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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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 16SrRNA**

PCR for detection of 16SrRNA 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 (10 pico moles) - 0.25μl, Reverse Primer (10 pico 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 4 min. 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 \textit{D. nodosus} serogroup by Multiplex PCR targeting \textit{fimA} gene

PCR for detection of \textit{fimA} gene of \textit{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 \textit{Dichelobacter nodosus} revealed by the amplification of 16SrRNA 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 \textit{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 16SrRNA 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 \textit{D. nodosus} infection.

| Primer | Primer sequence (5’–3’) | Sero-group | Product size (bp) | Reference |
|--------|-------------------------|------------|------------------|-----------|
| Forward | CGGGGTTATGTAGCT TGC | 16S r RNA | La Fontaine, \textit{et al.}, (1993) | 783 |
| Reverse | TCGGTACCGAGTATT TCTACCCAACACCT | | | |

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

| Primer | Primer sequence (5’–3’) | Sero-group | Product size (bp) | Reference |
|--------|-------------------------|------------|------------------|-----------|
| Forward primer | CCTTAATCGAACTCATGATTG | | | |
| Reverse Primer - A | AGTTTCGCTTTCCATTATATT | A | 415 | Dhungyel \textit{et al.} (2002) |
| Reverse Primer – B | CGGATCGCCAGCTTCTGTCTT | B | 283 | |
| Reverse Primer – C | AGAAGTGCCCTTGCCGTATTC | C | 325 | |
| Reverse Primer – D | TGCAACAAATTTCCCTCACC | D | 390 | |
| Reverse Primer – E | CACTTTGGATATCGATCAACTTG | E | 363 | |
| Reverse Primer – F | ACTGTTCGCGCTAGACCC | F | 241 | |
| Reverse Primer – G | CTTAGGGGTAAGTCTGCAAG | G | 279 | |
| Reverse primer – H | TGAGCAAGACCAAGTACCG | H | 409 | |
| Reverse Primer – I | CGATGCGTACGCATCTGGACC | I | 189 | |

Table 2: Primer sequence for detection of serogroups of \textit{D.nodosus} targeting \textit{fim-A} gene
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     | Basavayapalalem       | 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    | Ardhampala            | 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. 1](image1.png)

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

![Fig. 2](image2.png)
**Fig. 3** Serogroup ‘I’ and ‘H’ specific PCR products of *D. nodosus*

![Serogroup 'I' and 'H' specific PCR products of D. nodosus](image)

| Lane | Description |
|------|-------------|
| M    | 100bp Marker |
| PC   | Positive Control |
| NC   | Negative Control |
| 1    | 1890bp ‘I’ serogroup |
| 2    | 1890bp ‘I’ serogroup |
| 3    | 1890bp ‘I’ serogroup |
| 4    | 1890bp ‘I’ serogroup |
| 6    | 1890bp ‘I’ serogroup |
| 7    | 409bp ‘H’ serogroup |

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

![Serogroup 'E' specific PCR products of D. nodosus](image)

| Lane | Description |
|------|-------------|
| M    | 100bp Marker |
| NC   | Negative Control |
| 1    | 563bp ‘E’ serogroup |
| 2    | 563bp ‘E’ serogroup |
| 3    | 563bp ‘E’ serogroup |

**Fig. 5** Prevalence of *D. nodosus* serogroup

![Prevalence of D. nodosus serogroup](image)

| Serogroup | Prevalence |
|-----------|------------|
| I         | 61.60%     |
| A         | 27.90%     |
| E         | 6.90%      |
| H         | 2.30%      |
| B         | 1.20%      |
Serogrouping of *D. nodosus* by multiplex PCR

The samples positive by 16S rRNA 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, Nellore-27, Prakasam-12 and Mahaboobnagar-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, Nellore-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 (Prakasam). The prevalence of ‘B’, ‘I’, ‘A’, ‘E’ and ‘I’ was found to be 61.6 per 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, Nellore-21, Prakasam-16 and Mahaboobnagar-12) revealed specific amplicons of 783 bp size indicating the presence of *D. nodosus*. The overall prevalence of 16 per 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), 3.1 per 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 (61.6 per 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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**How to cite this article:**

Vijayalakshmi, S., D. Sreenivasulu, N. Vinodkumar, D. Raniprameela and Karthik, A. 2020. Molecular Characterization and Serogroup Prevalence of *Dichelobacter nodosus* from the Cases of Ovine Footrot in Andhra Pradesh and Telangana States of India. *Int.J.Curr.Microbiol.App.Sci.* 9(09): 14-21. doi: [https://doi.org/10.20546/ijcmas.2020.909.002](https://doi.org/10.20546/ijcmas.2020.909.002)