



First report on molecular characterization of *Streptococcus pluranimalium* from Sirohi goat

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ABSTRACT

The present study was done with an objective to identify the causative agent(s) involved in the causation of cutaneous abscessation in goat from semi-arid region of Rajasthan, India by using conventional bacteriological and PCR protocols targeting 16S ribosomal RNA gene. During January 2025, pus sample was collected from a large sized palpable cutaneous abscess of an adult female Sirohi goat and cultured on BHI agar plate. The amplicons obtained out of universal 16S ribosomal RNA using the chromosomal DNA isolated from the bacterial colonies grown on BHI agar plate, were cloned into *E. coli* based vector, and the confirmed recombinant plasmids were subjected for Sanger sequencing. The organism specific genes, *recN* and galactokinase were also targeted and sequenced after the gene cloning experiments. The obtained nucleotide sequences of both 16S ribosomal RNA and galactokinase genes of the identified bacterial species from goat were analysed using the bioinformatics tools. Upon sequence analysis, it was found that the obtained nucleotide sequences had 99 % identity with 16 S ribosomal RNA gene of *Streptococcus pluranimalium*. Both *recN* and galactokinase genes also had 100 % identity with that of *S. pluranimalium*. In the phylogenetic tree analysis, *S. pluranimalium* from Indian goat clustered with *S. pluranimalium* from other host species.

Keywords: 16S RNA PCR, Bioinformatics analysis, Cutaneous abscess, Sequencing, Sirohi goat, *Streptococcus pluranimalium*

During the year 1999, *Streptococcus pluranimalium* was identified as a new species of the genus *Streptococcus* and this Gram-positive bacterium was present in the various organs of different animal species (Devriese *et al.* 1999). *S. pluranimalium* has been associated with valvular endocarditis and septicaemia in broiler parents (Hedegaard *et al.* 2009), meningoencephalitis in a horse (Fu *et al.* 2021) and abortion in bovines (Yee *et al.* 2025). Twomey *et al.* (2012) characterized *S. pluranimalium* isolates from a wide range of animal species having various health issues. *S. pluranimalium* is known to have association with cerebral abscess and endocarditis (Duriseti and Fleisher 2019), and pneumonia (George *et al.* 2020) in humans. Pan *et al.* (2018) reported that this bacterial species could be transmitted through the infectious secretions from animals.

Palpable abscesses in the superficial lymph nodes are invariably observed among both sheep and goats being maintained at the semi-arid tropical region of Rajasthan, India. *Corynebacterium pseudotuberculosis* is considered as an etiological factor for the cutaneous abscess

formation among sheep and goats from the North-West region of India (Kumar *et al.* 2012 and 2013, Tripathi *et al.* 2016). In the present study area, the incidence of cutaneous abscess among goats is endemic, whereas, it is sporadic among sheep.

From the above said information, it is hypothesized that more than one etiological factor could be associated with cutaneous abscesses among goats from the semi-arid tropical region of Rajasthan, India. Keeping the above said information in view, the present study was carried out with the aim of the characterization of the etiological factor(s) associated with cutaneous abscesses from Indian goats based on the conventional microbiological examination, 16S ribosomal RNA PCR assay and the sequence analysis of the organism specific genes using the standard bioinformatics tools.

MATERIALS AND METHODS

Ethical approval: All animal experiments were carried out as per the approval from the Institute Animal Ethical Committee of ICAR-Central Sheep and Wool Research Institute, Avikanagar, Rajasthan, India.

Area of sampling: The sampling area falls in the semi-arid tropical region of Rajasthan, India, whose coordinates were 75°28'E longitude and 26°26'N latitude. The study

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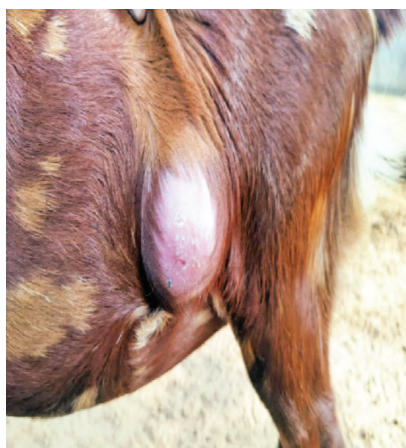


Fig.1 Sirohi goat exhibiting the cutaneous abscess in the abdominal region

area was located at an altitude of 320 m above mean sea level. During April to June, the average monthly temperature was about 42 °C. Rainfall is erratic and highly unpredictable and ranged between 200 to 500 mm per annum. Sirohi breed of goats were being maintained in an organized pattern of semi-intensive system of management.

Collection of samples and isolation of bacteria: During the third week of January 2025, the goat flocks were surveyed for the presence of cutaneous abscesses, if any. While doing the physical examination, an adult Sirohi female goat (Animal No. SG9024 – aged 2 years and 3 months) was diagnosed with a prominent cutaneous abscess with ripening state in the abdominal region (Fig.1). Pus material was collected aseptically from the abscess using a sterile disposable syringe and needle (18 gauge) and transferred on ice to the laboratory for further processing. As per the protocol described by Sunder *et al.* (2024), at the beginning, for the purpose of enrichment of the bacteria, the pus sample was inoculated into brain heart infusion broth (HiMedia, Thane, India) and kept overnight at 37 °C in an orbital shaker. Eventually, the growth was spread onto brain heart infusion agar (HiMedia, Thane, India) plates containing Nalidixic acid (4mg/litre) and Fosfomycin (200mg/litre) and incubated at 37 °C for 48h (Sohier *et al.*

2017). Colonies were examined for color, consistency, and stained with Gram's stain.

Chromosomal DNA extraction from the bacterial colonies: Chromosomal DNA was extracted from the bacterial colonies over the BHI agar plates using the commercial kit, Qiagen DNA Mini kit (Cat.No.# 51304) according to the manufacturer's instructions. The purity and concentration of the chromosomal DNA were approximately checked by running 0.8 % agarose gel electrophoresis.

Polymerase Chain Reaction: Reaction volumes for the PCR of 50 µl were used and contained 5 µl of 10X buffer with 15 mM MgCl₂, 10 mM of each dNTPs, 100 pmol of each oligonucleotide primer, 100 ng of chromosomal DNA isolated from the bacterial colonies and 3U Taq DNA polymerase. The complete details of the primers used for the amplification of the genes viz., 16S ribosomal RNA, CP40 endoglycosidase of *C. pseudotuberculosis*, recN and galactokinase of *Streptococcus pluranimalium* were given in Table 1.

The reaction mixture was subjected to initial denaturation of the template at 95°C for 5 min in a thermal cycler (BioRad, USA). Cycling conditions for PCR were 35 cycles of 60 s at 94°C, 60 s at 55°C (for CP40 protein gene of *C. pseudotuberculosis*, recN and galactokinase genes of *S. pluranimalium*) and 59°C (for 16S ribosomal RNA gene) and 60 s at 72°C, followed by a final extension for 10 min at 72°C. The PCR reaction mix without the template DNA was included as a negative control in all the PCR experiments.

Cloning and sequencing of the bacterial genes: Resultant PCR products were separated on 1.2% agarose gels containing ethidium bromide (10 mg/ml), and visualized under UV light. They were purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) and cloned into pDrive vector (Qiagen) using the protocol according to manufacturer's instructions. The plasmids were transformed into One Shot™ Mach1™-T1R Chemically Competent E. coli (Cat.No. # C8620-03, Invitrogen). Colonies harbouring the recombinant plasmid were inoculated into LB (Luria Bertani) broth containing

Table1. Details of the primers

Name of the gene	Primer ID	Primer sequence (5' -3')	Reference
16S Ribosomal RNA (Universal primer)	27-F	AGAGTTTGATCACTGGCTCAG	Tarhane and Büyük (2024)
	1492-R	ACGGCTTTACCTTGTTACGACTT	
Endoglycosidase	ENGL Y-F	CGACAAGCTTATGCATAATT CTCCTCGATCA (HindIII)	GenBank Accession No. KY041980
	ENGL Y-R	CGCACTCGAG TCTAGAACCAGTTGGCTTTCC (XhoI)	
DNA recombination/ repair protein (recN)	Sp- recNF3	GCTTTCGCCGATATTAAAGC	Kitamura <i>et al.</i> (2023)
	Sp- recNR3	ATCTCCTCTTCAAGAGAACG	
Galactokinase	GLK-F	ATGGAATCACAAGACTTAAAACAAGCT	GenBank Accession No. CP022601.1
	GLK-R	TTAGCTTCTTTCTAGGACATGTGAGCC	

Ampicillin (50 µg /ml) and incubated at 37°C overnight with horizontal shaking. The plasmid DNA was extracted from culture using Monarch® Plasmid Miniprep Kit (New England Biolabs Inc.). The recombinant plasmids were confirmed by PCR using the gene specific primers as well as by single restriction enzyme digestion involving EcoRI. The sequencing of three positive clones was carried out in both directions using Sanger sequencing method by M/s. Eurofins Genomics India Pvt. Ltd., Bengaluru-560048, Karnataka, India and the firm used both M13 forward and M13 Reverse primers for the sequencing experiments.

Sequence analysis: Using BLAST (Biological Local Alignment Search Tool) software of NCBI (Altschul *et al.* 1990), the nucleotide sequences provided by M/s. Eurofins Genomics India Pvt. Ltd., Bengaluru-560048, Karnataka, India, were analysed. Multiple Sequence Alignment was created by ClustalO 1.2.4. using UniProt. Percent Identity Matrix was calculated created by Clustal2.1. Phylogenetic tree was also constructed by CLustal 2.1 using PHYLIP as the format and the Neighbour Joining method as the clustering method (Madeira *et al.* 2024).

RESULTS AND DISCUSSION

The present study was aimed to find out the causative agent(s) incriminated in the cutaneous abscess formation in goats from semi-arid tropical region of Rajasthan, India. Based on the earlier report (Kumar *et al.* 2012), it was speculated that for the present study also, *Corynebacterium pseudotuberculosis* could be the etiological agent for the cutaneous abscess formation among Sirohi goats from the above said agroclimatic zone of Rajasthan, India. The protocols used for the isolation of *C. pseudotuberculosis*

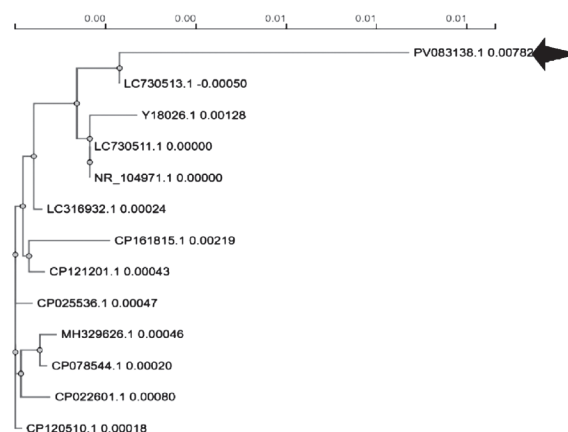


Fig. 2. Phylogenetic tree based on 16S ribosomal RNA gene of *S. pluranimalium* isolates from different geographical areas of the world

were also followed for the pus sample collected from the Sirohi goat in the current study. Upon initial screening of the bacterial colonies grown on BHI agar plate in Gram staining experiments, the organisms were found to be Gram positive cocci. The subsequent conventional PCR reaction involving the universal bacterial 16S ribosomal RNA gene primer pair and the eventual gene sequencing experiments revealed that the sequences obtained were having the highest identity with 16S ribosomal RNA gene of *S. pluranimalium* (1526 bp) in BLAST analysis. Phylogenetic tree was eventually constructed by including the 16S ribosomal RNA gene sequences of *S. pluranimalium* from Indian goat and the corresponding nucleotide sequences of *S. pluranimalium* available in the NCBI database. Analysis of

Table 2. Percent nucleotide identity of 16S ribosomal RNA gene of *Streptococcus pluranimalium* from Indian goat with that of from various geographical areas of the world

Sl. No.	Name of the bacterial species	Isolate and country	Host	GenBank Accession No.	Per cent nucleotide identity
1.	<i>Streptococcus pluranimalium</i>	SG9024, Avikanagar, India	Pus from the cutaneous abscess of Sirohi goat	PV083138.1	Present study
2.	<i>Streptococcus pluranimalium</i>	SP28 and China	Pig	CP121201.1	98.75
3.	<i>Streptococcus pluranimalium</i>	14A0014 and Switzerland	fetal abomasal content of bovine	CP022601.1	98.69
4.	<i>Streptococcus pluranimalium</i>	TH11417 and China	Milk	CP025536.1	98.69
5.	<i>Streptococcus pluranimalium</i>	SS-15 and China	Nasal mucosal swab of swine	CP161815.1	98.69
6.	<i>Streptococcus pluranimalium</i>	SP21-2 and China	fresh faeces	CP120510.1	98.69
7.	<i>Streptococcus pluranimalium</i>	Colony612 and Thailand	Food	CP078544.1	98.62
8.	<i>Streptococcus pluranimalium</i>	TRG7 and Thailand	Lung of pig	MH329626.1	98.62
9.	<i>Streptococcus pluranimalium</i>	DTK517 and Japan	Vagina of a cow with <i>Klebsiella</i> infections	LC730513.1	99.14
10.	<i>Streptococcus pluranimalium</i>	MOA141 and Japan	Tonsil of calf	LC316932.1	99.14
11.	<i>Streptococcus pluranimalium</i>	DTK430 and Japan	Aborted bovine fetus	LC730511.1	99.14
12.	<i>Streptococcus pluranimalium</i>	T70	Not available	NR_104971.1	99.14
13.	<i>Streptococcus pluranimalium</i>	LMG 14257	Not available	Y18026.1	98.41

Table 3. Percent nucleotide and amino acid identities of Galactokinase gene of *Streptococcus pluranimalium* from Indian goat

Sl.No.	Name of the bacterial species	Isolate and country	Host	GenBank Accession No.	Percent identity	
					nucleotide	Amino acid
1.	<i>Streptococcus pluranimalium</i>	SG9024, Avikanagar, India	Pus from the cutaneous abscess of Sirohi goat	PV702343.1	This report	This report
2.	<i>Streptococcus pluranimalium</i>	TH11417 and China	Milk	CP025536.1	97.87	97.95
3.	<i>Streptococcus pluranimalium</i>	Colony612 and Thailand	Food	CP078544.1	97.61	97.18
4.	<i>Streptococcus pluranimalium</i>	SS-15 and China	Nasal mucosal swab of Swine	CP161815.1	97.53	97.18
5.	<i>Streptococcus pluranimalium</i>	SP28 and China	Pig	CP121201.1	97.44	97.18
6.	<i>Streptococcus pluranimalium</i>	SP21-2 and China	fresh faeces	CP120510.1	97.36	97.18
7.	<i>Streptococcus pluranimalium</i>	14A0014 and Switzerland	fetal abomasal content of bovine	CP022601.1	96.93	96.67
8.	<i>Streptococcus halotolerans</i>	HTS9 and China	respiratory tract tissue of <i>Marmota himalayana</i>	CP014835.1	79.36	87.86
9.	<i>Streptococcus gallolyticus</i> subsp. <i>Gallolyticus</i>	37 and France	Blood sample	OZ254910.1	77.33	86.39
10.	<i>Streptococcus ruminicola</i>	CNU_G2 and South Korea	Rumen fluid	CP046919.1	77.10	84.55
11.	<i>Streptococcus suis</i>	Ssuis_MA4 and United Kingdom	Tonsil of Pig	CP084908.1	76.00	84.97

the phylogenetic tree also confirmed that *S. pluranimalium* from Indian goat clustered with *S. pluranimalium* isolates from various geographical areas of the world (Fig. 2).

Further, while using the primer pair specific for the endoglycosidase gene of *C. pseudotuberculosis* in the traditional PCR experiment, a portion of the gene encoding LivH-2 protein of *S. pluranimalium* (around 438bp) was unexpectedly amplified from the template chromosomal DNA extracted from the bacterial colonies grown on BHI agar plate. The traditional PCR experiments targeting both 16S ribosomal RNA and endoglycosidase gene of *C. pseudotuberculosis* were performed simultaneously.

Additionally, in order to confirm the specificity of the unusual presence of *S. pluranimalium* in the present study, two more set of organism specific primers targeting recN gene (Kitamura *et al.* 2023) and Galactokinase gene (GenBank Accession No. CP022601.1) were also included.

In accordance to the earlier report (Kitamura *et al.* 2023), while employing *S. pluranimalium*-specific PCR assay, an amplicon of size around 700 bp representing the partial nucleotide sequences of *recN* gene was also obtained in the present study. After nucleotide trimming, only 567bp fragment covering *recN* gene (without stop codon) of *S. pluranimalium* from the pus material collected from Sirohi goats having cutaneous abscess was deposited in the GenBank. *S. pluranimalium*-specific PCR assay developed by Kitamura *et al.* (2023) would, therefore, be the gold standard for the precise identification of *S. pluranimalium*

in the biological samples from the mammalian species at the molecular level. Indeed, instead of the usage of 60°C as described by Kitamura *et al.* (2023), the annealing temperature of 55°C yielded a single PCR amplified DNA fragment of around 700bp in the present study.

In the case of Galactokinase gene, the full open reading frame (1173bp) was amplified from the pus material from the Sirohi goat. The determined nucleotide sequences of 16S ribosomal RNA, LivH-2 protein, *recN* protein and galactokinase of *S. pluranimalium* from Indian goat, obtained out of gene sequencing experiments were, then, submitted to GenBank under the accession Nos. PV083138, PV102482, PV533655 and PV702343, respectively.

The details of the percent nucleotide identity of 16 S ribosomal gene of *S. pluranimalium* from Indian goat with that of from different hosts were provided in Table 2. On the other hand, the per cent level of sharing both nucleotide and amino acid identities of *S. pluranimalium* from Indian goat with that of from other streptococcal species was given in Table 3. While looking into the multiple alignment of amino acids of galactokinase gene of various streptococcal species, *S. pluranimalium* from Indian goat had an amino acid substitution at two positions, i.e., D352N and A372T (Fig.3). These two peculiar amino acid substitutions in galactokinase protein of *S. pluranimalium* from Indian goat could be species specific, playing a pivotal role in the pathogenesis of *S. pluranimalium* in goats.

Nucleotide alignments and amino acid alignment were

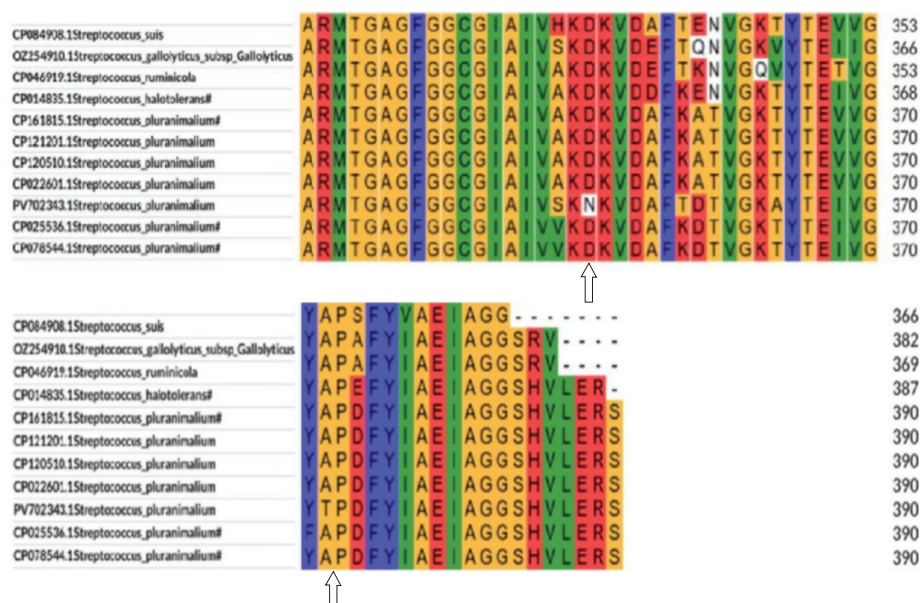


Fig. 3 Amino acid Alignment of galactokinase gene of *S. pluranimalium* Avikanagar isolate (PV702343.1) with that of other streptococcal species from various hosts. The arrow symbols denote the substitutions of amino acids at 352 position (D352N) and at 372 positions (A372T) of galactokinase of *S. pluranimalium* from Indian goat

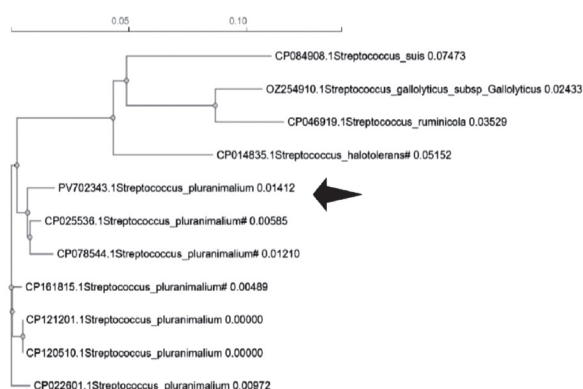


Fig. 4. Phylogenetic tree based on amino acids of the galactokinase gene of *S. pluranimalium* isolates from different geographical areas of the world

considered for the construction of phylogenetic trees for 16 S ribosomal gene (Fig.2) and galactokinase gene (Fig.4) of *S. pluranimalium*, respectively. In both phylogenetic trees, *S. pluranimalium* from Indian goat clustered with *S. pluranimalium* from other host species.

Further, the laboratory involved is continuously working on the isolation and identification of the etiological agent(s) involved in the causation of cutaneous and visceral abscessation in small ruminants from the Western part of India and as of now, almost from each goat, unexpectedly, a new bacterial species has been isolated from the pus samples every time. This could be due to the following two major factors: 1. Flare- up of the normal commensal microbes of the host owing to the immunosuppression caused by *Corynebacterium pseudotuberculosis* and 2. The developmental stage of the abscess at the time of collection of the pus.

From the major findings of the present study, the

following points need to be carried out from the future prospectives point of view.

- Examination of a large number of samples from the goats and comparison of the data with that of sheep from the same geographical area of Rajasthan, India.
- Development of CRISPR tool-based diagnostics for identification of major etiological agents involved in the causation of cutaneous and visceral abscessation in small ruminants
- Formulation of the ideal therapeutic measures for the control of the said infectious issues among the animals

Based on the salient findings of the present study, it is concluded that *Streptococcus pluranimalium* has been isolated from the pus in the cutaneous abscess of Sirohi goat. To our knowledge, this is the first report on the isolation and molecular characterization based on the genes encoding 16S ribosomal RNA, LivH-2 protein, recN protein and galactokinase of *S. pluranimalium* from Indian goat.

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