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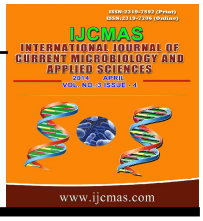

Original Research Article

Identification of anaerobic and concomitant aerobic bacterial etiologies in caprine footrot

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A B S T R A C T

Footrot is a mixed bacterial infection, caused by the synergistic action of highly fastidious anaerobes to easily cultivable aerobes. The present study was conducted to identify the bacterial microflora involved in footrot lesions in goats. A total of 78 samples were collected from goats in Kerala. They were subjected to a combination of identification techniques like PCR, for detection of *Dichelobacter nodosus* and *Fusobacterium necrophorum*, and isolation and identification for aerobic bacteria involved in the lesions. Among the 78 samples, 12 were positive for *D. nodosus* and 8 were positive for *F. necrophorum*. Total Viable Count (TVC) of the aerobes isolated from infected animals were done from healthy as well as infected animals and the values were subjected to statistical analysis using independent t- test to identify the organisms whose TVC significantly differed between the two groups. Results revealed that only *Staphylococcus* spp. and *Streptococcus* spp. were associated with footrot condition in goats.

Keywords

Footrot;
Goats*;*
D. nodosus;
F. necrophorum,
Staphylococcus spp;
Streptococcus spp

Introduction

Footrot is a contagious, debilitating and economically important disease of sheep and goats (Stewart *et al*., 1984; Tadich and Hernandez, 2000). The disease is prevalent worldwide and has severe economic impact in countries that have temperate climates and moderate to heavy rainfall (Stewart, 1989). *Dichelobacter nodosus* (*D. nodosus*) is the causative agent of footrot in goats (Egerton *et al*., 1969). The typical condition for the invasion of *D. nodosus* is created by the secondary bacterium, called as *Fusobacterium necrophorum* (*F. necrophorum*), which is a ubiquitous pathogen in soil and faeces. Both of them are Gram negative, fastidious, slow growing strict anaerobes, making the identification of these organisms by isolation very tedious and time consuming (Gradin and Schmitz, 1977; Langworth, 1977). Therefore, even if cultural isolation of the organisms is confirmative of the disease, absence of

growth of the bacteria in media cannot be considered as negative. Thus, specific and sensitive detection methods employing molecular techniques are necessary for the easy and confirmative diagnosis of *D. nodosus* and *F. necrophorum*.

The role of other aerobic bacteria commonly seen in soil, skin, faeces and the surrounding environment cannot be neglected in development of footrot lesions in goats. Aerobic bacteria such as *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli* and *Archanobacterium pyogenes* (*A. pyogenes*) can also be involved in footrot (Hudson, 1982; Teshale, 2005). Even though these bacteria cannot initiate footrot, they may increase the severity and incidence of the footrot lesion caused by *D. nodosus* and *F. necrophorum* (Currin *et al*., 2009). Thus, the organisms responsible for footrot may range from easily cultivable ones to highly fastidious organisms. Therefore, a combination of cultural identification along with molecular techniques like PCR should be employed for a comprehensive, sensitive and specific detection of organisms associated with foot lesions in goats.

A very few footrot cases have been reported from the Southern states of India. The only reported incidence of footrot from Kerala is by Thomas and coworkers in 2011. Graham and Egerton (1968) and Stewart *et al* (1984) reported the importance of a warm and wet weather for disease outbreaks. Kerala has a wet and maritime tropical climate influenced by the seasonal heavy rains of the southwest summer monsoon and northeast winter monsoon, which is very congenial for foorot outbreak. Overall economic impact of footrot was estimated to the tune of 0.3 million dollar annually to sheep farming in central Kashmir in India (Rather *et al*., 2011).

The present study was conducted to detect the aerobic and anaerobic bacterial pathogens involved in footrot lesions in goats in Kerala.

Molecular technique employing PCR was used for the detection of *D. nodosus* and *F. necrophorum*, while cultural isolation and identification was used for the detection of easily cultivable aerobes. PCR using species specific 16 S rRNA and *lkt*A primer pairs were found to be very specific for the detection of *D. nodosus* and *F. necrophorum* respectively (Bennett *et al*., 2009a; Hickford *et al*., 2010; La Fontaine *et al*., 1993; Moore *et al*., 2005 and Zhou *et al*., 2009) To avoid the false detection of the commensal aerobes seen in soil, faeces and skin as footrot pathogen, isolation of the aerobes from footrot lesions were done and Total Viable Count (TVC) of those aerobes from healthy and infected animals were studied. They were compared using independent t- test to confirm the association of the tested aerobes with footrot lesion.

Materials and Methods

Collection of samples and extraction of genomic DNA

A total of 78 samples were collected from naturally infected goats having foot lesions and lameness in Kerala. The exudates from the lesions were collected using sterile cotton swabs in duplicates. Genomic DNA was extracted from all the 78 samples using crude method (Wani *et al.*, 2007)

PCR detection of *D. nodosus*

PCR detection of *D. nodosus* was carried out by using species specific 16S rRNA primer pairs, 5'CGGGGTTATGTAGCTTGC3' (forward) and 5'TCGGTACCGAGTATTTCTACCCAA CACCT3' (reverse) (La Fontaine *et al*., 1993). The PCR amplifications were performed in 25 µl volumes. The final concentration contained 2 µl of template, 2.5 µl of 10X *Taq* buffer (10 mM Tris-HCl (pH 9.0), 50mM KCl, 15mM MgCl), 25 pM each of forward and reverse primers, 200 µM of each deoxyribonucleotide triphosphate and 1 IU *Taq* DNA polymerase. The amplification consisted of an initial denaturing step of 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 58°C for 30 s and 72°C for 30 s, with a final extension step at 72°C for 5 min, in a thermal cycler (Eppendorf). A negative control without template DNA was included for each reaction.

PCR detection of *F. necrophorum*

Fusobacterium necrophorum was detected by PCR amplification of leukotoxin A (*lkt*A) gene using primers 5'AATCGGAGTAGTAGGTTCTG-3' (Forward) and 5'CTTTGGTAACTG CCACTGC3' (Reverse) (Zhou *et al*., 2009). The PCR amplifications were performed in 25 µl volumes. The final concentration contained 2 µl of template DNA, 2.5 µl of 10 X *Taq* buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 15 mM MgCl), 25 pM of each primer, 200 µM of each deoxy- ribonucleotide triphosphate and 1 IU *Taq* DNA polymerase. Thermal profile for amplification of *lkt*A gene consisted of denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 58°C for 40 s and 72°C for 30 s, with a final extension step at 72°C for 10 min. A negative control without template DNA was included for each reaction.

Analysis and sequencing of PCR products

The PCR products were subjected to agarose gel electrophoresis and the gels were visualized under ultraviolet illumination (Hoefer, USA) and photographed using gel- documentation system (Bio-rad laboratories, USA).

Purified PCR products of the 16S rRNA gene of *D. nodosus* and *lkt*A gene of *F. necrophorum* were sequenced. The sequences of each PCR products were subjected to sequence similarity search using Basic Local Alignment Search Tool (BLAST) provided by the National Centre for Biotechnology Information (NCBI) and were submitted to GenBank.

Isolation of aerobic bacteria

Hoof swabs from all the 78 samples were inoculated on Brain Heart Infusion Agar (BHIA). The plates were incubated at 37°C for 24-48 hours. Following incubation, all colonies were stained and pure colonies were subcultured on to new sterile BHIA plates. The bacterial isolates were identified based on the morphological, cultural and biochemical characteristics (Barrow and Feltham, 1993). The TVC of the isolated species of bacteria were attempted from healthy and infected animals.

Total Viable Count

Two groups of goats were selected for assessing the TVC of the bacteria. First group comprised of seven healthy animals,

aged between 4 and 6 years and were housed in a single pen. For the second group, 7 infected animals housed in two adjacent pens, aged between 4 and 6 years were selected.

Hoof wash of all the 7 animals from both groups were collected using 100 ml of sterile Phosphate Buffered Saline (PBS). 1 ml of the collected hoof wash was serially diluted up to 10^{-5} using sterile PBS. To determine TVCs of *E. coli*, *Staphylococcus* spp*, Streptococcus* spp. *and Corynebacterium* spp., 1 ml of each dilution was plated on Eosin Methylene Blue agar (Okonko *et al*., 2008), Baird-Parker agar (Ruben and Fairoze, 2011), KF streptococcal agar (Nanu *et al*., 2007), and Cystine-Tellurite blood agar plates, respectively using spread plate method. The plates were incubated at 37^{o}C for 24 hours. The isolates obtained on the plates were identified based on established conventional cultural and morphological characters (Buchman and Gibbons, 1974). Total number of bacterial colonies of each species for different dilutions were counted separately. The plate which showed 30 - 300 colonies of each species were selected for calculating the dilution factor.

The formula for calculating TVC is as follows:

TVC of bacteria / milliliter of the hoof wash = number of colonies × dilution factor

Statistical analysis

TVC of each species of bacteria/milliliter of the hoof wash of the healthy animals (control group) were compared with infected (treatment group) using independent t- test, to check whether there is significant difference between the TVC of *E. coli*, *Staphylococcus* spp., *Streptococcus* spp. *and Corynebacterium* spp. between these two groups.

The bacterial species which showed significant difference of TVC between healthy and infected groups by statistical analysis were only attempted for identification upto species level.

Pathogenicity testing of the isolates

An 18h broth culture of the *Staphylococcus* spp. and *Streptococcus* spp. containing approximately 3 × 10^{8} organisms/ml was inoculated (0.1 ml) intra-peritoneally to 6 Swiss albino mice each. 2 mice were kept as control which were mock inoculated with sterile PBS (pH 7.4). All the mice were observed for 7 days post inoculation for the development of symptoms or lesions.

Results and Discussion

Among the 78 samples screened, 12 were positive for *D. nodosus and* 8 were positive for *F. necrophorum* which were confirmed by the presence of a 783 base pairs (bp) and 404 bp amplicon, respectively in the amplified product during agarose gel electrophoresis. In the negative controls, amplification product was not detected. Result of the representative samples are shown in figure 1 and figure 2. Of the 8 positive cases of *F. necrophorum*, 7 were found to have concomitant infection with *D. nodosus.*

The animals from which *D. nodosus* and *F. necrophorum* could be detected either alone or as mixed infection showed symptoms ranged from lameness to separation of horn with foul smelling necrotic exudate. It was observed that only

adult animals above four years of age were affected with footrot. Around 90% of the cases were reported during the months of October to December and June.

The nucleotide sequence of 16S rRNA and *lkt*A amplicons obtained after sequencing revealed 98% and 97% identity with *D. nodosus* and *F. necrophorum* nucleotide databases, respectively and thus proving the specificity of primers. The obtained sequences were submitted to GenBank to get the accession numbers **JX648295** for *Fusobacterium necrophorum* LktA (*lkt*A) gene and **JX648293** for *Dichelobacter nodosus* 16S ribosomal RNA gene.

The first stage and second stage biochemical characterization of the aerobes isolated from the footrot lesions revealed the presence of *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp. and *E. coli.* Therefore, TVC of these 4 species of bacteria was attempted from the healthy and infected group of goats to obtain values as shown in Table 1. Statistical analysis of the obtained data of each species of bacteria using independent t- test revealed that there is no significant variation in the TVC of *Corynebacterium* spp. and *E. coli* between the treatment (infected) and control (healthy) group (Table 2). However, there existed significant variation in the TVC of *Staphylococcus* spp. and *Streptococcus* spp. between the 2 groups. Therefore, it was inferred that the number of *Staphylococcus* spp. and *Streptococcus* spp. were significantly high in the hoof of goats affected with hoof lesions than the goats with healthy hooves. Hence, the species level characterization of these organisms was carried out.

21 isolates of staphylococci and 5 isolates of streptococcus were obtained from the 12 positive cases of footrot. No bacteria could be isolated from the foot lesion of animal (animal no. CC 141) in which only *F. necrophorum* could be detected. Third stage biochemical reactions revealed that among the 21 isolates of staphylococci, 9 were *S. hyicus*, 6 were *S. intermedius*, 5 were *S. aureus* and the rest was *S. epidermidis.* All the *Streptococcus* spp. isolated were *S. pyogenes*.

None of the isolates were found to be pathogenic to mice, since there was no development of symptoms or death even after seven days post inoculation

PCR detection of *D. nodosus* and *F.necrophorum*

In the present study, out of the 78 samples examined, 12 gave positive result for *D. nodosus* using the 16S rRNA species specific primer pair. Positive samples were confirmed by the presence of an amplicon having size of 783 bp in the electrophoresed gel viewed under UV transilluminator. This result was in accordance with Hussain *et al* (2009), La Fontaine *et al* (1993), Moore *et al* (2005), Taku *et al* (2010) and Wani *et al* (2007). Similar results were also reported by Thomas *et al* (2011), which is the only previous reported case of footrot from Kerala.

It was observed that only adult animals above four years of age were affected with footrot infection. Even though similar observations were stated by Kaler et al (2010) and Quinn et al (2002), further studies have to be conducted to confirm the positive relationship between age and susceptibility to footrot in goats.

Around 90% of the cases were reported during the months of October to December and June during which North East and

Table.1 TVC of *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp. and *E. coli* in healthy and infected animals

Animal No.	TVC of *Staphylococcus* spp.		TVC of *Streptococcus* spp.		TVC of *Corynebacterium* spp.		TVC of *E. coli*	
	Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
1	32000	290000	3200	41000	73000	500000	310	600000
2	15000	320000	4000	55000	88000	540000	800	300
3	60000	120000	0	50000	0	80000	720	0
4	50000	690000	700	80000	63000	0	0	1100
5	19000	770000	4500	73000	59000	55000	0	0
6	62000	80000	2100	66000	0	0	300	420000
7	0	970000	0	42000	44000	0	580	300000

Table.2 Mean values of *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp. and *E. coli* of healthy and infected groups.

Bacterial species	N	Control (Mean ± SE)	Treatment (Mean ± SE)	t-value
Staphylococcus spp.	7	3.89 ± 0.65	5.52 ± 0.156	0.032*
Streptococcus spp.	7	2.42 ± 0.63	4.75 ± 0.04	0.010*
Corynebacterium spp.	7	3.43 ± 0.89	3.01 ± 1.07	0.768^{NS}
E.coli	7	1.83 ± 0.49	3.17 ± 0.96	0.263^{NS}

N= number of animals, SE= Standard Error, *$p \leq 0.05$ (Significant), NS= Not Significant

Fig.1 PCR amplification of 16S rRNA gene of *D. nodosus*. Lane 1, 2 and 3: positive samples; Lane 4: negative control

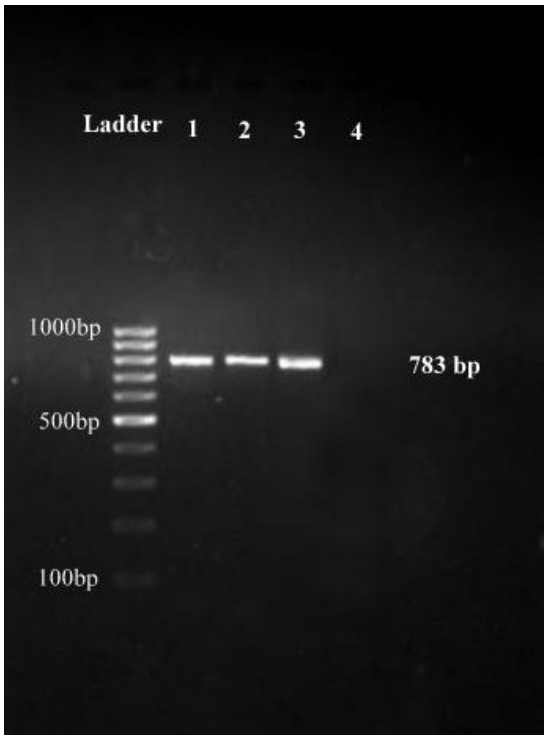
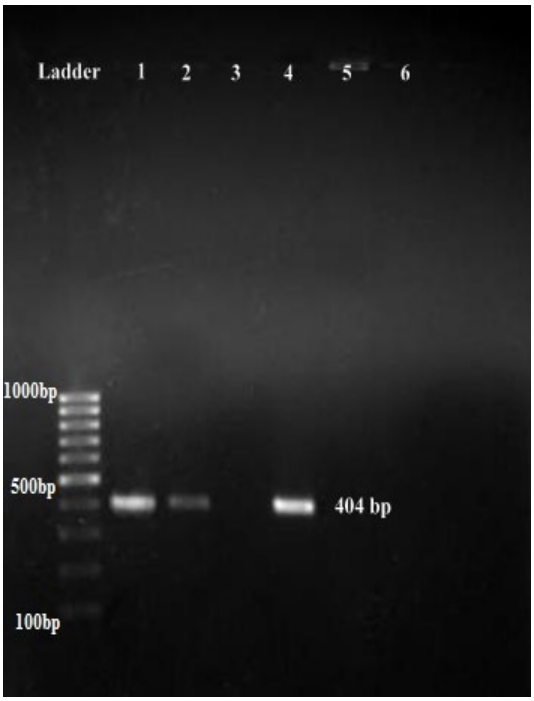


Fig. 2 PCR amplification of *lkt*A gene of *F. necrophorum.* Lane 1, 2 and 4: positive samples; Lane 3: negative sample; Lane 5: negative control



South West monsoons hits Kerala. The reason may be due to the wet and humid climate during the monsoon season that renders the interdigital skin of the animal constantly wet and soft that in turn make a congenial environment for the organisms to enter and elicit infection. Similar observations were reported by Graham and Egerton (1968) and Stewart et al (1984), wherein the importance of a warm and wet weather for disease outbreaks and an environmental temperature constantly above 10°C were key predisposing factors for the natural transmission of footrot.

Among the 78 samples screened, 8 were demonstrated to have *F. necrophorum* by *lkt*A PCR and seven of which were found to have concomitant infection with *D. nodosus*. These findings are in agreement with, Bennett *et al* (2009b), Hickford *et al* (2010) and Zhou *et al* (2009) who could detect the presence *F. necrophorum* in the hooves of lame cattle, sheep and goats by amplification of the *lkt*A gene. Zhou and coworkers (2009) observed that of the 29 samples screened, all were positive for *D. nodosus* whereas 27 cases had a mixed infection with *F. necrophorum.* A similar observation has been made in the present study also, in which about 58% of the clinical footrot cases were caused by mixed infection of both *D. nodosus* and *F. necrophorum*. Hickford *et al* (2010) reported the presence of *F. necrophorum* in a greater proportion of cases than *D. nodosus* in New Zealand dairy cattle.

Also in the present study 1 clinical sample had the presence of only *F. necrophorum*, without the presence of *D. nodosus.* Similar findings were reported in a study conducted by Bennet *et al* (2009a). They observed the presence of only *F. necrophorum* in 5% of samples collected from animals having symptomatic footrot.

Identification of aerobic bacteria associated with footrot

The first stage and second stage identification of the bacteria isolated from the hoof swab of the footrot lesion revealed the presence of *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp. and *E. coli*. Therefore, TVC of these 4 species of bacteria were only attempted from healthy and infected animals. Statistical analysis revealed that, there was no significant variation in the TVC of *E. coli* and *Corynebacterium* spp. between the control and treatment group. So it was concluded that, *Staphylococcus* spp. and *Streptococcus* spp. are the only aerobic bacteria that are significantly associated with footrot lesions in goats. The results obtained are in accordance with Hudson (1982) and Teshale (2005) who could successfully isolate staphylococci and streptococci from animals with footrot. Similar finding was also been made by Calvo-Bado *et al* (2011) who reported the association of *Staphylococcus* spp. with virulent footrot in sheep. The present study emphasizes the importance of commensal aerobes, in the development of footrot lesions in goats. The significantly high number of *Staphylococcus* spp. and *Streptococcus* spp. in footrot lesions positively indicates their association in footrot. *Trueperella pyogenes*, which was formerly known as *Arcanobacterium pyogenes* (*A. pyogenes*) was not identified from any of the samples, even though Santiago *et al* (2004) and Quinn *et al* (2002) reported the association of *A. pyogenes* with footrot. With regard to the pathogenicity testing of *Staphylococcus* and *Streptococcus* isolates, mice did not succumb to the intraperitoneal injections of *Sthaphylococcus hyicus*, *S. intermedius*, *S. aureus*, *S. epidermidis* and *Streptococcus pyogenes* obtained from clinical footrot lesions. This can be

attributed as the incapability of less pathogenic strains to elicit a systemic infection. But they may be capable to elicit or aggravate a local infection in the hoof of goats. So the association of these aerobes with footrot lesions cannot be neglected only due to their incapability to cause systemic infection in mice, because they were frequently isolated with substantially high numbers from all the footrot samples collected. This could be due to the augmentation of their growth in the congenial environment provided by the coexisting bacterial agents ensuing in the foot lesions or necrotic conditions. Detailed studies have to be conducted to know the actual role of aerobic commensal bacteria in footrot

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