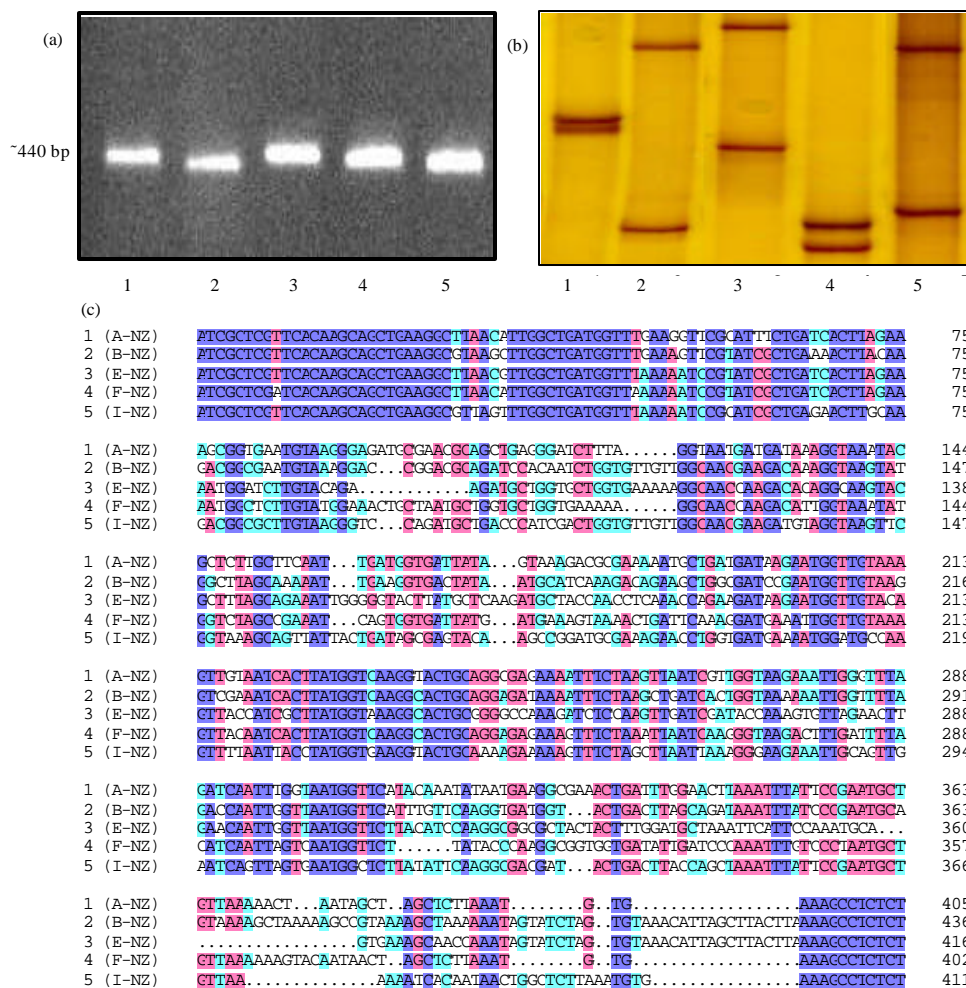


Fig. 1: a) Five different PCR products in agarose revealing minor variation in amplicon length; b) the amplicons show different PCR-SSCP patterns on an 8% polyacrylamide gel; c) PCR products were cloned and sent for sequencing. Sequences show the variable region of the *fim4* gene for sample 1-5

Table 1: Footrot Samples (FS) and serotypes detected

Sample name	1st examination of swab		No. of cloned amplimers examined	No. of unique strains identified by PCR-SSCP	Serotypes identified by DNA sequencing
	Gram strain	PCR			
FS-1	+, -	+	10	4	A, F
FS-2	+, -	+	7	2	E, F
FS-3	+	+	4	1	E, F
FS-4	+	+	5	2	B, I
FS-5	+, -	+	5	1	I
FS-6	+	+	10	3	B
FS-7	+, -	+	6	6	B, C, G, M
FS-8	+, -	+	10	5	A, B, E, F, I
FS-9	+	+	5	1	I
FS-10	+, -	+	5	1	B, F
FS-11	+	+	15	4	B, E
FS-12	+, -	+	5	4	E, E
FS-13	+, -	+	10	3	C, M
FS-14	+, -	+	5	2	F, E
FS-15	+	+	10	7	B, C, F, M

DNA amplicons were cloned and screened and 52 of these were chosen following PCR-SSCP (Table 1). The PCR-SSCP patterns generated from those samples suggested that they contained at least two or three different *D. nodosus* strains that were different to those previously identified New Zealand strains. The new strains were from serogroups A-I (Fig. 2). These PCR-SSCP pattern differences were investigated in more detail. Five similar size *D. nodosus* amplicons were analyzed confirmed by replicating the PCR amplification (Fig. 1a).

The amplicons producing each of these five PCR-SSCP patterns (Fig. 1b) were chosen for subsequent cloning and DNA sequencing and this confirmed that the *fimA* gene sequences from each of the colonies were different (Fig. 1c). The *fimA* gene inserted from clone one showed a high degree of sequence homology to the *fimA* sequence of serogroup A whereas clones 2-5 showed a high degree of sequence homology with *fimA* from serogroups B, E, F and I, respectively. Traditionally, the isolation and definitive identification of *D. nodosus* serotypes from footrot microflora is difficult and requires morphological characterisation of both the bacterium and colonies, gram staining and the use of other laboratory tests such as the elastase (Links and Morris, 1996) and gelatin tests (Palmer, 1993).

DNA sequencing can assist this identification but single colonies are needed prior to using this approach. Here, there is reveal how *fimA* gene specific PCR and SSCP can be used to quickly and easily detect and differentiated *D. nodosus* in DNA obtained directly from footrot lesions and without plating or colony isolation. The existence of considerable structural variation in the fimbrial gene of *D. nodosus* is well known (Ghimire *et al.*, 1998; Gurung *et al.*, 2006). The results confirmed that as little as a single nucleotide difference in the *fimA* sequences of PCR amplimers can be detected by PCR-

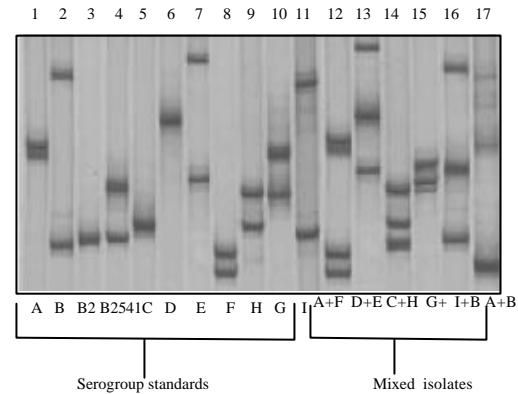


Fig. 2: PCR-SSCP analysis of the *fimA* gene of *D. nodosus*. Each line presents one genotype of *D. nodosus*. Lanes 1-11 are different serotypes from the control strains of *D. nodosus*. Lanes 12-17 represent mixed samples

SSCP. It is therefore, a simple and powerful way to identify, characterize and serotypes new *D. nodosus* with less time consuming methods. On the basis of PCR-SSCP and DNA sequencing, sixteen new *D. nodosus* strains belonging to eight different serogroups were detected in this research. Strains from serogroups H was not detected which contrasts the findings of Kingsley *et al.* (1986) who reported that serogroup D and H were the 2nd most common in New Zealand.

Moore *et al.* (2005) reported that serogroup H has retained its position of importance in England and Wales over the last 20 years. Thorley and Day (1986) who investigated 58 farms around the UK also identified serogroup H to be dominant. In contrast this study found that serogroup E was the most common serogroup in New Zealand and was present in around 30% of the samples taken from the four New Zealand farms. In the findings, the 2nd most common serogroup was serogroup B and the 3rd was serogroup A. These findings are consistent with those of Chetwin *et al.* (1991) who also reported the finding that serogroup E occurs in around 20% of New Zealand flocks. Coincidentally, serogroup E is the most common pathogenic serogroup in Nepal with a sheep industry that originated from imported rams from New Zealand. Serogroup E is reportedly uncommon in Australia (Claxton *et al.*, 1983), the United Kingdom (Kingsley *et al.*, 1986) and the United States of America (Gradin *et al.*, 1993).

Additionally, a single swab confirmed the presence of multiple strains of *D. nodosus* on a single hoof. The finding of multiple strain infections on the same hoof is supported by other research. Gradin *et al.* (1993) reported the presence of one or two serotypes of

D. nodosus on individual sheep/goat hooves. In the survey of Claxton *et al.* (1983), up to 4 serogroups per hoof were isolated from 38 out of 292 sheep and he also suggested that the more colonies that were examined per foot, the larger the number of serogroups that were detected. Moore *et al.* (2005) has reported that mixed serogroup infections are occur in both flocks and on individual feet.

CONCLUSION

Samples taken from the same farm and same sheep but different feet were found to contain different *D. nodosus* strains. This implies that the virulence of a *D. nodosus* strain varies for individual sheep or that the interaction between the host, pathogen and environment is complex. If this is the case then previous attempts to determine and classify the virulence of *D. nodosus* strains by both *in vitro* and *in vivo* investigations have possibly over-simplified the complexity of footrot as a disease.

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