

Virulence Characterization of *Dichelobacter nodosus* from Clinical Cases of Ovine Footrot

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ABSTRACT

Dichelobacter nodosus from ovine footrot was characterized for *pgr* and *intA* genes. Out of 260 samples collected from clinical cases of ovine footrot only 107 were found positive for *D. nodosus* by species-specific 16S rRNA-PCR. The overall prevalence of footrot based on clinical symptoms was estimated to be 10.57%. Serological diversity studies based on *fimA* gene revealed the presence of two serogroups of *D. nodosus* viz. B and E only. Out of 107 *D. nodosus* positive samples, 101 (94.39%) were positive for serogroup B, while remaining six (5.60%) samples showed the presence of serogroup E. None of the samples revealed mixed infection with two or more serogroups. The samples positive for 16S rRNA were subjected to detection of the *intA* genes. A total of 67 (62.61%) samples were found to carry the *intA* gene. Since 63.36% of serogroup B positive samples and 50.00% of serogroup E positive samples carried *intA* gene, there appeared to be no bias towards either of the serogroups. Ten *intA* positive and 10 *intA* negative samples were randomly selected and subjected to detection of *pgr* genes. *pgrA* gene was present in all the *intA* positive and *intA* negative samples screened, suggesting no correlation between the occurrence of the *intA* gene and *pgrA* variants. However, *pgr* analysis appeared much more reliable than *intA* gene analysis for detection of virulent strains of *D. nodosus*. Considerable heterogeneity in *pgrA* variants offers significant potential as a molecular tool in future epidemiological studies.

Keywords: *D. nodosus*; Serogroups; *intA* gene; *pgrA* gene; Footrot; Sheep; Goats; Caprine; Ovine.

INTRODUCTION

Footrot caused by *Dichelobacter nodosus* (*D. nodosus*) is a highly contagious disease of sheep and goats characterized by lameness, ulceration and sometimes separation of the hoof from the foot. Although the disease is of world-wide distribution, areas with temperate climate are often affected with significant impact on sheep farming. The clinical manifestations of footrot are dependent on both environmental conditions and the virulence of *D. nodosus* strain (1). Laboratory tests like protease thermostability /gelatin-gel test (2), detection of *intA* (3) and detection of genomic islands *vap* and *vrl* (4) have been developed to classify virulence of *D. nodosus* isolates. But these tests do not always correlate, suggesting

that either they do not test the absolute markers for virulence or that virulence is complex and linked to more than one process. However, only recently a gene encoding a putative large, repetitive secreted protein designated as *pgr* (proline-glycine repeats) was found in virulent strains of *D. nodosus*, suggesting that this gene might encode a virulence factor that could be involved in adhesion to the extracellular matrix (5). Recently, Calvo-Bado *et al.* (6) determined the diversity of the *pgr* gene and reported its two variants, *pgrA*, and *pgrB* based on sequence analysis of the genes.

The aim of the current study was to determine the prevalence of footrot in sheep, to characterize *D. nodosus* on the basis of virulence markers viz. *intA* gene and *pgr* gene

variants, correlate these two and to determine a better marker for virulence determination in *D. nodosus*. Moreover, serogrouping was also carried out to ascertain the predominant serogroup in Kashmir.

MATERIALS AND METHODS

A total of 2458 sheep from private flocks of south Kashmir were clinically screened for footrot and 260 swab samples were collected randomly from clinically affected sheep. The samples were collected from active lesions that developed between the horn of the hoof and the sensitive underlying tissues.

DNA Extraction

Suspension of the material present on the swabs was made in 100 µl of sterile phosphate buffered saline (PBS) using 1.5 ml tubes by gentle vortexing. The suspension was allowed to boil for 5 min followed by chilling on ice for 10 min and centrifugation at 10,000g for 1 min. The supernatant was used as DNA template for PCR reaction.

Detection of 16SrRNA gene by PCR

PCR amplifications were performed in 25 ml in 0.2 ml thin walled PCR tubes (Eppendorf, Germany). The PCR mixture contained a final concentration of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 3 mM MgCl₂, 0.5 mM concentration of each primer, 0.2 mM concentrations of each 20-deoxynucleo-

side 50-triphosphate and 1.0 U of Taq DNA polymerase (Promega Madison WI, USA). Oligonucleotide primers (Table 1) were also procured from Chromos Biotech Pvt. Ltd, Bengaluru, India.

The amplification cycles in a thermal cycler (Eppendorf, Germany) was carried out as per the cycling condition described earlier (7). Two microliters of sterilized distilled water served as negative control. The PCR products were electrophoresed on 0.8% agarose gels, stained with ethidium bromide and visualized under ultraviolet (UV) illumination and photographed with gel documentation system (GDS 8000 system, UVP, UK).

Serogrouping of *D. nodosus*

Samples positive by 16S rRNA were subjected to multiplex PCR using *fimA* gene specific common forward primer and nine (A-I) serogroup specific reverse primers (8). All the conditions were kept similar to those for 16S rRNA except an increased concentration of forward primer (2.5 times) as compared to reverse primers. The nucleotide sequence of primers used for serogrouping is provided in Table 2.

Detection of Virulence associated *intA* Gene

Samples positive for 16S rRNA were screened for the presence of *intA* gene as described previously (3) with minor modifications. The concentration of primers (5' ACA TCA TGC GAC TCA CTG AC 3' and 5'TCT CTG GTC GGT

Table 1: Details of primers used for detection *D. nodosus*.

Primer name	Primer sequence (5' - 3')	Target gene	Reference
Forward	CGGGGTATGTAGCTTGC	16S rRNA	La Fontaine <i>et al.</i> (7)
Reverse	TCGGTACCGAGTATTTCTACCCAACACCT		

Table 2: Details of primers used for serogrouping of *D. nodosus*

Primer name	Primer sequence (5' - 3')	Target gene	Serogroup	Reference
FP	CCTTAATCGAACTCATGATTG	<i>fimA</i>	-	Dhungyel <i>et al.</i> (8)
RA	AGTTTCGCCCTTCATTATATTT	<i>fimA</i>	A	
RB	CGGATCGCCAGCTTCTGTCTT	<i>fimA</i>	B	
RC	AGAAGTGCCTTTGCCGTATTC	<i>fimA</i>	C	
RD	TGCAACAATATTTCCCTCATC	<i>fimA</i>	D	
RE	CACTTTGGTATCGATCAACTTGG	<i>fimA</i>	E	
RF	ACTGATTTCCGGCTAGACC	<i>fimA</i>	F	
RG	CTTAGGGGTAAGTCCTGCAAG	<i>fimA</i>	G	
RH	TGAGCAAGACCAAGTAGC	<i>fimA</i>	H	
RI	CGATGGGTGAGCATCTGGACC	<i>fimA</i>	I	

CGT ACA AT 3') was 0.5 mM while the amplification was carried out at initial denaturation at 94°C for 2 min followed by 31 cycles of denaturation at 94°C for 1.5 min, annealing at 60°C 1 min and extension at 72°C for 2 min. Final extension was carried out at 72°C for 5 min.

The amplicons obtained by using *intA* gene primers were purified using MinElute PCR Purification Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions and were sequenced commercially by Chromous Biotech, Bengaluru, India.

PCR assay to detect *pgrA* and *pgrB* variants

Twenty samples (ten *intA* positive and ten *intA* negative) were randomly selected and subjected to PCR for detection of *pgrA* and *pgrB* variants as described (6) using a final concentration of 10mM Tris-HCl, 50mM KCl, 3mM MgCl₂, 0.2mM concentrations of each dNTP and 1U of *Taq* DNA polymerase (Promega Madison WI, USA). The amplification was carried at initial denaturation at 95°C for 2 min followed by 34 cycles of 95°C for 1 min, 60°C (for *pgrA*) 55°C (*pgrB*) for 45 sec and 72°C for 2 min. Final exten-

sion was carried out at 72°C for 5 min. The concentration of *pgrA* primers (5-CCTGCACCATGCTTGTAAA-3 and 5-GCTGTTGGTGGTTTGG CTAT-3) and *pgrB* primers (5-AKCATCRGGAAAGGTGA-3 and 5-GACGGCATCAGCAGCA-3) was 0.5 mM.

The amplicons obtained by using *pgrA* gene primers were purified and sequenced commercially.

RESULTS AND DISCUSSION

Out of a total of 2458 sheep screened, 260 exhibited clinical signs characteristic of footrot thus indicating an overall prevalence of 10.57%. However, a lower prevalence has been reported in the United Kingdom (9, 10) and Bhutan (11). Out of these 260 samples only 107 were found positive for *D. nodosus* by PCR (Figure 1). The probable reason for this low detection rate by PCR could be due to the crude method of DNA extraction or because the samples were taken from animals in the process of healing. Moreover, the samples could not be taken from deeper areas of skin and hoof where anaerobic conditions favor the growth of *D. nodosus*.

The predominant serogroup was serogroup B found in

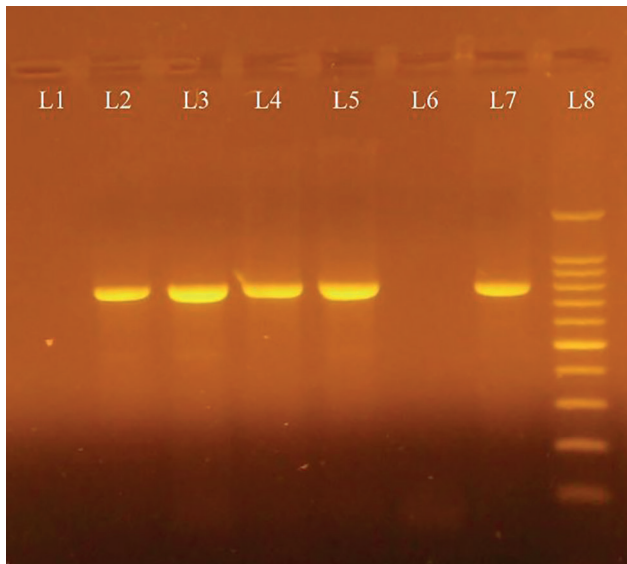


Figure 1: PCR Gel Image carried out for the detection of *D. nodosus* based on species-specific 16S rRNA gene. L1: Negative control having distilled water as template, L2-L5: foot-rot suspected samples taken from clinically affected animals showing an amplified product of 783 bp. L6: A foot-rot negative sample, L7: A Positive control having reference *D. nodosus* culture, Lane 8: 100 bp ladder.

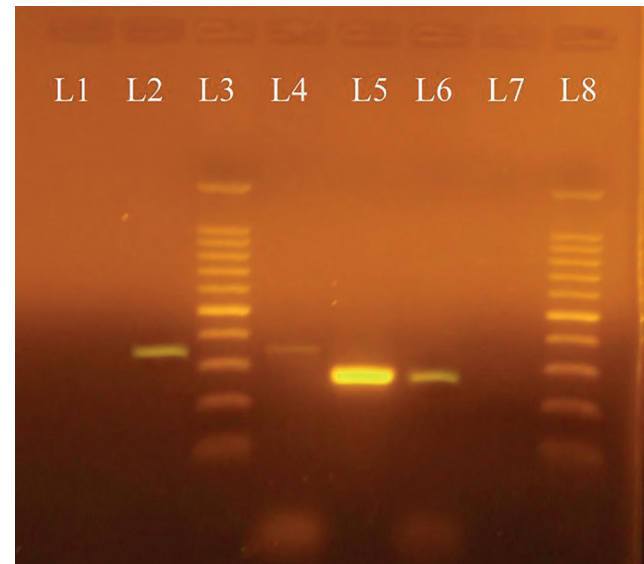


Figure 2: Multiplex PCR gel image for serotyping of *D. nodosus* based on *fimA* gene. L1: A Negative control for serogroup B having distilled water as template. L2: A serotype B Positive reference *D. nodosus* culture. L3: 100 bp ladder, L4: A foot-rot sample showing 325 bp product specific for serogroup B of *D. nodosus*, L3 & L8: 100 bp ladder, L5: A serotype E Positive reference *D. nodosus*. L6: A foot-rot sample showing 283 bp product specific for serogroup E of *D. nodosus*. L7: Negative control for serogroup B having distilled water as template.

101 (94.39%) samples. The only other serogroup detected was serogroup E (5.60%) (Figure 2). However, serogroup G and serogroup I could not be detected in the present study which is in contrast to studies carried out by Rather *et al.* (12). The predominance of serogroup B has been reported in various countries viz. Australia (13), Britain (14), New Zealand (15) and Bhutan (11). None of the samples were found positive for more than one serogroup which is quite contrary to a previous report (12) which reported the occurrence of a mixed infection in 31% of samples.

Out of 107 PCR positive samples, 67 (62.61%) were positive for *intA* gene. Similar results have been found by Rather *et al.* (12) who reported that 61.88% isolates from central Kashmir were *intA* positive thus designated as virulent. The distribution *intA* gene in both the serogroups (B and E) was almost equal, thus indicating no bias towards any serogroup. PCR-based test for the detection of *intA* was developed and validated by Cheetam *et al.* (16) who reported having designed primers to amplify a 530 bp fragment of the *intA* gene. However, we used the same pair of primers and could amplify only 501bp amplicon as shown in Figure 3. (GenBank accession number JN574475).

The diversity of *pgr* gene was studied with the aim to exploit it for detection of benign and virulent strains of *D.*

nodosus and for further comparison with *intA*. Out of twenty samples (10 *intA* positive and 10 *intA* negative) selected randomly, all were positive for *pgrA* as shown in Figure 4 and none could be found positive for *pgrB*. Unexpectedly the *pgrA*, variant was detected equivocally from all the *intA* positive and *intA* negative samples. Thus no conclusion of a correlation between the occurrence of *intA* gene and *pgrA* variant could be made.

Since all the samples were taken from clinically affected animals exhibiting a virulent form of the disease and 37.31% samples revealed the presence of *pgrA* gene without the *intA* gene, it can be concluded that *pgr* analysis is a much more reliable parameter than *intA* gene as far as the virulence of *D. nodosus* is concerned. Furthermore, we recorded considerable heterogeneity in the *pgrA* variant thus offering significant potential as a molecular tool in epidemiological studies. (GenBank accession no of *pgrA* gene JN601141)

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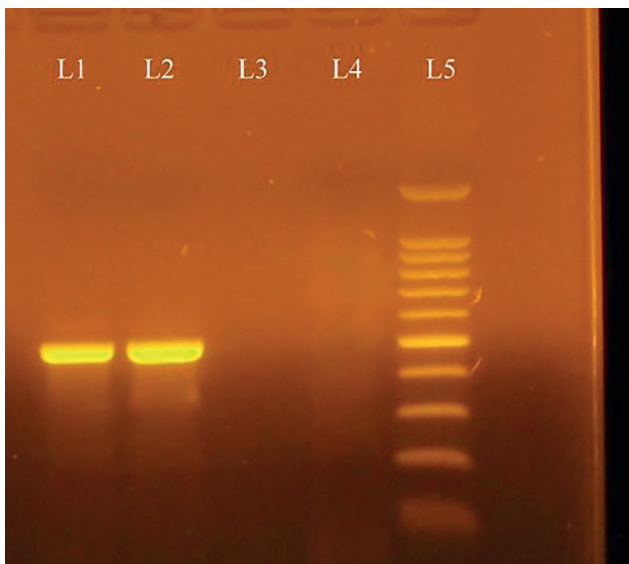


Figure 3: Gel image of *intA* gene PCR of *D. nodosus*. L1: A positive reference *D. nodosus* showing amplified product of 501 bp representing *intA* gene, L2: A footrot clinical sample showing presence of *intA* gene (501 bp), L3: A Negative control having distilled water as template, L4: a footrot sample not having *intA* gene, L5: 100 bp ladder.

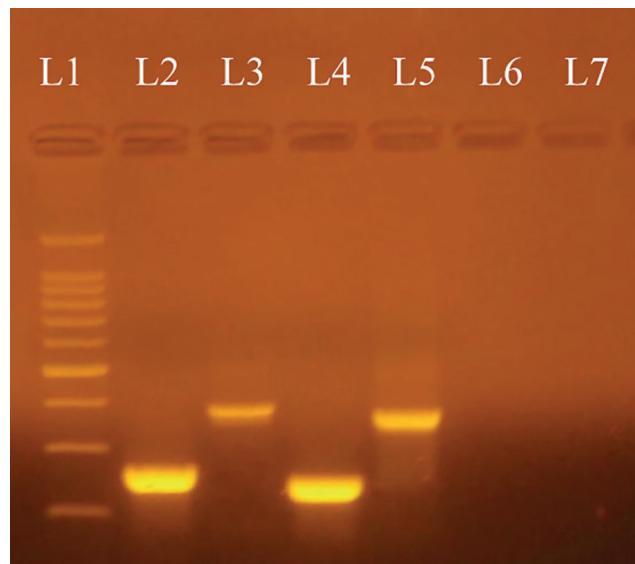


Figure 4: Gel Image of *pgrA* gene PCR for *D. nodosus*. L1: 100 bp ladder. L2 & L3: A positive reference culture showing variable size of *pgrA* gene, L4 & L5: two footrot samples showing the variability in size of *pgrA* gene. L6: A Negative Control having distilled water as template.

REFERENCES

1. Depiazzi, L. J. and Richards, R. B.: Motility in relation to virulence of *Bacteroides nodosus*. *Vet. Microbiol.* 10: 107-116, 1985.
2. Palmer, M. A.: A gelatin test to detect activity and stability of proteases produced by *Dichelobacter (Bacteroides) nodosus*. *Vet. Microbiol.* 36: 113-122, 1993
3. Cheetham, B. F., Tanjung, L. R., Sutherland, M., Druitt, J., Green, G., McFarlane, J., Bailey, G. D., Seaman, J. T. and Katz, M. E.: Improved diagnosis of footrot using *intA* gene. *Vet. Microbiol.* 116: 166-174, 2006.
4. 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.: Comparison of gene probe and conventional methods for the differentiation of ovine footrot isolates of *Dichelobacter nodosus*. *Vet. Microbiol.* 52: 127-141, 1996.
5. Myers, G. S., Parker, D., Al-Hasani, K., Kennan, R. M., Seemann, T., Ren, Q., Badger, J. H., Selengut, J. D., Deboy, R. T., Tettelin, H., Boyce, J. D., McCarl, V. P., Han, X., Nelson, W. C., Madupu, R., Mohamoud, Y., Holley, T., Fedorova, N., Khouri, H., Bottomley, S. P., Whittington, R. J., Adler, B., Songer, J. G., Rood, J. I. and Paulsen, I. T.: Genome sequence and identification of candidate vaccine antigens from the animal pathogen *Dichelobacter nodosus*. *N. Biotechnol.* 25: 569-575, 2007.
6. Calvo-Bado, L. A., Green, L. E., Medley, G. F., Ul-Hassan, A., Grogono-Thomas, R., Buller, N., Kaler, J., Russell, C. L., Kennan, R. M., Rood, J. I., and Wellington, E. M.: Detection and diversity of a putative novel heterogeneous polymorphic proline-glycine repeat (*Pgr*) protein in the footrot pathogen *Dichelobacter nodosus*. *Vet. Microbiol.* 3-4: 358-366, 2010.
7. La Fontaine, S., Egerton, J. R., and Rood, J. I.: Detection of *Dichelobacter nodosus* using species-specific oligonucleotides as PCR primers. *Vet. Microbiol.* 1-2: 101-117, 1993.
8. Dhungyel, O. P., Whittington, R. J. and Egerton, J. R.: Serogroup specific single and multiplex PCR with pre-enrichment culture and immuno-magnetic bead capture for identifying strains of *D. nodosus* in sheep with footrot prior to vaccination. *Mol. Cell Probes.* 16: 285-96, 2002.
9. Grogono, T. R. and Johnston, A. M.: A study of ovine lameness. MAFF Open Contract OC 5945K, DEFRA Publications, London, 1997.
10. Wassink, G. J., Grogono-Thomas, R., Moore, L. J. and Green, L. E.: Risk factors associated with the prevalence of footrot in sheep from 1999 to 2000. *Vet. Rec.* 152: 351-358, 2003.
11. Gurung, R. B., Tshering, P., Dhungyel, O. P. and Egerton, J. R.: Distribution and prevalence of footrot in Bhutan. *Vet. J.* 171: 346-351, 2006.
12. Rather, M. A., Wani, S. A., Hussain, I., Bhat, M. A., Kabli, Z. A., and Magray, S. N.: Determination of prevalence and economic impact of ovine footrot in central Kashmir India with isolation and molecular characterization of *Dichelobacter nodosus*. *Anaerobe.* 17: 73-77, 2011.
13. Claxton, P. D., Riberio, L. A. and Egerton, J. R.: Classification of *Bacteroides nodosus* by agglutination tests. *Aust. Vet. J.* 60: 331-334, 1983.
14. Hindmarsh, F. and Fraser, J.: Serogrouping of *Bacteroides nodosus* isolated from ovine footrot in Britain. *Vet. Rec.* 116: 187-188, 1985.
15. Kingsley, D. F., Hindmarsh, F. H., Liardet, D. M. and Chetwin, D. H.: Distribution of serogroups of *Bacteroides nodosus* with particular reference to New Zealand and the United Kingdom. In: Proceedings of a Workshop on Footrot in Ruminants Melbourne 1985 (Eds. D.J. Stewart, J.E. Peterson, N.M. McKern and D.L. Emery). CSIRO Division of Animal Health and Australian Wool Corporation, Glebe, 1986.
16. Cheetham, B. F., Tattersall, D. B., Bloomfield, G. A., Rood, J. I. and Katz, M. E.: Identification of a gene encoding a bacteriophage-related integrase in a vap region of the *Dichelobacter nodosus* genome. *Gene.* 162: 53-58, 1995.