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Characterization of *Dichelobacter nodosus* isolates *by* RAPD and SDS-PAGE

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Abstract
Ovine Footrot is a hoof disease manifested in the form of inter digital lesions of foot of sheep. Regularly in Andhra Pradesh, India the outbreaks of footrot is being reported in sheep causing severe economic loss to the sheep husbandry. *Dichelobacter nodosus*, an obligatory anaerobic, gram negative bacteria is the principal causative agent. On characterization of *D. nodosus* strains isolated in our study by RAPD analysis has shown the presence of 400 bp band common to virulent and intermediate strains and the same band is not seen in benign strains of D. nodosus. Similarly, on SDS-PAGE analysis, different protein band profile pattern was observed in different serogroups. 'I' and 'A' serogroups have common protein banding pattern. 'C' Serogroup revealed a different protein band pattern with an extra band at 120KDa. No protein band could be identified which can differentiate virulence of the strains.

Keywords: Footrot, virulence, characterization

1. Introduction
Dichelobacter nodosus is an obligatory anaerobic, Gram negative bacilli bacteria which is the principal agent causing ovine footrot, a debilitating disease of the small ruminants. Footrot is one of the economically significant disease of sheep particularly in Andhra Pradesh, India. The immunogenic fimbriae are considered as the major protective immunogens of host (Every *et al.*, 1982) [5] causing 'K' type agglutination. Claxton *et al.* (1983) [2] classified the bacteria in to ten distinguishable serogroups based on 'K' type agglutination (A, B, C, D, E, F, G, H, I and M). Based on cross absorption tests, they were further classified into 19 serotypes and are designated as A1, A2, B1, B2, B3, B4, C1, C2, D, E1, E2, F1, F2, G1, G2, H1, H2, I and M (Claxton *et al.*,1989 and McKern *et al.*,1989) [3, 8]. The protection in vaccinated sheep is serogroup specific (Egerton *et al.*, 1974) [4]. To take up footrot control programs in endemic areas through vaccination, it is essential to have the details of serogroup distribution in the area. *D. nodosus* isolation and identification from field samples is difficult because of its fastidious nature. Sreenivasulu *et al.* (2013) [9] initiated the work on isolation and characterization of *D. nodosus* in A.P and isolated *D. nodosus* serogroups 'B', 'I', 'A' and 'C'. The present study is carried out to characterize *D. nodosus* isolates by Random amplification of Polymorphic DNA (RAPD) and SDS -PAGE

2. Methodology
2.1 Isolates
Nine isolates out of which Three virulent isolates (JKS-05, ARD-I, &KDP-I) three intermediate isolates (MBNR-I, V-PET-I & PNP-I) and three benign strains (Bon-II-B, SKHT-I & Bon-I-B) isolated in our studies were used in the study.

2.2 Random amplification of polymorphic DNA (RAPD)
Nine Representative samples with six virulent and three benign isolates were subjected to RAPD in the study. RAPD analysis was carried out using HLWL-74 and HLWL-85 primers designed by Mazurier, *et al.*, 1992 [7] for assessing *C. jejuni* and *Listeria* species. The primer sequences was depicted in Table 1.

Table 1: In brief the reactions were carried out in a total volume 25 µl consisting

Taq buffer A (10X)	2.5 µl
dNTP mix 10 mm	1.25 µl
25mMMgCl$_2$	2.5 µl
Primer (either HLWL-74 and HLWL-85)	1 µl
TaqDNA polymerase	0.2 µl
Templet DNA	5 µl
DEPC water	12.5 µl

PCR was carried out in Kyratec Thermal cycler with the following cycling conditions, one cycle of denaturation at 94 ºC for 10 min, annealing for one min at 34 ºC and extension at 72 ºC for 4 min. Followed by 43 cycles 94 ºC for 1min, 34 ºC for 1min and extension at 72 ºC for 4min. PCR products were analyzed by electrophoresis in 2% agarose gels in TBE buffer containing ethidium bromide and viewed in UV transillumination system (Alpha imager)

2.3 Whole cell protein profiles of isolates by SDS-PAGE
The whole bacterial cell protein band profiles of the *D. nodosus* isolates were analysed by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) as per the methodology described by Laemmli (1970) [6].

2.3.1 Gel preparation
The gels used for resolving whole cell lysates are 12% resolving and 4% stacking gels. The vertical PAGE slab gel unit in the casting mould was assembled using spacers. Resolving gel was filled to a level of two centimetres from top and distilled water was gently added along the walls of gel sandwich. After polymerization of the gel, the water on the resolving gel was discarded and dried using filter paper. The comb was introduced in the sandwich and then the stacking gel was overlaid on the resolving gel and is allowed to polymerize.

2.3.2 Sample preparation
Bacterial cells were grown for five days in the TAS agar plates in anaerobic atmosphere. Cells were collected in 600 µl of sterile normal saline in a 1.5ml micro centrifuge tube and adjust the opacity to tube no 6 Mc Farland opacity standards. 40 µl of properly mixed bacterial cell suspension was added to 100 µl of the sample buffer with bromophenol blue and this suspension were stored at -20 ºC until required. Before usage the bacterial suspension in the buffer was boiled for three minutes and then cooled to room temperature. The bacterial suspension was centrifuged at 10000 rpm for 5 min. 5 µl was used to load the gels.

2.3.3 Loading and running of gels
After gel polymerization, the comb was removed from the gels without disturbing the well dividers. Electrode buffer is filled in both upper and lower tanks. The electrophoresis unit was connected to a power pack with 50V of current applied initially. The current supply was increased to 100 V, once the sample entered the resolving gel. As the dye reaches the bottom of the running gel, the current was brought to zero and the power was disconnected from unit.

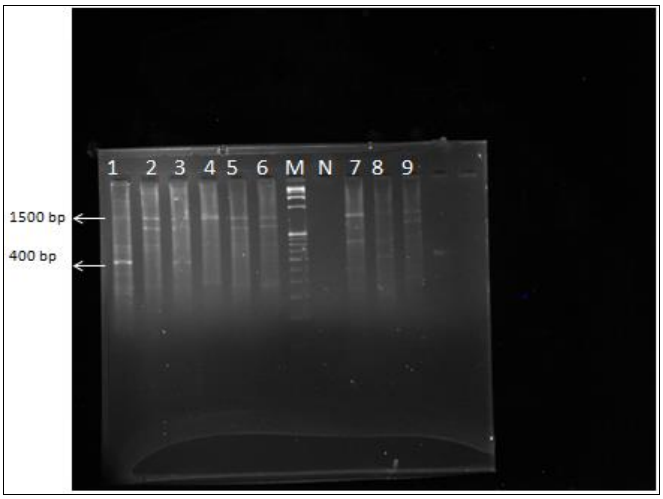
2.3.4 Staining and Distaining
After completion, the gel mould was dismantled carefully and immersed in a glass tray for two hours containing Coomassie brilliant blue stain. After staining, the gel was transferred to detaining solution until the gel background became clear. Later the gel was transferred to gel storage solution.

2.3.5 Molecular weight determination
The distance of migration from the well to the midpoint of each protein band of samples and marker proteins were measured. Standard curve was built by taking migration on X-axis and molecular weights on Y-axis for marker proteins on a graph paper. A straight line was drawn passing through points. The molecular weights of the proteins were calculated by standard curve.

3. Results
3.1 Random amplification of polymorphic DNA (RAPD)
D. nodosus isolates representing virulent, intermediate and benign groups were subjected for RAPD with HLWL-74 and HLWL-83. All the isolates used in the test revealed the similar pattern of bands at 1500 bp with HLWL-74 primer. The virulent and intermediate isolates showed a common band at 400 bp. However the band at 400 bp was not observed within benign isolates (Fig.1) The remaining bands are hazy without any significant differentiating capacity.
The samples subjected to RAPD with HLWL-84 showed a common banding pattern around 1100 bp and 650 bp in all the virulent, intermediate and benign strains (Fig.2). Significant differentiation could not observe among the strains used in the study.

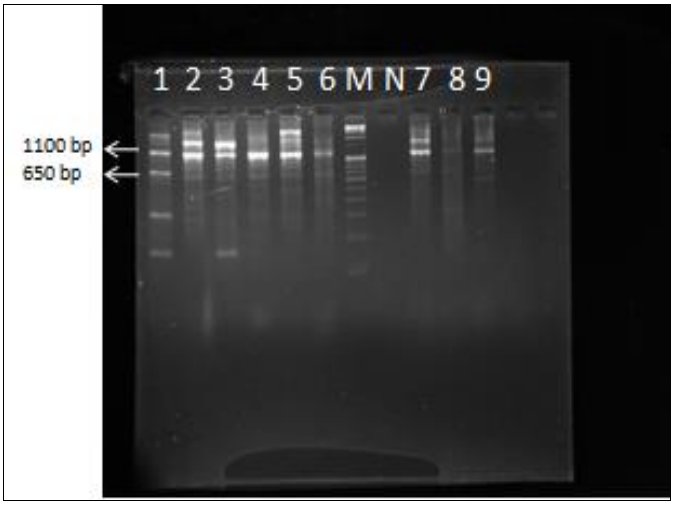
3.2 Whole cell protein profiles of *D. nodosus* isolates by SDS-PAGE
Differences in the protein banding pattern were noticed between serogroups ‘B’, ‘C’ and I. Many protein bands were common between the serogroups (Fig.3). All the isolates of ‘B’ serogroup belonging to virulent, intermediate and benign strains were found to have common banding pattern with an extra band at 180 KDa. A typical band pattern with an extra protein band at 120KDa is seen in ‘C’ serogroup. Serogroup I and A was found to have same protein profile patterns. No protein band could be identified which can differentiate virulence of the strains.

4. Discussion
D. nodosus isolates having different ranges of protease activities were subjected to RAPD using the primers HLWL-74 and HLWL-85 designed by Mazuier *et al.* (1992) [7]. RAPD revealed the diversified bands with both the primers, but a common banding pattern at 400 bp was observed for virulent isolates with primer HLWL-74. The band was absent in benign and intermediate isolates. However, the specificity of the result needs to be assessed by using a greater number of isolates.
SDS-PAGE analysis of whole cell bacterial proteins of the *D. nodosus* isolates showed the differences in the protein band pattern between serogroups. Serogroup I and A was found to have common protein profile patterns. Serogroup ‘C’ revealed a typical pattern with an extra protein band at 120 KDa. No protein band could be identified which can differentiate virulence of the strains. Buller (2005) [1] by conducting PFGE observed that the whole cell protein profiles of *D. nodosus* isolates in the same clonal group were generally similar, but the isolates from different clonal groups and with different zymogram profiles had different protein profiles.



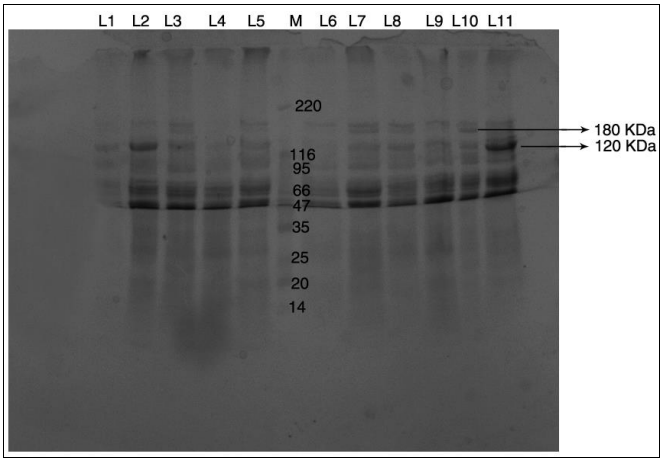
1. JKS-05 2. ARD-I, 3. KDP-I 4. MBNR-I, 5. V-PET-I
6. PNP-I 7. Bon-II-B 8. SKHT-I 9. Bon-I-B

Fig 1: RAPD result for *D. nodosus* isolates with primer HLWL-74



1. JKS-05 2. ARD-I, 3. KDP-I 4. MBNR-I, 5. V-PET-I 6. PNP-I 7. Bon-II-B 8. SKHT-I 9. Bon-I-B

Fig 2: RAPD result for *D. nodosus* isolates with primer HLWL-85



M: Marker, L1: V. Pet - 'I', L2: PNP - 'I', L3: ARD - II- 'B', L4: SKHT- 'I', L5: BON-II 'B', L6: KDP - 'I', L7: JKS - 02- 'B', L8: ARD-I 'B', L9: MON 'A', L10: BON-I 'B', L11: MON-'C'

Fig 3: SDS-PAGE analysis of *D. nodosus* Isolates

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