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Review Article

The Detection and Identification of Mixed Strains of Fusobacterium necrophorum in Foot Rot Lesions of Sheep in Kashmir, India

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Abstract

The objective of this study was to determine the prevalence and identification of *lktA* variant strains of *F. necrophorum* in the foot rot lesions of sheep. The detection of *F. necrophorum* was carried out by Polymerase Chain Reaction (PCR) targeting the leukotoxin (*lktA*) gene fragment and identification of *lktA* variant strains was done by PCR–Single Stranded Conformational Polymorphism (PCR-SSCP) and gene sequencing of the 450 swabs collected from foot rot lesions of sheep, 117 were positive for *F. necrophorum* of the 50 swabs collected from apparently asymptomatic sheep, only one was positive for *F. necrophorum*. The overall prevalence of *F. necrophorum* in foot rot affected sheep in Kashmir valley was 26%, with the highest prevalence of 34.8% recorded in samples from the districts of Kulgam and Pulwama, and the lowest (20%) in Baramulla district. PCR-SSCP of *lktA* gene fragment revealed the presence of three *lktA* variants, designated as JKS-F1/ F2/ F3, while two samples (1.70%) showed the presence of multiple *lktA* variant strains of *F. necrophorum* on a single foot rot affected sheep hoof. The JKS-F3 was the most frequent (75.4%), followed by JKS-F2 (14.4%) and JKS-F1 (8.4%) *lktA* variant, respectively. Of the three *lktA* variants identified, JKS-F3 was detected in 74 (86.0%) out of 86 samples taken from foot rot affected sheep with lesion score 4. The data suggest that JKS-F3 is the predominant and most adapted *lktA* variant of *F. necrophorum* and is associated with severe foot rot in sheep, hence a significant variant contributing to the severity and duration of the disease. This appears to be the first report on the presence of more than one *lktA* variant of *F. necrophorum* in a foot rot lesion of sheep.

Keywords: *Fusobacterium necrophorum*; Ovine foot rot; PCR-SSCP; *lktA* gene

Introduction

Foot rot is a contagious bacterial infection of the feet of sheep that causes lameness, and significant production and economic losses worldwide. The disease is characterized by an exudative inflammation, followed by necrosis of the epidermal tissue of the inter digital skin and hoof matrix, resulting in separation of the hoof from the underlying soft tissue. The affected animal's exhibit lameness, loss of body weight, reduced wool and meat production [1]. The infectious syndrome is caused by the synergistic action of several bacterial species, particularly Dichelobacter nodosus, and *Fusobacterium necrophorum* [2]. Both the anaerobes are found together at a significantly higher rate in severe foot rot lesions in sheep in which *D. nodosus* drives the pathogenesis of foot rot from initiation of Interdigital Dermatitis (ID) to Severe Foot Rot (SFR) and *F. necrophorum* contributing to the severity and duration of SFR [3,4].

F. necrophorum is a Gram negative, anaerobic, pleomorphic and non-spore-forming bacterium, which is more sensitive to oxygen than *D. nodosus* [5]. It is a normal resident of manure-contaminated environments. The *F. necrophorum* has two subspecies; *F. necrophorum* subsp. *necrophorum* (formally biovar A) and *F. necrophorum* subsp. *funduliforme* (formally biovar B). The subsp. *necrophorum* is more pathogenic due to a higher lipopolysaccharide content and higher production of leukotoxin [6]. Among a variety of virulence factors, the best described and major virulence factor is leukotoxin, encoded by *lktA* gene. The *lktA* gene has been used for the detection of *F. necrophorum* and to determine variation among its strains [7].

In India foot rot is endemic in the states of Jammu and Kashmir, Andhra Pradesh, Tamil Nadu, Uttar Pradesh and Himachal Pradesh [8,9]. Recently, its severity has increased and attained significant importance in sheep husbandry practices [10,11]. There is very little knowledge about the *lktA* variant(s) associated with severe foot rot in sheep (lesion score 4) and no study has described the most predominant *lktA* variant(s) of *F. necrophorum* in ovine foot rot lesions. The aim of the present investigation was to study the prevalence, isolation and identification of predominant *lktA* variant of *F. necrophorum* that is linked to severe foot rot in sheep.

Materials and Methods

Survey

The present survey was conducted from 2012 to 2016 to

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Table 1: Depicting samples and *lktA* variant distribution of *Fusobacterium necrophorum* vis-à-vis Severe Foot Rot (SFR) in sheep.

S. No.	No. of flocks inspected	No. of samples collected from footrot affected sheep	Samples positive for F.n*	No. of samples collected from healthy sheep	Samples positive for F.n	Distribution of *lktA* variants in positive samples JKS*- F1/F2/F3/MSI*	Distribution of *lktA* variants in SFR with LS*=4 JKS*- F1/F2/F3/MSI	Prevalence of F.n (%)
1	3	21	5	2	-	0 2 3 0	0 1 3 0	23.8
2	33	150	40	15	-	4 7 27 2	1 2 24 2	26.7
3	6	23	8	3	-	1 1 6 0	0 0 5 0	34.8
4	8	36	9	4	-	1 1 7 0	0 0 4 0	25
5	16	90	19	10	01	3 3 14 0	1 2 11 0	21.1
6	5	23	8	2	-	0 1 7 0	0 0 5 0	34.8
7	4	28	9	3	-	0 1 8 0	0 1 7 0	32.1
8	5	24	7	3	-	0 0 7 0	0 0 6 0	29.1
9	9	25	5	4	-	0 1 4 0	0 1 4 0	20
10	10	30	7	4	-	1 0 6 0	0 1 5 0	23.3
	99	450	117	50	01	10 17 89 2	2 8 74 2	26

**Fusobacterium necrophorum* (F.n)
*Accession numbers of the strains JKSF1/F2/ F3 are JF911484.1, JX104210.1 & JX104211.1, respectively
*MSI= Multiple Strain Infection
*LS= Lesion score

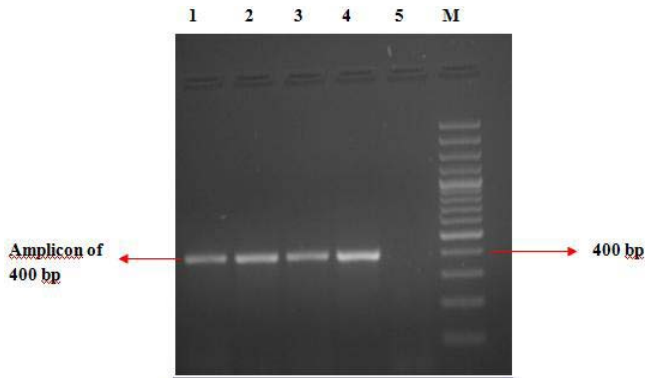

Figure 1: Detection of *lktA* gene fragment of *Fusobacterium necrophorum* by Polymerase Chain Reaction (PCR).
Lane 1 to 3 = Test samples; Lane 4 =Positive control; Lane 5 =Negative control; Lane M = 100 bp DNA ladder

determine the prevalence of *F. necrophorum* in foot rot-affected sheep of Kashmir valley. A two-stage simple random sampling procedure was followed. A complete list of all villages falling under the administrative control of these districts were obtained from district offices of respective districts and 5% of villages from each district were selected through a Simple Random Sampling (SRS) procedure. Subsequently, a complete list of household flocks in the selected villages was prepared and again simple random sampling (second stage sampling) was followed to select household flocks. Again 5% of household flocks were selected. These household flocks were finally visited to determine the prevalence of *F. necrophorum* in foot rot affected sheep.

Collection of clinical samples

A total of 500 swab samples (450 from symptomatic and 50 from healthy asymptomatic sheep) were collected from ninety nine foot rot affected flocks across the valley as detailed in (Table 1). The samples constituted duplicate swab samples of exudates of foot rot lesions with a lesion score of 2 (ID) to 4 (SFR i.e under running of the hard horn of the hoof) collected at the active lesion that developed between the horn of the hoof and the sensitive underlying tissue. One sample from each case was inoculated onto media for isolation and other used for DNA extraction for direct detection of *F. necrophorum*. Only one foot per sheep was sampled. The swabs used for DNA extraction were transported to laboratory in thioglycolate broth (Hi Media, Mumbai, India). The sampling was done by a single trained researcher to minimize the variation.

Extraction of DNA

The swab samples in thioglycolate broth were vortexed briefly to suspend swabbed material and swabs were then removed and broth subjected to centrifugation at 5000×g for 10 min. The supernatant was discarded and suspensions of the pelleted material were prepared in 1.5 ml micro centrifuge tubes in 150 µl of sterile Phosphate Buffered Saline (PBS) by gentle vortexing. The samples were boiled for 5 min, cooled on ice for 10 min and centrifuged at 10,000×g for 1 min. Two microlitres of the supernatant were used as the template for each PCR reaction.

Detection of *Fusobacterium necrophorum*

All the samples were subjected to the *lktA* gene specific PCR for detection of *F. necrophorum* as detailed by [12]. The PCR conditions consisted of initial denaturation at 94°C for 4min, followed by 35 cycles of 94°C for 30s, 60°C for 30s and 72°C for 40s. This was followed by final extension of 5 min at 72°C. The DNA from F. n. subsp. *necrophorum* strain, which was isolated by the Division of Veterinary Microbiology and Immunology and confirmed by sequencing of its *lktA* gene fragment, served as positive control.

Isolation and identification of *Fusobacterium necrophorum* from clinical samples

The fifty swab samples positive for *F. necrophorum* by PCR were inoculated on Brain-Heart-Infusion-Blood-Agar (BHIBA, Difco) containing 10% defibrinated sheep blood, 0.5% yeast extract, 0.01% magnesium sulphate and antimicrobials (vancomycin and neomycin) @ 5µg and 100 µg/ml of media, respectively. After inoculation, the

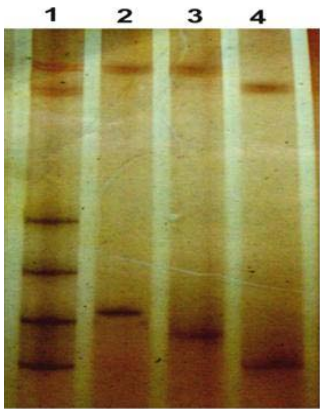

Figure 2: Strains of *Fusobacterium necrophorum* as observed by PCR – SSCP of *lktA* gene fragment.
Lane 1= Multiple strain infection; Lane 2= JKS-F1 strain; Lane 3= JKS-F2 strain; Lane 4= JKS-F3 strain.

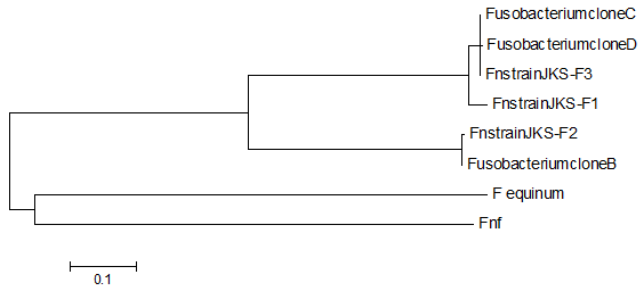

Figure 3: Neighbor-joining tree of *lktA* sequences. Tree was constructed using the *lktA* nucleotide sequences identified from ovine footrot lesions (JKS F1/F2/F3) and from already recorded sequences of *Fusobacterium necrophorum* clones (B-D), *F. equinum* and *F. necrophorum* subsp. *funduliforme* (indicated as Fnf), respectively.

plates were placed immediately in a 3.5 liter anaerobic jar (Oxoid, UK) with requisite number of AnaeroGaspack (Becton and Dickinson, USA). After 48-72 h of incubation at 37°C, suspected colonies which appeared flat, irregular, grayish and surrounded by zone of β-haemolysis were subcultured on the same medium until they were free from contaminating bacteria. Confirmation of the isolates as *F. necrophorum* was done by demonstration of the typical cellular morphology in Gram-stained smears, a biochemical test for lipolytic activity of isolates on egg yolk agar and detection of the *lktA* gene fragment by PCR as described above.

Analysis of strain variation of *Fusobacterium necrophorum* isolates through Single Strand Conformational Polymorphism (SSCP)

The *lktA* gene fragment of *F. necrophorum* was amplified by PCR as described earlier. A 5µl aliquot of each amplicon was mixed with 15µl loading dye (98% formamide, 10mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol), and after denaturation at 95°C for 5min, instantly, samples were snap-cooled on ice for 15 min. Electrophoresis was performed in an SE 600 Ruby electrophoresis unit(GE Healthcare) using a12% polyacrylamide gel (37.5:1) at 300 V for 18 h at 4°C in 0.5 X TBE buffer, and gels were silver stained as per the protocol of [13].

Cloning of variant *lktA* amplicons

Representative amplicons with unique SSCP patterns in gel were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The PCR products were ligated into the p-Drive Cloning Vector (Qiagen) according to the manufacturer's recommended protocol. The ligation mixture was used to transform DH5α Escherichia coli cells by electroporation. Five positive colonies for each transformation were picked up and transferred into Luria Bertani (LB) broth (Hi Media, India) then incubated with shaking (200rpm) overnight at 37°C in a rotary shaking incubator (JEIO TECH, Korea).

DNA sequencing and analysis

The plasmid DNA from selected colonies were extracted using a QIAprep® Miniprep Kit (Qiagen, Hilden, Germany) and the concentration of the DNA adjusted to 200 ng/ml. The DNA was sequenced commercially by Macrogen Inc., Korea, using M13 universal primers. Sequence alignments and translation were performed using Basic Local Alignment Search Tool (BLAST), Fast PCR (Primer Digital Ltd, Helsinki, Finland) and Clustal X (Genome Net, Japan) and the phylogenetic tree was constructed using software MEGA5.

Results

Prevalence and *lktA* variant distribution of *Fusobacterium necrophorum* in ovine foot rot

Out of 450 swab samples collected from foot rot affected sheep, 117 (26.0%) were detected positive as revealed by 400 base pair (bp) amplicon characteristic of the *lktA* gene fragment of *F. necrophorum* (Figure 1). Among 50 swabs collected from clinically healthy sheep only one sample carried *F. necrophorum*. Of the 118 *F. necrophorum* positive samples, 89 (75.4%) samples carried JKS-F3 *lktA* variant, while JKS-F1 & F2 variants were observed in 10 (8.4%) and 17 (14.4%) samples, respectively. Out of 117 positive samples, 86 (73.5%) were collected from affected sheep with lesion score 4, while rest of positive swabs (31) belonged to lesion scores of 2 and 3, respectively. Among samples with lesion score 4, 74 (86%) samples carried JKS-F3 *lktA* variant of *F. necrophorum* (Table 1).

Isolation of *Fusobacterium necrophorum* from clinical samples

Out of fifty samples tested positive for the *lktA* gene, 41 (82%) yielded the isolates in pure culture. After subsequent subculture on the same medium (BHIBA), pure colonies of *F. necrophorum* were obtained. The pure colonies of F.*necrophorum* produced characteristic lipolytic activity on egg yolk agar medium.

Strain variation of *Fusobacterium necrophorum*

The SSCP analysis of the *lktA* gene fragment of 118 samples (117 from foot rot affected and one from healthy asymptomatic sheep) found positive for *F. necrophorum*, revealed four different banding patterns (Figure 2) designated as JKS-F1, JKS-F2, JKS-F3 and JKS-F4, respectively. Three of these patterns revealed two bands each while the fourth banding pattern (JKS-F4) revealed unusually multiple (six) bands. This fourth banding pattern was observed in two samples only. To ascertain the status of this unusual banding pattern the amplified products of these two samples were cloned as described above. All the transformed colonies obtained from these two samples were grown

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JKS-F1          IGVVGSVGVGGNVGVGASSDTNIIKRNTKTRVGKNTTMSDGGFGEEAEITADSKQGISSF
JKS-F3          IGVVGSVGVGGNVGVGASSDTNIIKRNTKTRVGKNTTMSDGGFGEEAEITADSKQGISSF
JKS-F2          IGVVGSVGVGGNVGVGASSDTNIIERNTKTRVGK-TTMSDEGFGEEAEITADSKQGISSF
                ************************:********* ***** ******************


JKS-F1          GVGVAAAGVGAGVAGTVSVNQFAGKTEVDVEEAKILVKKAEITAKRYSSVAIGNAAVGVA
JKS-F3          GVGVAAAGVGAGVAGTVSVNQFAGKTEVDVEEAKILVKKAEITAKRYSSVAVGNAAVGVA
JKS-F2          GVGVAAAGAGAGVAGTVSVNQFAGKTEVDVEEAKILVKKAEITAKRYSSAAIGNAAVGVA
                ********.****************************************.*:********


JKS-F1          AKGAGIGAAVAVTK
JKS-F3          AKGAGIGAAVAVTK
JKS-F2          AKGAGIGAAVAVTK
                **************
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Figure 4: Alignment of putative amino acid sequence of three *lktA* variants (JKSF1/F2 and F3) of *Fusobacterium necrophorum* using the CLUSTAL 2.1 multiple sequence alignment tool.

separately, plasmid DNA extracted and subsequently subjected to PCR-SSCP analysis which revealed three banding patterns similar to JKS-F1, JKS-F2 and JKS-F3 indicating mixed strain infection of all the three *lktA* variants of *F. necrophorum* on a single infected sheep hoof. The DNA sequences of these representative amplicons have been deposited into the NCBI Gen Bank with the accession numbers depicted in (Table1).

Phylogenetic Analysis of *lktA* gene variants of *Fusobacterium necrophorum*

A comparison was made among different *lktA* nucleotide sequences of *F. necrophorum* recorded from foot rot lesions in Kashmir valley viz JKS-F1, JKS-F2 and JKS-F3, with the nucleotide sequences of already reported *lktA* variants B, C and D of *F. necrophorum* [12], *F. equinum* [14] and *F. necrophorum* subsp. *funduliforme* (Fnf) [15]. The neighbor joining tree was constructed using software MEGA5 [16]. The evolutionary history was inferred using the neighbor-joining method [17]. The optimal tree with the sum of branch length = 2.43515723 is shown in (Figure 3). There were a total of 400 positions in the final dataset. The average pair wise distance between sequences is 0.84. The newly identified *lktA* sequences from ovine foot rot in Kashmir valley were homologous to previously reported *lktA* sequences such as clones B, C and D of *F. necrophorum* [12]. The clone B of *F. necrophorum* showed 99% sequence homology to our JKS-F2, while clones C and D 100% and 99% to our JKS-F3, respectively. The neighbor joining tree of the *lktA* gene fragment sequence also revealed JKS-F1 to be a genetically distant variant of *F. necrophorum*, reported so far. A close genetic relationship between variant JKS-F2 with clone B and that of variant JKS-F3 with clone C and D of *Fusobacterium* species was also revealed. While *F. equinium* and F. n. subsp. *fundiliforme* (Fnf) shows that these were at some genetic distance from the rest. The amino acid sequences of three variants of *F. necrophorum* recorded from the Kashmir valley were analyzed using the Clustal-W (CLUSTAL 2.1 multiple sequence alignment) to generate a sequence alignment report as shown in (Figure 4).

Discussion

This paper describes the prevalence of *F. necrophorum* in foot rot affected sheep. The overall prevalence of *F. necrophorum* observed in the Kashmir valley was 26%, with the highest prevalence of 34.8% in Pulwama and Kulgam districts each and the lowest prevalence of 20% in district Baramulla. The high prevalence suggests that *F. necrophorum* is frequently associated with foot rot lesions as compared to clinically healthy sheep in which only one out of fifty swabs were detected positive for *F. necrophorum*. The highest prevalence of *F. necrophorum* in ovine foot rot in the districts of Pulwama and Kulgam could be because the pastures around the villages of the districts are favorable for the persistence of the organism, due to the manure contaminated environment (as *F. necrophorum* is a commensal in the alimentary tract and is shed in faeces), water logging and marshy areas. However, it is notable that a large portion of swabs (74.0%) were negative for *F. necrophorum*. This would suggest either that PCR is not sensitive enough to detect *F. necrophorum* at lower cell count and/or due to the presence of PCR inhibitory substances in some swabs soiled with dirt and dust that may act as a barrier to the successful amplification of DNA. Secondly, cotton swabs allow sampling from the surface but not from deeper areas of skin and hoof where anaerobic conditions favour more growth of *F. necrophorum*.

The current study also reveals three different *lktA* variants of *F. necrophorum* in the hooves of sheep affected with foot rot. Out of 450 swab samples collected from the lesions of foot rot affected sheep, only two samples carried all the three *lktA* variants of *F. necrophorum* in the same infected ovine hoof. This seems to be first report of its kind. This is in contrast with the findings of [12] who reported single strain infection of *F. necrophorum* in cattle, sheep and goats in New Zealand. However, mixed serogroup/ strain infections are quite common in case of *D. nodosus* where up to seven serogroups may be present on a single hoof [18,19]. The JKS-F3 was the most frequent *lktA* variant strain (75.4.0%) detected and was found to be associated with severe foot rot (86%) lesions in sheep. Its high frequency in ovine foot rot is likely to be due to its greater degree of adaptation to sheep in comparison to other two *lktA* variants of *F. necrophorum*. These findings show that mixed strain infections, although observed in only two samples is possible in case of *F. necrophorum* like *D. nodosus* in which multiple serogroups are detected on single foot rot affected hoof. It was also observed that in swabs in which JKS-F3 *lktA* variant was present, the affected sheep had severe foot rot with lesion score mostly 4 in comparison to other foot rot affected sheep in which either of the two other strains were present. These results highlight the higher degree of adaptation and virulence of JKS-F3 *lktA* variant of *F. necrophorum* in sheep, contributing to severity and duration of foot rot lesion in comparison to other two *lktA* variants. The reason for this is unknown and further studies in more flocks and different areas would be useful to confirm these findings.

The three *lktA* variants of *F. necrophorum* reported in the current study, isolated from sheep may represent different species of *Fusobacterium* as suggested by [12] as well. Further studies in this direction may confirm this opinion or otherwise. A similar phenomenon has been previously reported for "*F. necrophorum*" strains isolated from horses, with the reclassification of *F. equinum* as a new species based on phylogenetic analysis of the 16S rRNAgene, DNA-DNA hybridization and phenotypic characterization [20].

We have previously reported [19,21] that samples in which both the anaerobes were detected together, were more likely to come from sheep that had severe foot rot with lesion scores mostly 4 in comparison to other foot rot affected sheep in which *F. necrophorum*

was absent or found alone. The present study reveals predominant and adapted *lktA* variant (JKS-F3) of *F. necrophorum* that is associated with severe foot rot in sheep, therefore, contributing to the severity and duration of disease. These findings may further contribute towards understanding the pathogenesis of ovine foot rot, in order to develop appropriate treatment and prevention measures. Hence, this *lktA* variant of *F. necrophorum* need also to be considered while managing an outbreak, or maintaining a quarantine or future strategies to combat foot rot.

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