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Phenotypic Virulence Characterization of *Dichelobacter nodosus* from the Cases of Ovine Footrot in Andhra Pradesh and Telangana states of India

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A B S T R A C T

Footrot is a contagious disease affecting the interdigital epidermis and living tissues of digits of sheep and goats. The disease is being reported in Andhra Pradesh (AP) and Telangana states of India regularly causing significant economic loss to the sheep husbandry. The causative agent is the anaerobic, gram negative bacteria, *Dichelobacter nodosus*. A total of sixteen isolates of *D.nodosus* have been recovered from footrot positive clinical samples collected from AP and Telangana state and further confirmed by PCR targeting 16Sr RNA gene. The isolates were further processed for virulence characterization by Gelatin gel test and elastase test. Gelatin gel test revealed 56.5% of isolates as virulent, 31.25% of isolates as Intermediate and 12.25% of isolates as benign. Whereas elastase test identified only 18.75% of isolates as virulent, 12.5 % of isolates as intermediate, 12.5% of isolates as benign, the rest 56.25% of the isolates did not exhibit any elastase activity.

Introduction

Footrot is highly contagious disease of sheep and goats caused by an obligatory anaerobic bacteria *Dichelobacter nodosus.* Ovine footrot in India was reported for the first time in India by Wani *et al.*, 2004. Virulence of the *D.nodosus* is an important factor in developing clinical footrot disease in sheep and goats. Therefore it was important to establish knowledge of virulence of the *D.nodosus* and the corresponding manifestations of footrot. *D.nodosus* were categorized as virulent, intermediate and benign based on the corresponding clinical forms of the disease (Stewart *et al., 1*989).

Sometimes the clinical diagnosis of virulent footrot is not easy, particularly in the early stages of an outbreak, or during hot dry

conditions when footrot cannot express fully. Therefore laboratory tests like elastase and gelatin gel tests which measures protease activities have been developed to confirm the clinical diagnosis. *Dichelobacter nodosus* produces different types of proteases like gelatinase, elastase fibrogenase, collagenase and caesinase (Kortt *et al., 1*994). The degree of virulence of various *D.nodosus* strains are mainly due to differences in expression of the subtilisin-like extracellular proteases AprV2/ B2, AprV5/B5 and BprV/B (Stauble *et al.*, 2014). Among them the expression of two proteases known as virulent AprV2 and AprB2 was found to fully correlate with the clinical status clinical status. The elastase test measures the quantitative activity of proteases, while the gelatin gel test measures a difference in protease thermostability between the strains of *D.nodosus* (Liu and Yong, 1993; Palmer 1993). Thermostable strains retain their ability to hydrolyze gelatin even after heating at 68^{o}C for 16 min where as benign strains produced proteases that are susceptible to heat and did not retain gelatinase activity (Palmer 1993). The gelatin gel test has the advantage of being a more rapid test. It takes 2-3 days for testing of protease activity. Whereas, the elastase test requires 21 days to study elastase activity (Links and Morris, 1996). The aim of the study is to characterize *D.nodosus* isolates with respect to virulence. In the present investigation gelatin test and elastase test were used to study the virulence of *D.nodosus* isolates recovered in the study.

Materials and Methods

Collection of samples

A total of 338 foot swabs were collected from 13 villages of Chittoor, Nellore, Praksam and Mehaboobnagar districts of Andhra Pradesh and Telangana state from sheep with foot lesions (Fig. 1 and 2).

Material from foot lesions were collected aseptically from individual hooves using sterile cotton swabs and inoculated on Trypticase arginine serine (TAS) agar with 4% hoof powder. The samples were processed for isolation and identification of *Dichelobacter nodosus.*

Preparation of TAS agar

TAS agar comprising Trypticase Peptone-1.5 g, Protease Peptone 0.5 g Beefextract-0.5g, Yeast extract-0.2g, Magnesium sulphate-0.2g, BactoAgar -3.0 g. Hoof powder was added at the rate of 4% and 2% for preparation of primary isolation and maintenance media respectively was prepared. Then medium was autoclaved at 15lb pressure for 15 minutes. After autoclaving, it was allowed to cool and 50X serine arginine solution were added at the rate of 2.5 ml per 100 ml of agar. and dispersed into sterile Petri plates and subjected to sterility test before usage.

Preparation of 50x arginine serine solution

Serine-3.75 g and Arginine -12.50g were weighed and added to 50ml of sterile triple distilled water and mixed until dissolved. Then the solution was sterilized by 0.2µ Millipore filter and stored at -20ºC for further use.

Inoculation into TAS

Samples collected from footrot clinical lesions were inoculated on to 4% Hoofagar plates. These plates were placed in an anaerobic jar (Oxoid) with gaspacks (BD, difco) and incubated at 37^{o}C. After five days of incubation, suspected colonies were sub cultured on the 2% Hoofagar plates. The presence of *D.nodosus* in the colonies was confirmed by PCR targeting 16S*r* RNA.

Polymerase chain reaction for detection of *D. nodosus*

DNA from the suspected colonies of *D.nodosus* was extracted by boiling method. PCR for detection of 16S*r*RNA gene of *D.nodosus was* carried out as per the method of Wani *et al.*, (2004, 2007). Details of the primer sequence are enlisted in Table 1.

PCR amplification was performed in 200µl PCR tubes with a reaction mixture 25µl comprising 10x Taq buffer A- 2.5µl, 10 mM dNTP mix - 2µl, $MgCl_2$ 25 mm - 1.5µl, Taq DNA polymerase (3U/ µl) - 0.3µl, Forward Primer (10pico moles)- 0.25µl, Reverse Primer(10picomoles)-0.25µl, DNA Template-2µl, DEPC water - 16.25µl.

The tubes were then spun for 10 seconds and PCR was carried out in Thermal cycler (Kyratec) with Cycling conditions of intial denaturation at 94^{o}C for 2min, five cycles of denaturation for 30 sec at 94 ^{o}C, annealing for 30sec at 62^{o}C and extension at 72^{o}C for 4m, 25 cycles of denaturation for 30 sec at 94 ^{o}C, annealing for 30sec at 62^{o}C and extension at 72^{o}C for 30sec and final extension at 72^{o}C for 4min. The JKS-02 strain maintained and characterized in the department was used as positive control.

Detection of *D. nodosus* sero group by multiplex PCR

The positive samples for *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 used in the study.

Agarose gel electrophoresis of PCR product in 2% agarose

Amplified products were analyzed by agarose gel electrophoresis. under U.V trans-illuminator and photographed with Gel Documentation System (Alpha Innotech, AlphaImager HP).

Virulence characterization of *D. nodosus* isolates

Gelatin gel test

The gelatin gel test was carried out as per the method developed by Palmer (1993) based on the principle that extracellular proteases produced by virulent strains of *D. nodosus* are more heat stable than those produced by benign strains. Briefly *D. nodosus* isolates were grown in TAS broth separately for 2-4 days to achieve a concentration of 1X10^{8}cells/ml, measured by spectrophotometric reading.

500µl of this broth cultures was diluted with 2 ml of Hepes buffer, mixed well and an aliquots of 20µl was placed into the one of the three wells of an agarose gel containing gelatin in a petriplates. The remaining quantity in dilution was incubated at 68^{0}C for 8 min in water bath and aliquot comprising 20µl was placed in second well.

The sample was further incubated for 8 min at 68^{0} C and 20µl of it placed into the last well. The gels were incubated overnight in a moist chamber at 37^{0}C, after which undigested gelatin was precipitated by flooding with hot (60^{0}-70^{0}C) saturated ammonium sulphate solution. The zone of proteolysis indicated by clearing around the wells was measured by scale and recorded in nearest millimeters.

The percentage of thermostabilty was calculated as

$$\% \text{ Thermo stability} = \frac{\text{Zone size of broth culture heated at 67°C for 16min}}{\text{Zone size of broth culture unheated}} \text{ X } 100$$

All the 16 isolates in the study were subjected to Gelatin gel test and the results are recorded.

Elastase test

The elastase activity of *D.nodosus* isolates was measured by Elastin agar test as described by stewart (1979). Isolates were cultured in TAS agar with 2% hoof powder before inoculation in elastin agar comprising Trypticase peptone -1.5 g, Protease peptone - 0.5 g, Beef extract - 0.5g, Yeastextract-0.2g, L-arginine HCl-0.5g, DLserine-0.15g $MgSO_4.7H_2O$-0.2g, Calcium chloride (anhydrous) -0.15 g, Bacto agar (Difco) -1.5 g, Elastin powder (bovine neck ligament, insoluble, Sigma)-0.3 g. All the reagents except the Bacto agar and the elastin powder are dissolved in 100ml of DW in a sterile conical flask containing a magnetic bar.

The pH was adjusted to 7.8-8.0 by adding 10 M NaOH. Agar and elastin powders are dissolved while stirring on a magnetic stirrer until (about 30min) the elastin is well dispersed. Then the medium was autoclaved for 20 minutes at 121ºC and Cooled to 50ºC. The solution was thoroughly mixed to evenly disperse the elastin particles. The medium is poured aseptically into sterile petridishes and stored in an anaerobic atmosphere at 4ºC.

Inoculation

An elastase agar plate was marked into tridents. A loopful of culture was streaked in a line about 2-3 cm in the middle of each trident and similarly the other two tridents with positive and negative controls.

Plates are incubated in an anaerobic jar with as gas pack at 37ºC. Plates were examined on 7^{th} day, 14^{th} day, 21^{st} day and 28^{th} day.

Results and Discussion

Isolation of *Dichelobacter nodosus*

The 4% hoof agar plates inoculated with suspected foot swab material, incubated under anaerobic conditions at 37°C for seven days showed the multiple colonies along the line of streaking. *D.nodosus* colonies mixed with others were identified based on the colony morphology which has diffuse ground glass appearance particularly around the colony edge and grow out and away from streak line (Fig. 3). A total of eight isolates have been isolated in the study. The smears made out of the pure colonies revealed the presence of gram negative rod shaped, slightly curved organisms with terminal swollen ends characteristic of *D.nodosus* (Fig. 4). Along with this eight isolates, six isolates (Serogroup 'I'-4, Serogroup 'A'-1 and Serogroup 'C'-1) which were already isolated previously were used in the study. A positive control JKS-20 serogroup 'B', SUKAST was also used in the study

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

The DNA extracted from all the eight isolates revealed specific amplicons of 783bp size (Fig. 5) suggestive of *D.nodosus* infection. The samples positive by 16SrDNA PCR were subjected to Multiplex PCR. Out of 8 isolates, five isolates revealed specific amplicons of 283bp size suggestive of 'B' serogroup (Fig. 6) (Nellore-3 and Mehaboobnagar-2) and three isolates revealed specific amplicons of 189 bp size suggestive of 'I' serogroup (Fig. 7) (Chittoor-3). The eight isolates along with seven isolates (Seroogroup 'B'-1, Serogroup 'I'-4, Serogroup 'A'-1 and Serogroup 'C'-1)

which were already isolated previously were used in the study.

Protease thermostability test (Gelatin gel test)

The thermo stability of proteases from the isolates was calculated by dividing the zone size of TAS broth culture heated 67^{o}C for 16 min by that of unheated control (0 min). The diameter of the zone of clearance around each well was measured in nearest millimeters. The isolates with percentage of thermo stability between 0 and 10% were considered as benign, those between 11-60% as intermediate and those above 60% are considered as virulent. The results were depicted in table 3. Out of 15 isolates tested for gelatin gel test, 8 isolates were found to be virulent with % thermostability >60, 5 isolates were found to be intermediate with % thermostability 11-60 and two isolates were found benign with % thermostability <10 (Fig. 8).

Elastase test

Out of 15 isolates tested two isoltes JKS-02 and Bon I were found to degrade elastin within seven days and are considered as virulent. Che-I and Mon-I degraded elastin in 7-14 days and are considered as intermediate, where as the Ard-I, Yplm-I degraded elastin in 14-21 days and are considered as benign and the rest of isolates did not show in elastase activity. The results are shown in Table 4 and Fig. 9.

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

Primer	Primer sequence (5'-3')	Target gene	Reference	Product size (bp)
Forward	CGGGGTTATGTAGCTTGC	16S r RNA	LA Fontaine, *etal.*,(1993)	783
Reverse	TCGGTACCGAGTATTTCT ACCCAACACCT			

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 rimer – G	CTTAGGGGTAAGTCCTGCAAG	G	279	
Reverse primer – H	TGAGCAAGACCAAGTAGC	H	409	
Reverse Primer – I	**CGATGGGTCAGCATCTGGACC**	**I**	**189**	

Table.3 Details of *D.nodosus* isolates showing virulence activity on Gelatin gel test

S.No	Isolate	Unheated (zone of clearance mm)	Heated (8min)		Heated (16min)		Virulence
			Zone of clearence	% Thermo stability	Zone of clearance	% Thermo stability	
1	JKS-B	21	18	86%	15	75%	Virulent
2	Ard I-B	18	15	83%	12	67%	Virulent
3	Ard II-B	14	8	57%	6	43%	Intermediate
4	MBNR-B	16	9	56%	7	44%	Intermediate
5	Bon I-B	22	18	82%	15	68%	Virulent
6	Bon II-B	15	5	33%	1	6%	Benign
7	Vpet-I	16	8	50%	5	31%	Intermediate
8	SKHT-I	15	5	33%	1.5	10%	Benign
9	Cha-I	18	14	78%	12	67%	virulent
10	Man-I	17	11	65%	9	53%	Intermediate
11	PNP-I	18	11	61%	8	44%	Intermediate
12	Yplm-I	21	17	81%	15	71%	Virulent
13	Bplm-I	22	18	82%	15	68%	Virulent
14	Man-A	23	21	91%	18	78%	Virulent
15	Man-C	21	18	86%	15	71%	Virulent

Table.4 Results of Elastase test

S.No	Isolate	Elastase activity			No elastase activity
		7^{th} day	14^{th} day	21-28 days	
1	JKS-B	+			
2	Ard I-B			+	
3	Ard II-B				+
4	MBNR-B				+
5	Bon I-B	+			
6	Bon II-B				+
7	Vpet-I				+
8	SKHT-I				+
9	Che-I		+		
10	Man-I				+
11	PNP-I				+
12	Yplm-I			+	
13	Bplm-I				+
14	Man-A		+		
15	Man-C				+

Fig.1 Sheep showing typical lameness

Fig.2 Hoof showing severe lesion of virulent footrot

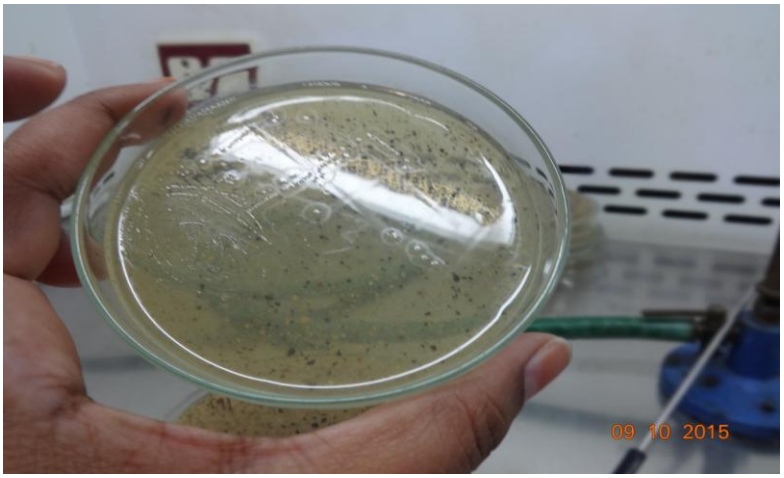
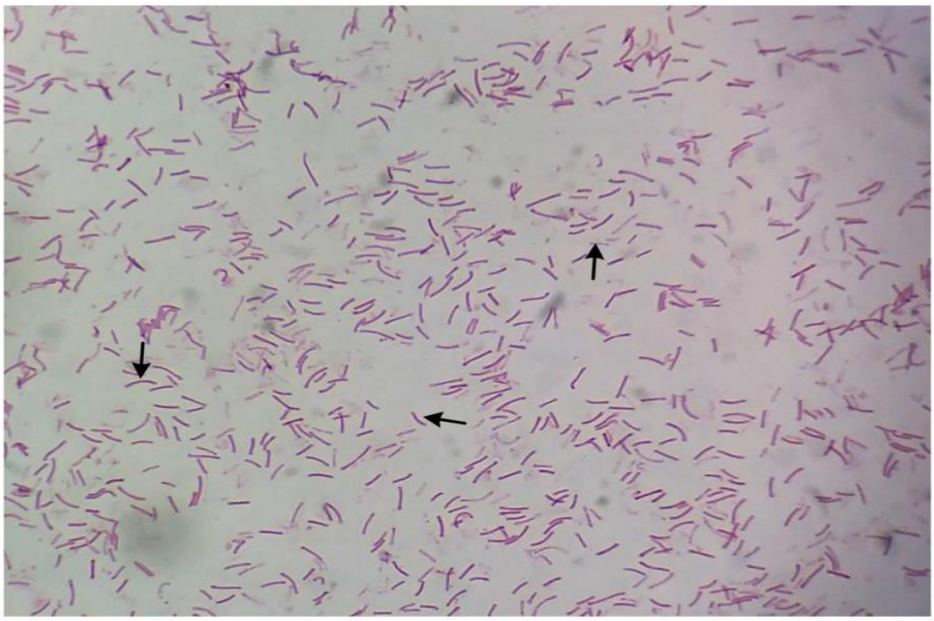
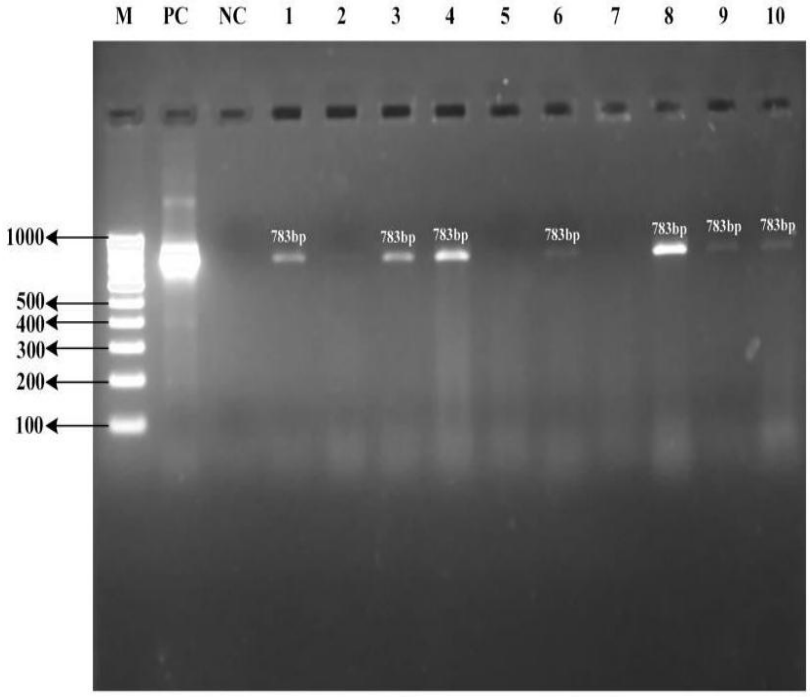


Fig.3 Pure colonies of *D.nodosus* in 2% Hoofagar



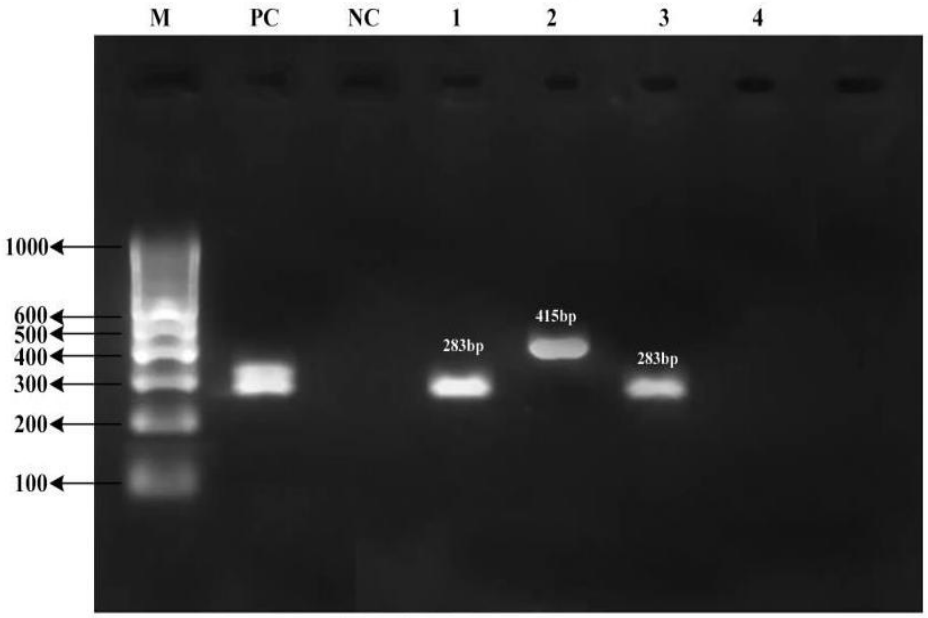
Arrow heads showing Large gram negative curved rods with swollen ends

Fig.4 Gram staining of *D.nodosus*



Lane M – 100 bp Marker
PC – Positive Control
NC – Negative Control
Lane 1, 3, 4, 6, 8, 9, 10 – 783 bp PCR amplified product

Fig.5 Amplification of 16SrRNA gene of *D.nodosus*



Lane M – 100 bp Marker
PC – Positive Control
NC – Negative Control
Lane 1, 3 – 283 bp 'B' serogroup
Lane 2 – 415 bp 'A' serogroup

Fig.6 Serogroup 'B' and 'A' specific PCR products of *D.nodosus*

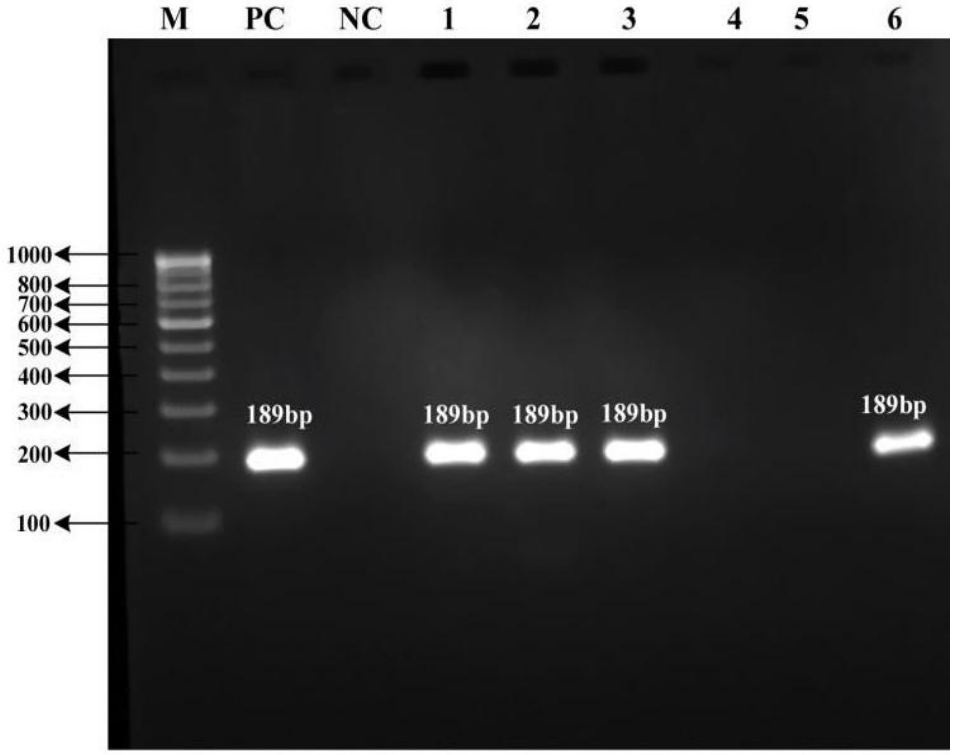
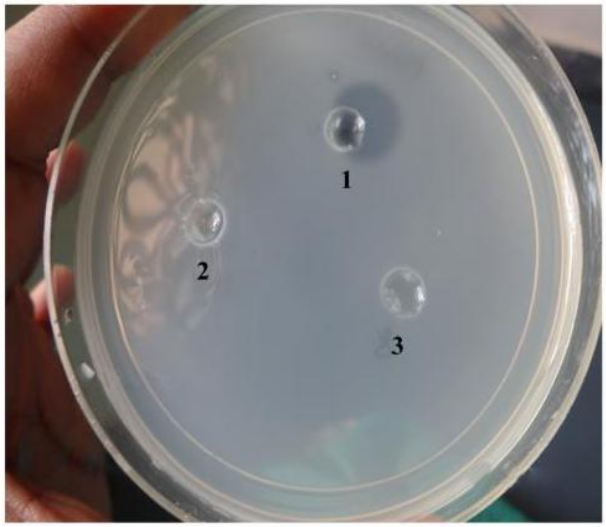


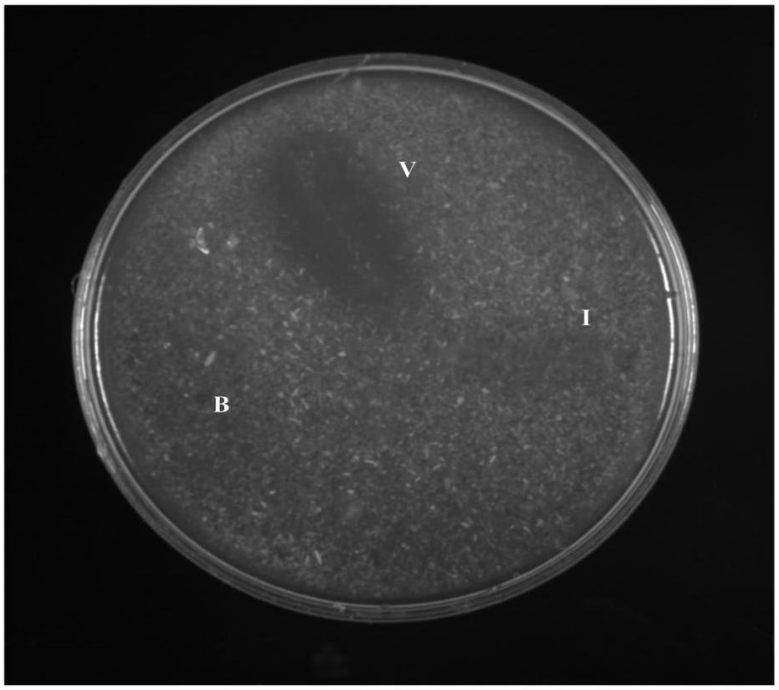
Fig.7 Serogroup 'I' specific PCR products of *D.nodosus*

Clearance Zone of TAS broth cultures
1- Unheated
2- Heated for 8 min
3- Heated for 16 min



Clearance Zone of TAS broth cultures
1- Unheated
2- Heated for 8 min
3- Heated for 16 min

Fig.8 Results of gelatin gel test
a. Presence of thermostable proteases b. Presence of Thermolabile proteases



Zone of hydrolysis of Elastin
V-Virulent Strains (>7 days)
I- Intermediate Strains (7-14 days)
B-Benign Strains (>21 days)

Fig.9 Results of elastase test

In the present study gelatin gel test and elastase test were used to study the virulence of *D.nodosus* isolates. Gelatin gel test revealed that 53% of the isolates were found to be virulent followed by 33% of the strains are intermediate and 14% of the strains are benign. Whereas the elastase test performed on the same isolates, 13% of isolates are found to be virulent, 13% isolates are found to be intermediate and 13% isolates found to be benign. Whereas the rest of 61% isolates did not exhibit any elastase activity. Similar results were reported by Palmer, (1993) and Liu *et al.,* (1993).

Egerton *et al.,* (2000) and Stewart (1989) stated that the phenotypic expression of *D.nodosus* is often influenced by factors that affect the growth and viability of the bacteria such as seasonal and local conditions. According to Liu *et al.,* (1994) the diagnostic test based on the measurement of phenotypic characterization of *D.nodosus* are variable with factors and conditions that affect the growth of these bacteria. In addition a slight change of the conditions in these tests might also cause some variation in test results like the usage of different brands and batches of elastin might also has a bearing on the results of elastase test.

The *D.nodosus* isolates defined as virulent by Gelatin gel test belongs to four different serogroups. Whereas the intermediate and benign strains belonged to two serogrups only. The greater serogroup diversity of virulent isolates indicates a greater genetic variation among virulent variants. These results were completely contradictory to the results reported by Gilhus *et al.,* (2013) in Norway where the greater serogroup diversity is seen in benign strains which belongs to eight different serogroups. The agreement between elastase test and the gelatin test did not tally in the present study. There is a difference in the number of isolates proved to be virulent in Gelatin gel test (53%) and Elastase test (13%). Similarly with intermediate in Gelatin gel test (33%) and elastase test (13%). However there is slight correlation with benign strains of 13% each. 61% of isolates did not show any activity of elastase.

Links and Morris, (1996) and Gillhus *et al.,* (2013) worked on the similar lines and reported that there is agreement between elastase test and Gelatin gel test. The difference in agreement between GG test and elastase test in the present study may be that the isolates produced a thermostable protease which is different from elastase. *Dichelobacter nodosus* produces different types of proteases like gelatinase, fibrogenase, collagenase, caesinase and elastase (Egerton, 2000; Kortt *et al., 1*994). Kennan *et al.,* 2001 reported that the analysis of mutants of extracellular protease genes has shown that AprV2 thermostable protease is responsible for extracellular protease activity. AprV2 gene codes for elastases.

This difference in the proteases secreted by the local isolates might be the reason for existence of strains of *D.nodosus* in tropical climates where the temperature may reach to 47^{o}C in summer which was reported earlier by Sreenivasulu *et al.,* (2012). It is also possible that changes in protease thermostability are due to a conformational change in the protein and is not a genetic abnormality, or is not one that can be detected by the typing methods used here. Previous work has indicated that the difference between a protease (V2) from a virulent isolate and a protease (B2) from a benign isolate was due to a single amino acid change. It was hypothesised that the protease genes may have diverged from a common ancestral gene (Riffkin *et al.,* 1995).

Other researchers suggest that the isoenzyme bands arise from three or four closely-related genes that code for protease (Moses *et al.,*

1995). Stauble *et al.*, (2014) developed a competitive real time PCR for detection and demonstration of virulent and benign *D.nodosus* targeting AprV2 and BprV2 which is a rapid and sensitive diagnostic tool for the early detection and virulotyping of *D.nodosus* directly from simple, non-invasive interdigital swabs of sheep. Locher *et al.*, (2015) stated that the competitive PCR developed for detection of AprV2/B2 genes of *D.nodosus* will be very useful for nationwide fotrot control programme.

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