

Prevalence of ovine footrot in Kashmir, India and molecular characterization of *Dichelobacter nodosus*

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ABSTRACT

In a study conducted in 4 districts of south Kashmir the prevalence of ovine footrot was found to be 16.41%, with the highest prevalence of 19.43% in district Kulgam and lowest of 12.21% in district Pulwama. While 18.65 and 17.45% prevalence of footrot was observed in districts Anantnag and Shopian respectively. Overall economic impact of footrot was estimated to the tune of Rs 36.79 million annually to the sheep production in south Kashmir. Out of 400 samples collected from footrot lesions of naturally infected sheep, 224 (56%) samples were detected *D. nodosus* positive. Out of these, 185 (82.58%) isolates were belonging to serogroup B of *D. nodosus*, 3 (1.33%) serogroup C, 21 (9.37%) serogroup E, 5 (2.23%) serogroup I and 10 (4.46%) had mixed infection of 2 serogroups B and E. Serogroup C was detected for the first time in India. Out of 224 *D. nodosus* isolates, 138 (61.60%) possessed *intA* gene, thus were considered as virulent strains. Serogroup-wise, *intA* gene was found in 123 (66.48%) isolates of serogroup B, 11 (52.38%) of E and 4 (80.0%) of I. It was interesting to note that none of the sample from serogroup C was positive for *intA* gene, indicating benign status of disease.

Key words: *Dichelobacter nodosus*, Footrot, PCR, Serogrouping, Sheep

Footrot is a specific contagious disease of the feet of sheep and goats, although it has been reported in cattle, horses, pigs, deer and mouflon. It is an infectious syndrome caused by the synergistic action of certain bacterial species, of which *Dichelobacter nodosus* is the main transmitting agent (Thomas 1964). The current Australian classification system classified *D. nodosus* into 10 serogroups (A–I and M) (Claxton 1986, Ghimire *et al.* 1998). Currently, identification of the *D. nodosus* is being carried out by polymerase chain reaction (PCR) using 16S rDNA specific primers (La Fontaine *et al.* 1993) while its serogrouping is being carried out by multiplex PCR (m-PCR) (Dhungyel *et al.* 2002) using serogroup-specific primers without the need to culture. Despite its worldwide presence, the disease has significant economic impact in those sheep farming countries that have temperate climates and moderate to heavy rainfall, such as Australia and New Zealand. Muddy pastures, frequent rains and injury to feet, predispose animals to the infection. Nieuwhof and Bishop

(2005) reported that the most prevalent cause of lameness in sheep in U.K is footrot and results in economic loss of £24 million per annum. Three different clinical forms of the disease have been described as virulent, intermediate and benign (Stewart 1989) which are caused by the corresponding strains of the *D. nodosus*. In order to differentiate *D. nodosus* strains into virulent, intermediate and benign, initially elastase test (Stewart 1979) and subsequently gelatin gel test by Palmer (1993) was used. Cheetham *et al.* (2006) described a PCR assay, for detection of a virulent-specific genetic element the integrase (*intA*) gene, which was found to be superior to earlier methods. They showed that there is a high correlation between the presence of the *intA* gene and the ability of the strain to cause virulent footrot. The disease has become enzootic throughout the state of Jammu and Kashmir, India for the last 18 years or more. Of late its severity increased and attained significant importance in sheep husbandry practices. Hence present investigation was undertaken to determine the prevalence and economic impact of footrot as well as to determine the serological diversity and strain differentiation of *D. nodosus* in south Kashmir.

MATERIALS AND METHODS

Survey: The present survey was conducted from

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September 2007 to May 2008 to determine the prevalence of footrot in south Kashmir comprising 4 districts (Table 1). A 2-stage simple random sampling procedure was followed. A complete list of all villages falling under the administrative control of these districts were obtained from district offices of respective districts and 5% of villages from each district were selected as per convenience, through simple random sampling (SRS) procedure. Subsequently, complete list of household flocks in the selected villages were prepared and again simple random sampling (second stage sampling) was followed to determine the identity of the house hold flocks. Again 5% of household flocks were selected as per convenience. These household flocks were finally visited to determine the prevalence of footrot in south Kashmir. The serological diversity study of *D. nodosus* was conducted on 400 swab samples from sheep affected with footrot.

Collection of samples: A total of 400 exudate samples of footrot lesions with a lesion score of 2 (interdigital dermatitis) to 4 (underrunning of the hard horn of the hoof) (Egerton and Roberts 1971) were collected from 100 naturally infected sheep from each of the 4 districts of south Kashmir, viz. Anantnag, Kulgam, Shopian and Pulwama, by regular visits during the period of study. Samples were collected on sterile cotton swabs in duplicate, one for inoculation onto media and another for DNA extraction for direct detection of *D. nodosus*.

Table 1. Number of villages in districts of south Kashmir

District	Number of villages	5% of villages selected through SRS
Anantnag	312	16
Kulgam	329	16
Shopian	272	14
Pulwama	333	17
Total	1246	63

Extraction of bacterial DNA: Suspensions of the suspected material from the swabs were prepared in 1.5 ml microcentrifuge tubes in 100 µl of sterile phosphate buffered saline (PBS) by gentle vortexing. After removing the swabs, the samples were boiled for 5 min, cooled on ice for 10 min and centrifuged at $10,000 \times g$ for 1 min. Similarly, from the culture plates suspected colonies with characteristic morphology were directly suspended into 100 µl sterile PBS and processed for the extraction of DNA as before. Two microlitres of the supernatant was used as the template for each PCR reaction.

Detection of 16S rRNA gene of *D. nodosus* by PCR: For the detection of 16S rRNA gene of *D. nodosus*, PCR conditions and primers were essentially the same as described earlier (La Fontaine *et al.* 1993). Positive control DNA, kindly supplied by Dr. O. P. Dhungyel, Faculty of Veterinary Medicine, University of Sydney, Cambden, NSW 2570, Australia, was included in the PCR. Distilled water served as negative control.

Isolation and serogrouping of *D. nodosus*: For isolation of *D. nodosus*, all the swab samples were streaked at the place of collection on tripticase-arginine-serine (TAS) agar with 4% hoof powder (Thorley 1976). The hoof powder was prepared from dried hooves collected from healthy slaughtered sheep. After inoculation, the plates were placed immediately in an anaerobic jar with Anaero Gaspack (HiMedia, Mumbai, India) and carried to the laboratory. The jar was placed in incubator at 37°C within 2–3 h. After 5 days of incubation, suspected colonies were subcultured repeatedly on the same medium containing 2% hoof powder until pure colonies of *D. nodosus* were obtained. Plates, which did not show any characteristics growth of *D. nodosus*, even after initial 5 days of incubation, were incubated for another 5 days before being discarded. Confirmation of the isolates as *D. nodosus* was done by demonstration of the typical cellular morphology in Gram stained smears and detection of species-specific 16S rRNA gene by PCR as described

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

Primer name	Primer sequence (5' – 3')	Primer concentration	Target gene	Serogroup	References
Forward	CGGGGTTATGTAGCTTGC	0.25 mM each	16S rDNA	–	LaFontaine <i>et al.</i> (1993)
Reverse	TCGGTACCGAGTATTTCTACCCAACACCT	–	–	–	
FP	CCTTAATCGAACTCATGATTG	0.625 mM	<i>fimA</i>	–	Dhungyel <i>et al.</i> (2002)
RA	AGTTTCGCCCTTCATTATTT	0.25 mM	<i>fimA</i>	A	
RB	CGGATCGCCAGCTTCTGTCTT	0.25 mM	<i>fimA</i>	B	
RC	AGAAGTGCCTTTGCCGTATTC	0.25 mM	<i>fimA</i>	C	
RD	TGCAACAATATTTCCCTCATC	0.25 mM	<i>fimA</i>	D	
RE	CACTTTGGTATCGATCAACTTGG	0.25 mM	<i>FimA</i>	E	
RF	ACTGATTTCGGCTAGACC	0.25 mM	<i>FimA</i>	F	
RG	CTTAGGGGTAAGTCCTGCAAG	0.25 mM	<i>FimA</i>	G	
RH	TGAGCAAGACCAAGTAGC	0.25 mM	<i>FimA</i>	H	
RI	CGATGGGTCAGCATCTGGACC	0.25 mM	<i>FimA</i>	I	

above except the hoof medium was used as a negative control. For serogrouping multiplex PCR was carried out by using nine (A–I) serogroup specific primers (Table 2) as described earlier (Dhungyel *et al.* 2002). Serogroup specific DNA kindly supplied by Dr O P Dhungyel, was used as positive control in PCR.

Strain differentiation of *D. nodosus* into virulent and benign: To ascertain the virulent or benign status, all the *D. nodosus* isolates were screened for presence of *intA* gene by PCR as described by Cheetham *et al.* (2006) with minor modifications. The concentration of the primer pair (5'ACA TCA TGC GAC TCA CTG AC3' and 5'TCT CTG GTC GGT CGT ACA AT 3') was 0.25 µM while concentration of MgCl₂ was 2.0 mM. The amplification was done for 31 cycles, each cycle consisting of 1.5 min at 94°C, 1 min at 60°C and 2 min at 72°C. This was followed by a final extension of 5 min at 72°C.

RESULTS AND DISCUSSION

Prevalence of footrot in south Kashmir: The prevalence of footrot as revealed during the period of study in south Kashmir is depicted in Table 3. The overall prevalence (16.41%) of footrot observed in present work is higher than that observed in other parts of world like 8–10% in United Kingdom (Grogono and Johnston 1997, Wassink *et al.* 2003) and 3.1% in Bhutan (Gurung *et al.* 2006a). However, Gurung *et al.* (2006a) reported 9 times higher prevalence of the disease in migratory flocks. The highest prevalence in district Kulgam among the 4 districts of south Kashmir could be because pastures around the villages of the district are more favourable for the persistence of the diseases due to water-logging. No work regarding prevalence of footrot from any other part of India is available to compare these results. However, very recently, in Andhra Pradesh (India) footrot like lesions have been reported in sheep and cattle by Prof. Sreenivasulu (*personal communication*). Considering 10% production loss (Marshall *et al.* 1991), average treatment cost of Rs 20/sheep, 40 kg average body weight/sheep and Rs 90/kg live weight, then it amounts to Rs 36.79 million loss at current prevalence rate of footrot in south Kashmir due to footrot, which has a population of 5.9 lakh sheep (Jammu and Kashmir Livestock Census 2008). This is the

first-ever estimate of its kind from India. This estimate is similar to those of Marshall *et al.* (1991) who estimated that footrot resulted in a loss of A\$ 43 million per annum in New South Wales (Australia) in terms of loss of production, treatment, control, prevention and eradication of virulent footrot. Nieuwhof and Bishop (2005) also reported economic loss to the tune of £24 million per annum due to footrot in United Kingdom. This huge economic impact of the disease stresses the need for development of immediate control measures including a vaccine.

Serological diversity of *D. nodosus* in south Kashmir: To date, it is a common practice to prepare the commercial footrot vaccine containing representative serogroups available locally. This serogroup-specific vaccination is widely used for successful control and eradication of virulent footrot in different countries including Nepal and Bhutan (Egerton *et al.* 2002, Gurung *et al.* 2006b). Thus for the development of an efficacious vaccine against virulent footrot, it is essential to know the serological diversity as well as virulent status of the strains of the *D. nodosus*.

Out of 400 swab samples collected, 224 (56.0%) samples detected positive for *D. nodosus* by the amplification of 783 bp product of 16S rRNA gene of *D. nodosus* (Fig. 2).

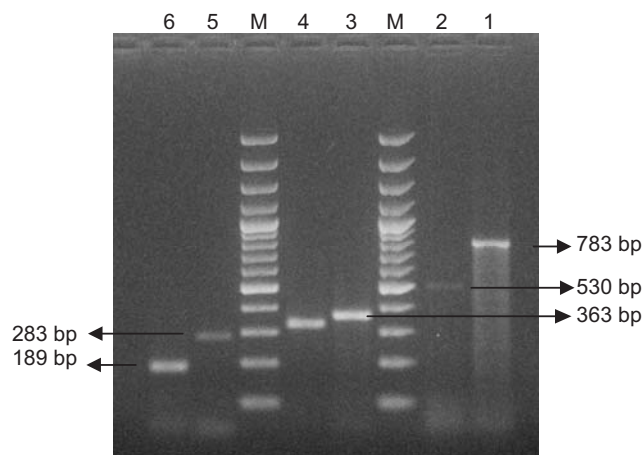


Fig. 1. Detection, serogrouping and virulence of *D. nodosus* isolates by PCR. Lane M: 100 bp DNA marker Lane 1: 16S rRNA gene specific band Lane 2: *intA* gene positive sample Lane 3, 4, 5 and 6: serogroup E, C, B and I specific band, respectively.

Table 3. Prevalence of footrot in districts of south Kashmir

District	Number of H.H.Fs in the selected villages	5% of H.H.Fs selected through SRS	No. of sheep in the selected flocks	No. affected sheep	Prevalence of footrot
Anantnag	312	16	1238	231	18.65
Kulgam	329	16	1708	332	19.43
Shopian	272	14	779	136	17.45
Pulwama	333	17	2088	255	12.21
Total	1246	63	5813	954	16.41

SRS, Simple random sampling; H.H.Fs, Household flocks.

Table 4. District-wise serological diversity of *Dichelobacter nodosus* in south Kashmir

District	Samples collected	Samples positive for <i>D. nodosus</i>	Serogroup					Predominant serogroup	<i>intA</i> positive isolates	Serogroup-wise distribution of <i>intA</i> gene			
			B	C	E	I	B+E			B	C	E	I
Anantnag	100	55	44	–	7	–	4	B (80.00%)	31	27	–	4	–
Pulwama	100	62	48	–	10	–	4	B (77.41%)	28	24	–	4	–
Shopian	100	53	48	–	–	5	–	B (90.56%)	40	36	–	–	4
Kulgam	100	54	45	3	4	–	2	B (83.34%)	39	36	–	3	–
Total	400	224	185	3	21	5	10		138	123		11	4

Serogrouping of *D. nodosus* positive samples by m-PCR using nine (A–I) serogroup specific primers revealed the presence of 4 serogroups (B, C, E and I) as shown in Table 4 and Fig. 1.

Out of 224 positive samples, 185 (82.58%) carried serogroup B, 21 (9.37%) serogroup E, 5 (2.23%) serogroup I, 3 (1.33%) serogroup C while remaining 10 (4.46%) samples showed mixed infection of both serogroups B and E. This corroborates with earlier findings of Wani *et al.* (2004 and 2007), who also reported serogroup B as the predominant serogroup besides detecting serogroup E and I in small geographical area of Kashmir. Similarly, Claxton *et al.* (1983) reported the predominant prevalence of serogroup B in Australia, Hindmarsh and Fraser (1985) in Great Britain, Kingsley *et al.* (1986) in New Zealand and Gurung *et al.* (2006a) in Bhutan. This is first report of the occurrence of serogroup C of *D. nodosus* in India even though it was found in only 3 (1.33%) samples. Kingsley *et al.* (1986) also reported that only 2% of *D. nodosus* strains belonged to serogroup C in Great Britain.

Mixed infection with 2 different serogroups (4.46%) detected in the present study corroborates with the findings of earlier investigators (Moore *et al.* 2005, Hussain *et al.* 2009). However, Hindmarsh and Fraser (1985) reported a higher prevalence (13.8%) in Great Britain. The significance of mixed infection may be explained by the fact that *D. nodosus* can undergo serogroup conversion as a result of natural transformation (Kennan *et al.* 2003). This also explains why a serogroup specific vaccine fails under these typical field conditions.

Virulent and benign status of *D. nodosus*: Out of 224 *D. nodosus* isolates, 138 (61.60%) were found to carry the *intA* gene while the remaining 86 isolates did not possess *intA* gene as shown in Table 4 (Fig. 1). Serogroupwise, 123 (89.13%) isolates belonging to serogroup B, 11 (7.97%) to serogroup E and 4 (2.89%) to serogroup I carried *intA* gene. Overall 61.60% of *D. nodosus* isolates were designated as virulent strains based on the presence of *intA* gene (Cheetham *et al.* 2006).

Thus the present investigation unveils that there is heavy economic impact due to high prevalence of ovine footrot and serogroup B of *D. nodosus* is the predominant serogroup

followed by serogroup E. Hence, these 2 serogroups could be incorporated in the formulation of an effective bivalent vaccine intended to combat the menace in south Kashmir.

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