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Molecular Characterization and Serogroup Prevalence of *Dichelobacter nodosus* from the Cases of Ovine Footrot in Andhra Pradesh and Telangana States of India

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ABSTRACT

Keywords

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A total of 338 foot swabs were collected from different districts of AP and Telangana state and were subjected to PCR targeting 16S rRNA gene of *D. nodosus*. Out of 338 samples, 86 samples were found to be positive for PCR suggesting the presence of *D. nodosus*. Further all the PCR positive samples were subjected to multiplex-PCR for serogrouping. Out of 86 positive samples, 53 found to be belonged to 'B' serogroup followed by 24 'I' serogroup, 6 'A' serogroup, 2 'E' serogroup and one sample 'H' serogroup. The overall prevalence of the *D. nodosus* was calculated as 16%. The prevalence of *D. nodosus* serogroups 'B', 'I', 'A', 'E' and 'H' was 61.6%, 27.9%, 6.9%, 2.3% and 1.2% respectively.

Introduction

Footrot is a contagious disease of feet of ruminants commonly seen in sheep and goats and a milder form in cattle. It occurs worldwide. It is a complex disease resulting from bacterial infection in which *Dichelobacter nodosus* (Dewhirst *et al.,* 1990) is the essential transmitting agent. The disease is a chronic bacterial infection causing inflammation of the epidermal tissues of the hoof with under running of the horn progressing from an initial interdigital dermatitis to separation of horn from the soft tissues of the hoof with varying degrees of lameness. *D.nodosus* is a Gram negative, obligate anaerobic bacillus lives only in the diseased feet of the animals and survives for 7 to 14 days in faeces, soil or pasture (Stewart and Claxton, 1993). Fimbriae of *D.nodosus* are highly immunogenic for sheep and are the major host-protective immunogens (Every and Skerman, 1982). They are responsible for the K-type agglutination, based on which the

field isolates of *D.nodosus* are classified into at least 10 distinguishable serogroups designated as A, B, C, D, E, F, G, H, I and M (Claxton *et al.,* 1983; Chetwin *et al.,* 1991). During the rainy season, the disease attains epidemic proportions and cause threat to the sheep industry in southern states of India. Even though various treatment regimes are tried in field with low degree of success, but control through an effective vaccination holds the key. Hence the present study was carried out with an objective to study the serogroup prevalence in Andhrapradesh..

Materials and Methods

Collection of foot swabs

Foot swabs were collected from sheep showing foot lesions in various districts of Andhra Pradesh and Telangana states of India. Material from foot lesions were collected ascetically from individual hooves using sterile cotton swabs. Immediately the ends of swabs were fractured off into 1.5ml micro centrifuge tubes containing 100µl of sterile distilled water.

Extraction of DNA from foot swabs

DNA extraction was carried out by boiling method**.** The clinical material present on the swabs was extracted by gentle vortexing of micro centrifuge tubes containing swabs in sterile water. After removing the swabs, the samples were boiled for 5 min at 100°C. Later these samples were subjected for centrifugation at 10000xg for 10 min in a refrigerated centrifuge. Two microlitre of the supernatant was used as a template for PCR.

Detection of *D. nodosus* by PCR targeting 16S*r*RNA

PCR for detection of 16S*r*RNA gene of *D. nodosus* was carried out as per the method of Wani *et al.,* (2004, 2007). Oligonucleotide primers designed by La Fontaine, *et al.,*(1993) were used in the study and obtained from Eurofins Genomics India Pvt. Ltd, Bangalore.

Details of the primer sequence are enlisted in Table 1. PCR amplification was performed in 200µl PCR tubes (Tarson, India) with a reaction mixture comprising of 25µl of 10x Taq buffer A - 2.5µl, 10 mM dNTP mix - 2µl, MgCl2 25 mM - 1.5µl,Taq DNA polymerase (3U/ µl) - 0.3µl, Forward Primer (10pico moles) - 0.25µl, Reverse Primer (10pico moles) - 0.25µl DNA Template - 2µl, DEPC water - 16.25µl, The tubes were then spun for 10 sec and PCR was carried out in Thermal cycler (Kyratec) with cycling conditions of initial denaturation at 94° C for 2 min, five cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 62°C and extension at 72°C for 4 min, 25 cycles of denaturation for 30 sec at 94°C, annealing for 30sec at 58°C and extension at 72°C for 30 sec and final extension at 72°C for 4min. The JKS-02 *D.nodosus* strain maintained in the department of Microbiology, C.V.Sc, Tirupati was used as positive control.

Agarose gel electrophoresis of PCR product in 2% agarose

Amplified products were analyzed by agarose gel electrophoresis in 2 per cent agarose gels in TBE buffer containing ethidium bromide. Molecular size marker 100 bp (DNA ladder, Genei, Bangalore) was loaded to the first well.

The electrophoresis was carried out at 60 volts for 90 min to detect PCR amplified product of *Dichelobacter nodosus* targeting 16S rRNA. Sterile distilled water was used as negative control. The gels were viewed under UV transillumination system (Alpha imager, Gel documentation system).

Detection of *D. nodosus* serogroup by Multiplex PCR targeting *fim*A gene

PCR for detection of *fim*A gene of *D. nodosus* was carried out as per the method of Wani *et al.,* (2004, 2007). The primers designed by Dhungyel *et al.,* (2002). The positive samples for *Dichelobacter nodosus* revealed by the amplification of 16S*r*RNA gene were subjected to multiplex PCR for serogrouping using serogroup specific primers (Table 2) with a common forward and nine different reverse primers. Method of DNA extraction, enzymes, buffers and PCR conditions used in the test were similar to that of PCR for detection of 16SrRNA except an increased concentration of Forward primer (2.5 times) than reverse primer. Agarose gel electrophoresis of PCR products was carried out similar to that of the procedure followed for detection of 16SrRNA.

Results and Discussion

Identification of *D.nodosus* by 16S rRNA PCR

DNA was extracted from the foot swabs by rapid boiling method and 2µl of supernatant was taken as template for each PCR reaction. Out of 338 (Chittoor-200,Nelloore-59, Prakasam-42 and Mahaboobnagar-37) samples subjected to 16S*r*RNA PCR, 86 samples (Chittoor-37, Nelloore-21, Prakasam-16 and Mehaboobnagar-12) revealed specific amplicons of 783bp size (Fig. 1 and Table 3) suggestive of *D. nodosus* infection.

Table.1 Primer sequence used for detection of 16SrRNA gene

W	Primer sequence (5'-3')	Target gene	Reference	Product size (bp)
Forward	CGGGGTTATGTAGCT TGC	16S r RNA	La Fontaine, *et al.,*(1993)	783
Reverse	TCGGTACCGAGTATT TCTACCCAACACCT			

Table.2 Primer sequence for detection of serogroups of *D.nodosus* targeting *fim-A* gene

Primer	Primer sequence (5'–3')	Sero-group	Product size (bp)	Reference
Forward primer	CCTTAATCGAACTCATGATTG			**Dhungyel *et al.* (2002)**
Reverse Primer - A	AGTTTCGCCTTCATTATATTT	A	415	
Reverse Primer – B	CGGATCGCCAGCTTCTGTCTT	B	283	
Reverse Primer – C	AGAAGTGCCTTTGCCGTATTC	C	325	
Reverse Primer – D	TGCAACAATATTTCCCTCATC	D	390	
Reverse Primer –E	CACTTTGGTATCGATCAACTTGG	E	363	
Reverse Primer – F	ACTGATTTCGGCTAGACC	F	241	
Reverse Primer – G	CTTAGGGGTAAGTCCTGCAAG	G	279	
Reverse primer – H	TGAGCAAGACCAAGTAGC	H	409	
Reverse Primer – I	**CGATGGGTCAGCATCTGGACC**	**I**	**189**	

Table.3 Results of PCR and Multiplex PCR for detection and serogrouping of *D.nodosus*

S. No	Place of collection	District	No. of Foot swabs collected	No. of Samples positive for 16s r RNA gene	Serogrouping by detection of *fimA* gene				
					‘B’	‘I’	‘A’	‘E’	‘H’
1	RV kandriga,P.Pet	Chittoor	25	7	2	5	-	-	-
2	Yerravaripalem	Chittoor	40	11	2	6	1	2	-
3	Mallavaram, Renigunta	Chittoor	15	Negative	-	-	-	-	-
4	Pileru	Chittoor	10	Negative	-	-	-	-	-
5	Basavayaplalem	Chittoor	32	9	3	6	-	-	-
6	Katuru	Chittoor	42	10	5	3	2	-	-
7	Karakambadi	Chittoor	12	Negative	-	-	-	-	-
8	MD puttur	Chittoor	10	Negative	-	-	-	-	-
9	Yerpedu	Chittoor	14	Negative	-	-	-	-	-
10	Ardhamala	Nelloore	33	11	9	-	2	-	-
11	Bonupalli, Naidupet	Nelloore	26	10	18	2	-	-	-
12	Jaladanki	Prakasam	42	16	12	2	1	-	1
13	Mahaboob-nagar rural	Mahaboob-nagar	37	12	12	-	-	-	-
Total			**338**	**86**	**53**	**24**	**6**	**2**	**1**

Fig.1 Amplification of 16Sr RNA gene of *D.nodosus* from clinical samples of ovine foot rot

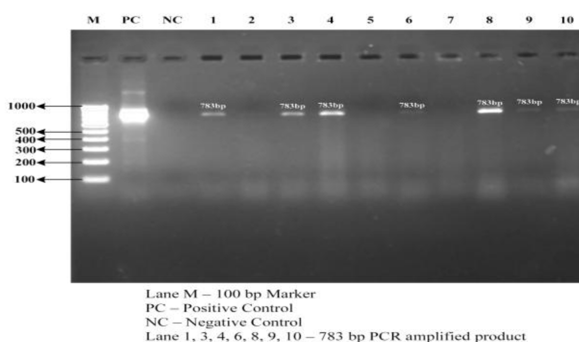


Fig.2 Serogroup ‘B’ and ‘A’ specific PCR products of *D.nodosus*

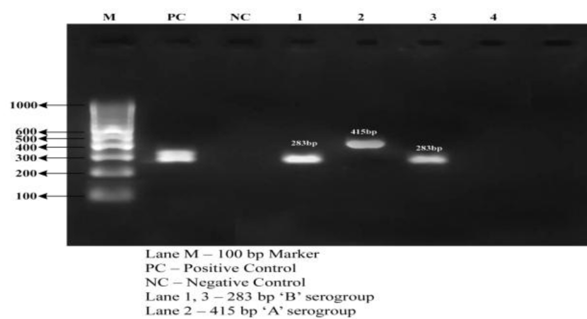


Fig.3 Serogroup 'I' and 'H' specific PCR products of *D.nodosus*

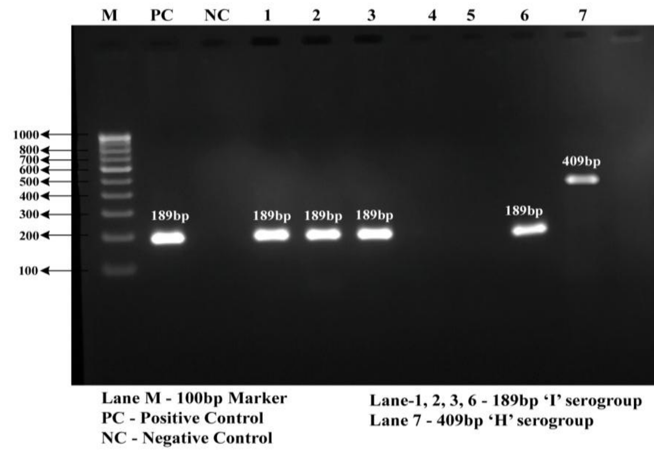


Fig.4 Serogroup 'E' specific PCR products of *D.nodosus*

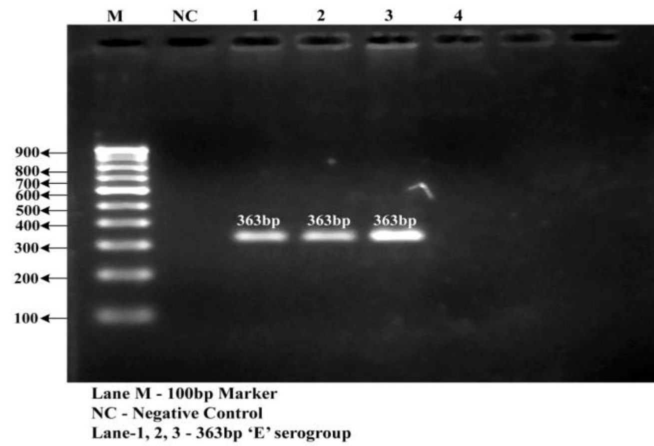
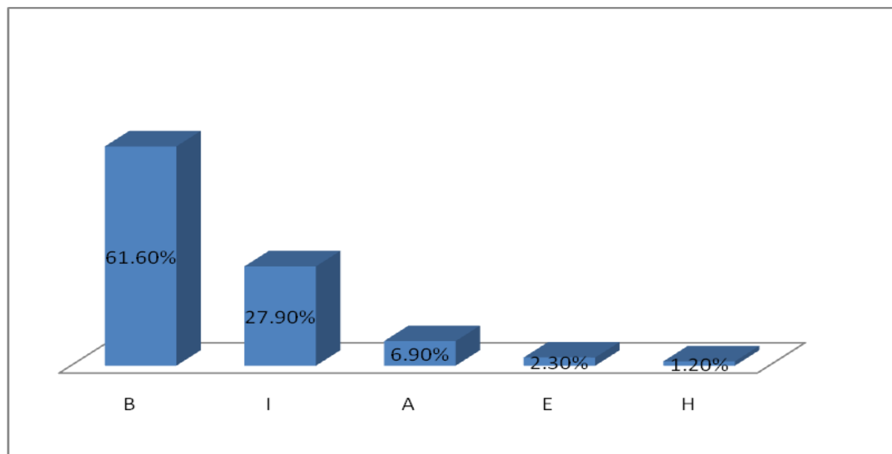


Fig.5 Prevalence of *D.nodosus* serogroup



Serogrouping of *D.nodosus* by multiplex PCR

The samples positive by 16S *r*RNA PCR were subjected to Multiplex PCR. The results are shown in Table 3. Out of 86 samples, 53 revealed specific amplicons of 283 bp size suggestive of 'B' serogroup (Fig. 2) (Chittoor-12, Nelloore-27, Prakasam-12 and Mehaboobnagar-12), 24 revealed specific amplicons of 189 bp size suggestive of 'I' serogroup (Fig. 3) (Chittoor-20, Nellore-2, Prakasam-2), 6 revealed specific amplicons of 415 bp size suggestive of 'A' serogroup (Fig. 2) (Chittoor-3, Nelloore-2, Prakasam-1) 2 revealed specific amplicons of 363 bp size suggestive of 'E' serogroup (Fig. 4) (Chittoor-2) and one sample revealed specific amplicons of 409 bp size suggestive of 'H' serogroup (Fig. 3) (Prakasam). The prevalence of 'B', 'I', 'A', 'E' and 'I' was found to be 61.6per cent, 27.9 per cent, 6.9 per cent, 2.3 per cent and 1.2 per cent respectively (Fig. 5).

Out of 338 samples subjected to 16SrRNA PCR, 86 samples (Chittoor-37, Nelloore-21, Prakasam-16 and Mahaboobnagar-12) revealed specific amplicons of 783 bp size indicating the presence of *D.nodosus*. The overall prevalence of 16per cent was observed in the present study, which was higher than that reported by Sreenivasulu *et al.,* (2013) in Andhra Pradesh and Farooq *et al.,* (2010) in Jammu and Kashmir. The overall prevalence was higher than that reported in other parts of world like 8-10per cent in UK (Wassink *et al.,* 2003), 3.1per cent in Bhutan (Gurung *et al.,* 2006). The higher prevalence reported in the study may be attributed to heavy rain fall recorded in AP and Telangana state during the year 2014-15.

In the present investigation the prevalence of serogroup 'B' was predominant (61.6per cent), followed by serogroups 'I' (27.9%), 'A' (6.9%), 'E' (2.3%) and 'H' (1.2%). The predominance of serogroup varies from country to country. The predominance of serogroup 'B' was also reported earlier in AP (Sreenivasulu *et al.,* 2013), in Kashmir (Farooq *et al.,* 2010), and in Bhutan (Gurung *et al.,* 2006). It is significant to note that high prevalence of 'I' serogroup was recorded in this region when compared to other parts of the world. Prevalence of 'I' serogroup was reported as 3.5 per cent by Moore *et al.,* (2005) in England and Wales, 4.47 per cent by Hussain *et al.,* in Kashmir, India and 2 per cent by Gillhus *et al.,* (2013) in Norway.

The predominance of serogroup 'B' followed by serogroup 'I' was also reported earlier by Sreenivasulu *et al.,* (2013) which is unique to this region. Similar pattern of occurrence of footrot has not yet been reported so far from the other footrot affected countries. In Kashmir the predominance of serogroup 'B' followed by 'E' was reported by Wani *et al.,* (2007) , Farooq *et al.,* (2010) and Rather *et al.*(2011). Studies carried out by Ghimire and Egerton (1996) recorded the prevalence of three serogroups 'E', 'B' and 'C' in which E was found to be predominant in Nepal. Mattick *et al.,* (1991) based on sequence analysis and the presence of hypervariations in *fimA* gene grouped *D.nodosus* into three subtypes, subtype I (A,E, F), subtype II (B, I) and Subtype III (G, C). 80 per cent of the *D.nodosus* detected in the present investigation belonged to the subtype II ('B' and 'I').

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