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Simultaneous Detection and Virulence Characterisation of *Dichelobacter nodosus* from Ovine Footrot by Multiplex PCR

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Abstract: Out of 262 swab samples from feet of clinically footrot affected sheep from Jammu and Kashmir, 135 (52.0%) detected positive for *Dichelobacter nodosus* by *16S rRNA* gene specific PCR. Out of these135 positive samples, 82 (61.74%) tested positive for *intA* gene carried by virulent strains. A mulplex PCR for simultaneous detection and virulence characterization of *D. nodosus* in clinical samples was therefore devised. The test detected 77 (57.03%) samples positive for virulent *D. nodosus*. The results were comparable and the success of the multiplex PCR was established. Out of 30 randomly selected isolates subjected to gelatin gel test, 24 isolates with *intA* gene produced thermostable protease while six isolates without *intA* gene revealed the production of thermolabile protease. This indicated a good co-relation between presence of *intA* gene and gelatine gel test in determination of the *D. nodosus* virulence.

Key words: Footrot, Gelatin gel test, *intA* gene, multiplex PCR, 16S rRNA, India

INTRODUCTION

Footrot is a highly contagious bacterial disease of ruminants particularly of sheep and goats caused by the synergistic action of many bacteria of which *Dichelobacter nodosus* (*D. nodosus*) is the essential causative agent (Thomas, 1964). Based on clinical expression two main forms are recognised; benign and virulent. The classification of the *D. nodosus* organism into virulent or benign is based on production of thermostable protease by virulent strains or heat labile protease by benign strains (Palmer, 1993). Since, virulent form of footrot is of major economic concern, it is the target of eradication. The organism is a strict anaerobe and has fastidious growth requirements thus, the diagnosis and virulence characterization of the disease based on isolation of the organism is an extremely difficult task (Gradin and Schmithz, 1977). However, PCR based detection of the organism in the clinical sample makes the task of its diagnosis easy (Fontaine *et al*., 1993). Similarly Cheetham *et al*. (2006) described PCR based assay for the integrase gene (*intA*) and concluded that there is high correlation between intA and ability of the organism to cause virulent footrot. Even though the disease is present in several parts of India, it has become enzootic throughout the state of Jammu and Kashmir. There are several reports of its occurrence and economic impact from different parts of the state (Wani *et al*., 2007; Farooq *et al*., 2010; Taku *et al*., 2010). In these studies, it is of interest to note that serogroup B is the only strain identifiable in most flocks in Jammu and Kashmir and infection with multiple serogroups is rare. The present study records the simultaneous detection and virulence characterization of footrot in Jammu and Kashmir.

MATERIALS AND METHODS

Collection of samples: Total 262 samples were collected from feet of clinically affected sheep from house hold flocks in Pulwama, Anantnag and Shopian districts of Jammu and Kashmir. The site selected for sample collection was the active lesion that developed between the horn of the hoof and the sensitive underlying tissues. Samples were collected on sterile cotton swabs (HiMedia, India) from each animal for detection and virulence characterisation of *D. nodosus*.

Extraction of bacterial DNA: The material present on the swab was suspended in 100 μL of sterile Phosphate Buffered Saline (PBS) in 1.5 mL microcentrifuge tubes by gentle vortexing. The samples were boiled for 10 min, cooled on ice for 10 min and centrifuged at 10,000×g for 1 min. Total of 2 μL of this supernatant was used as template for each PCR reaction.

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Detection of *16S rRNA* gene of *Dichelobacter nodosus* by PCR: PCR amplifications were performed in 25 μL in 0.2 mL thin walled PCR tubes. The PCR mixture contained a final concentration of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 3 mM $MgCl_2$, 0.5 μM concentration of each primer, 0.2 mM concentrations of each 2'-deoxynucleoside 5'-triphosphate and 1.0 U of Taq DNA. Amplification was carried out as per the cycling condition described by Fontaine *et al.* (1993) and Every (1982). The PCR products were electrophoresed in 0.8% agarose gels, stained with ethidium bromide visualized under Ultraviolet (UV) illumination and photographed using gel documentation.

Virulence characterisation of *Dichelobacter nodosus:* To ascertain the virulent or benign status, all the samples positive for *D. nodosus* were screened for presence of *intA* gene by PCR as described by Cheetham *et al.* (2006) and Farooq *et al.* (2010) 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 $MgCl_2$ was 2.0 mM. The amplification cycles consisted of initial denaturation at 94°C for 2 min followed by 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. The PCR products were analysed in 1.5% agarose gels, stained with ethidium bromide, visualized under ultraviolet illumination and photographed with gel documentation system.

Gelatin gel protease thermostability assay: *Dichelobacter nodosus* was isolated from 30 randomly selected 16S rRNA positive samples. These isolates were screened for *intA* gene and then subjected to gelatin gel protease thermostability assay for virulence determination. The test was carried out as per the procedure described by Palmer (1993).

Multiplex PCR for simultaneous detection and virulence characterization of *Dichelobacter nodosus:* In order to detect the presence of *D. nodosus* as well as to determine the virulent status simultaneously in a single PCR assay, samples that were positive for 16S rRNA were subjected to multiplex PCR using primers for both 16S rRNA and integrase A (*intA*) gene. PCR conditions were similar to that used for *16S rRNA* gene amplification. Final confirmation of the PCR products was done by agarose gel electrophoresis.

RESULTS

Detection of *Dichelobacter nodosus* : Out of 262 samples that were subjected to 16S rRNA PCR for detection of *D. nodosus* directly from clinical sample, 135 (52.0%) were found to be positive as they revealed an amplicon of 783 bp (Fig. 1).

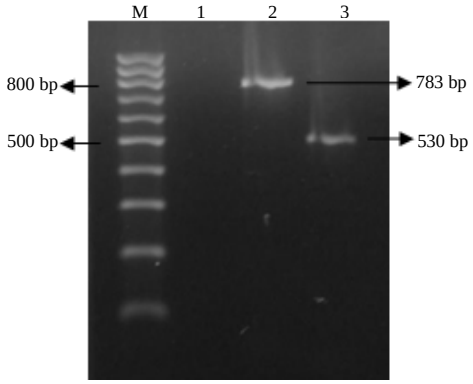

Fig. 1: Detection and virulence of *D. nodosus* in clinical samples by PCR. Lane M: 100 bp DNA marker; Lane 1: Negative control; Lane 21: *16S rRNA* gene specific band; Lane 3: *intA* gene positive sample

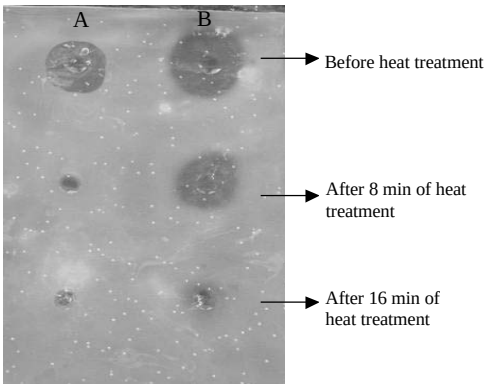

Fig. 2: Gelatin gel protease thermostability assay of *Dichelobacter nodosus* isolates; Strain A: Benign; Strain B: Virulent

Virulence characterisation of *Dichelobacter nodosus:* Out of 135 samples positive for *D. nodosus* by species specific 16S rRNA PCR, 82 (61.74%) produced an amplicon of 530 bp specific to *intA* gene (Fig. 1).

Gelatin gel test: Out of 30 representative isolates subjected to gelatin gel protease thermostability assay, 24 isolates with *intA* gene were also able to produce thermostable protease while remaining four isolates without *intA* gene revealed the production of heat-labile protease (Fig. 2).

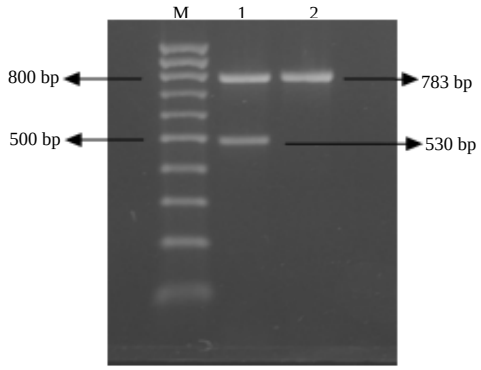

Fig. 3: Multiplex PCR for detection of virulent footrot. Lane M: 100 bp marker; Lane 1: 16S rRNA and *intA* gene specific products; Lane 2: 16S rRNA specific product

Multiplex PCR for simultaneous detection and virulence characterization of *D. nodosus* in clinical samples: Out of 135, *16S rRNA* gene positive samples subjected to multiplex PCR for simultaneous detection and virulence characterization of *D. nodosus*, 77 (57.0%) revealed the presence of two bands, one specific to *D. nodosus 16S rRNA* gene (783) bp and other specific for *intA* gene (530 bp). Remaining 58 samples showed the presence of *16S rRNA* gene specific amplicons only (Fig. 3).

DISCUSSION

The sheep and goat population in the state of Jammu and Kashmir is 3.4 and 2.0 million, respectively (Govt. of India, Ministry of Agriculture, Department of Animal Husbandry, Dairying and Fisheries in 2003). Due to the favourable temperate climatic conditions footrot has become enzootic in the sheep population causing great economic loss to sheep production, particularly in view of the lack of effective vaccine (Farooq *et al*., 2010). Out of the two clinical forms of the disease it is the virulent form which causes severe disease and is often the target of costly control programme. Thus, reliable methods for the diagnosis and virulence characterization of the organism are needed. The present reseach was therefore, under taken to work out an efficient and rapid diagnostic strategy for footrot as well as to determine the virulent or benign status of *D. nodosus* directly in clinical samples.

When used in isolation, 16S rRNA based PCR could detect only 52.0% of clinical cases. These findings correspond to findings of Hussain *et al*.. (2009) who reported 52.34% sensitivity and Farooq *et al*. (2010) who could detect 56.0% of cases by this method. There can be several reasons for low sensitivity of the test. Most obvious reason seems the presence of non specific PCR inhibitors in the samples. Hoof horn, soil particles and humic acid in the samples. Belloy *et al*. (2007) were not able to detect *D. nodosus* in ibex and mouflon by using direct PCR as described by Fontaine *et al*. (1993). Similar observations were made by Moore for the detection of *D. nodosus* from clinical samples from sheep. Presence of humic acid in swabs contaminated with faecal material may act as a barrier to the successful amplification of the target DNA as it is known to be a powerful inhibitor of Taq polymerase (Tsai and Olson, 1992). Other possible reasons could be the crude method of DNA extraction, low copy number of the organism or presence of other disease with clinical picture similar to footrot.

Genotypic virulence characterization of *D. nodosus* like detection of *intA* gene (Cheetham *et al*., 2006) offers some advantages over phenotypic characterization using elastase test (Egerton *et al*., 1969), electrophoretic zymogram test (Every, 1982; Kortt *et al*., 1982; Gordon *et al*., 1985), gelatin gel test (Palmer, 1993) and monoclonal antibody ELISA (Riffkin *et al*., 1995) which often require the culture of organism. In the present study 61.74% samples amplified *intA* gene specific amplicon thus were declared to carry the virulent strains of *D. nodosus*. Similar results from south Kashmir were reported by Farooq *et al*. (2010). However, Hussain *et al*. (2009) reported that 72.28% of the isolates from Kashmir carried *intA* gene. Virulence was also tested by gelatin gel thermostability test. A strong correlation between the presence of *intA* gene and the protease thermostability of *D. nodosus* isolates in the gelatin gel test was observed. This is in agreement with the findings of Cheetham *et al*. (2006) who also observed a high correlation between the presence of the *intA* gene and the thermostability of *D. nodosus* strains in the gelatin gel test.

CONCLUSION

In the present study multiplex PCR for simultaneous detection and virulence determination of *D. nodosus* was attempted. Out of 135 *16S rRNA* gene positive samples, 77 (57.25%) samples revealed the presence of two bands of 783 and 530 bp indicating the presence of virulent strains of *D. nodosus*. Five samples (3.70%) which were positive for intA failed to reveal the same upon multiplex PCR. This small percentage of false negative can be neglected in view of the time advantage provided by multiplex PCR. Since, no research has been done regarding this, no data is available to compare these findings.

Apart from establishing the occurrence of virulent footrot in Jammu and Kashmir, this study also explores the possibility of multiplex PCR for detection of virulent footrot without the need for doing manually cumbersome gelatine gel protease thermosatbility test. This test saves time and effort and thus may help in eradication and control programme.

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