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Isolation and molecular characterization of lumpy skin disease virus from recent outbreaks in Andhra Pradesh, India 2023

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Abstract
Lumpy skin disease (LSD) is a contagious viral illness that mostly affects the cattle. Trade limitations result from the disease's prevalence in a region, according to the World Organisation for Animal Health (OIE), which classifies it as a disease that must be reported. Given the economic, public health and social implications of LSD, it is essential to prioritize its characterization molecularly to safeguard the livestock. In the current investigation, a total of 79 samples (36 skin scabs, 21 nasal swabs, 18 blood samples and 4 faecal samples) from 52 clinically infected animals in different regions of Andhra Pradesh (AP) were collected. All these samples were used for the detection of Lumpy skin disease virus (LSDV) by polymerase chain reaction (PCR) targeting the p32 envelope protein gene (LSDV074) and LSD specific fusion gene (LSDV117). Out of 36 skin scabs 32 samples (88.89%), 7 out of 21 nasal samples (33.33%) and 1 out of 4 faecal samples (25%) were found positive for capripox generic PCR. LSDV specific fusion (F) gene was detected in 27 skin scabs (75%), 3 nasal swabs (14.29%) and 1 faecal sample (25%). All the blood samples were found negative for both genes. The genomic region of F gene (LSDV117) from six field isolates representing different regions of Andhra Pradesh was sequenced and the phylogenetic analysis revealed that some of the isolates were similar to Kenya, KSGP 0240 strain, Odisha, and Tamil Nadu strains whereas remaining isolates were clustered separately. Further the virus was isolated in primary lamb testicular cells (PLT) with characteristic cytopathic effect (CPE) produced in the first blind passage and confirmed by PCR targeting the fusion gene.

Keywords: LSDV, fusion gene, p32 gene, phylogenetic analysis, virus isolation

1. Introduction
LSD is a notifiable transboundary disease because of its rapid spread across the countries and economic losses to livestock sector. LSD was endemic in Africa and the Middle East until 2015, when it spread to the Caucasus, Balkans, and the southern Russian Federation (Tuppurainen *et al*., 2017) [27]. Odisha state in India, reported the sickness for the first time ever in 2019 (Sudhakar *et al*., 2020) [23].

LSDV is a linear, ovoid, enveloped double stranded DNA virus belongs to the genus capripoxvirus, within Chordopoxvirinae subfamily of the Poxviridae family. It is antigenically related with sheeppox and goatpox viruses. The 151 kbp-long LSDV genomic sequence, which contains 156 putative genes, is made up of a core coding region that is bordered by identical 2.4 kbp-inverted terminal repeats (OIE, 2021) [18]. This illness is characterised by formation of generalised firm papules and nodules of 0.5-5.0 cm size all over the body, including the head, legs, neck, udder, scrotum, perineum, and buccal mucosa. Other symptoms include pyrexia, enlarged superficial lymph nodes, nasal discharge, anorexia, watery eyes, and decreased milk production. (Tageldin *et al*., 2014; Sudhakar *et al*., 2020) [24, 23]. Arthropod vectors are the mechanical means through which this disease is spread. The severity of LSD varies from subclinical infection to death, depending on the virus strain, prevalence of the vector, age, and immunological condition of animal. It also has a high morbidity rate and a low to moderate mortality rate (OIE, 2018) [17].

Lumpy skin disease has the potential to cause economic losses, impact livelihoods and food security, threaten animal welfare, and have potential zoonotic implications. Managing and controlling the disease is essential to protect livestock populations, ensure food production, and safeguard public health and the global economy. This study aimed to isolate LSDV in primary lamb testis cells and to provide molecular characterization of LSDV from different regions of Andhra Pradesh.

Fig 1: (A-D): Clinical manifestations of LSD infected cattle in study area of Andhra Pradesh, India. A) Generalised skin nodules in indigenous calf, B) Ulcerative skin lesions on muzzle, C) Skin lesions with scabs and ulcers, D) Ruptured nodules created a deep -seated wound in the fetlock region

2. Materials and Methods

2.1 Ethical approval

This investigation requires collection of biological samples from the cattle. Without employing anaesthetic, skin scabs, nasal swabs and blood samples of three ml each were taken from the LSD infected cattle in accordance with established procedures. NTRCVSc, Gannavaram, granted the permission to collect the biological samples. A valid consent form was obtained from the animal owners prior to sample collection.

2.2 Area of study

The investigation was conducted in various regions of Andhra Pradesh (Fig. 1) where the extreme hot and humid weather is present which is ideal for the growth of vector-borne diseases.

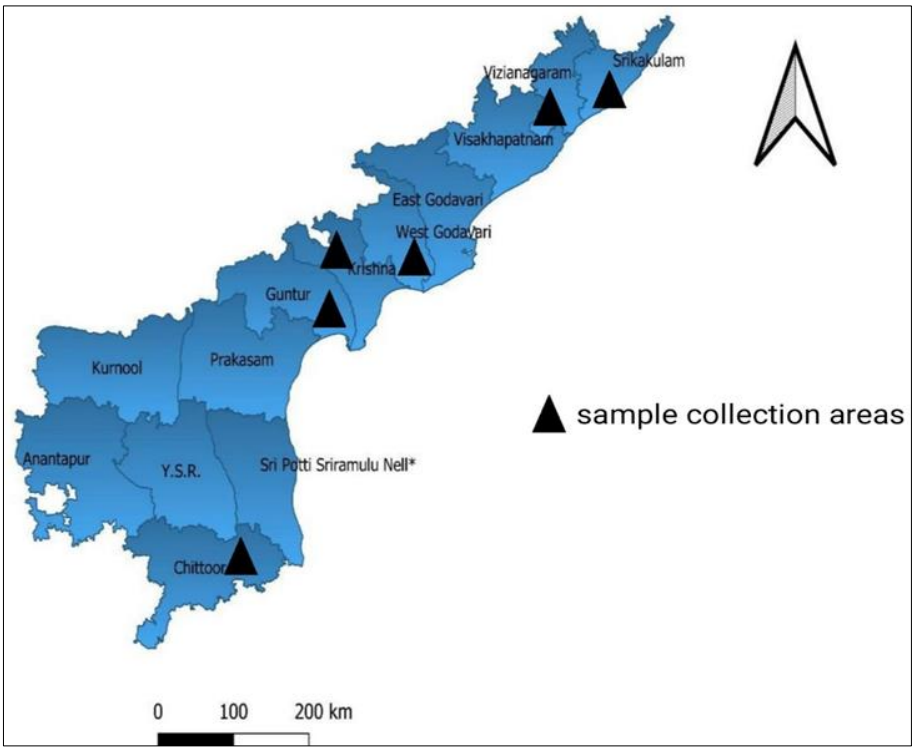

Fig 2: Map of Andhra Pradesh showing sample collection areas. This is created using QGIS 3.24 version software

2.3 Sample collection

A total of 79 samples (36 skin scabs, 21 nasal swabs, 18 blood samples and 4 faecal samples) were collected from 52 clinically infected cattle showing symptoms of pyrexia, skin nodules, enlarged lymph nodes, anorexia, nasal discharges and decrease in milk production representing different districts of Andhra Pradesh. The specifics of samples collected were listed in Table 1. The skin scabs were collected in 50% glycerol saline, nasal swabs in sterile PBS, whole blood with anticoagulant and faecal samples in the form of rectal swabs using sterilized swabs containing Phosphate Buffer Saline (PBS). The samples were collected aseptically and transferred in an ice box to Department of Veterinary Microbiology, NTRCVSc, Gannavaram. The skin scabs, nasal samples, and faecal samples were stored at −80 °C and blood at 4 °C for further analysis.

Table 1: Details of sample collected from clinically infected cattle in different regions of Andhra Pradesh

Sl. No	Sample collected Region in AP	No. of animals examined	Type of sample collected	No. of samples collected
1.	Srikakulam	6	Skin scabs	6
			Nasal swabs	1
			EDTA blood	1
2.	Vizianagaram	18	Skin scabs	9
			Nasal swabs	7
			EDTA blood	12
3.	Tanuku	6	Skin scabs	4
			Nasal swabs	6
			EDTA blood	0
4.	Krishna	14	Skin scabs	9
			Nasal swabs	6
			EDTA blood	5
			Faecal samples	4
5.	Machilipatnam	2	Skin scabs	2
			Nasal swabs	0
			EDTA blood	0
6.	Guntur	4	Skin scabs	4
			Nasal swabs	1
			EDTA blood	0
7.	Palamaner	2	Skin scabs	2
			Nasal swabs	0
			EDTA blood	0
	Total	52		79

2.4 Molecular detection

2.4.1 Sample processing

The clinical samples collected from cattle were processed at Department of Veterinary Microbiology, NTRCVSc, Gannavaram. About 10 mg of scab tissue was homogenised in mortar and pestle using 1ml phosphate buffer saline (PBS), centrifuged @1000 g for 10min, and the supernatant was collected. This supernatant is used for viral DNA extraction. For faecal samples, the swabs were rinsed in PBS and taken out followed by centrifugation @ 6,000 rpm for 15 min at 4 °C. The supernatant collected was used for DNA extraction.

2.4.2 Viral DNA extraction

The viral DNA was extracted from scab tissues, nasal swabs and blood by using GeNei™ TRIsoln reagent in accordance with the manufacturer's recommendations. Boiling method was used for viral DNA extraction from faecal samples. The DNA was eluted in 50 µl of TE buffer, and the Nanodrop Spectrophotometer 200C (Thermo Scientific, USA) was used to determine the DNA concentration. The DNA samples (Optical density ratio of 1.8 to 2) were stored at −80 °C until further use. As a positive control for capripoxvirus, viral DNA from the goatpox vaccine was employed. DNA from field-obtained scab tissue that tested positive for LSDV (nucleotide sequencing) was used as a positive control in the following experiments.

2.4.3 Amplification of p32 (LSDV074) and F gene (LSDV117) by PCR

All the DNA samples were first tested for detection of p32 gene using OIE recommended primers (Table 2). For the PCR, 12.5µl of GoTaq® Green Master Mix, 2X [GoTaq® DNA Polymerase in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2 (Promega, USA)], 1.25 µl of both forward and reverse primers (20 pmol/µl), 150ng of DNA with final volume of 25µl reaction was prepared using nuclease free water. The assay was carried out in Proflex PCR systems, applied biosystems, under standardized cycling conditions (Table 3).

For detection of LSDV specific fusion gene, primers and cycling conditions used were mentioned in Table 2 and 3 respectively. With the help of the BIO-RAD gel documentation system, the PCR products were electrophoretically separated on a 1.5% agarose gel and then visualized under UV illumination.

2.5 Nucleotide sequencing and Phylogenetic analysis

The PCR products of six field isolates namely SKLM-1S,VZM-7S, VZM-16S,MCP-2S, KSN-4S, and PMR-1S positive for F gene representing different regions of Andhra Pradesh were sequenced (Barcode Biosciences Private Limited, Bangalore, Karnataka, India). The nucleotide sequences were edited by aligning with the reference sequences of LSDV strains that were retrieved from the GenBank using Codon Code Aligner software.

MEGA 11 was used to do the Phylogenetic analysis using ClustalW, and a maximum likelihood tree was constructed with a bootstrap value of 1000 replicates. These six isolates' partial F gene nucleotide sequences have been submitted to GenBank.

Table 2: List of primers used in this study

LSDV Gene	Primer sequence	Amplicon size	Reference
p32	F: 5′- TCCGAGCTCTTTCCTGATTTTTCTTACTAT -3′ R: 5′- TATGGTACCTAAATTATATACGTAAATAAC - 3′	192bp	(Ireland and Binepal, 1998; Tuppurainen *et al*., 2005; OIE, 2021) [11, 26, 18]
F gene	F: 5′- ACTAGTGGATCCATGGACAGAGCTTTATCA -3′ R:5′- GCTGCAGGAATTCTCATAGTGTTGTACTTCG -3′	472bp	(Sudhakar *et al*., 2019; Gupta *et al*., 2022; Sethi *et al*., 2022) [23, 10, 21]

Table 3: Standardized thermal cycling conditions used in this study

Steps	Standardized thermal cyclical conditions	
	P 32	F gene
Initial Denaturation	94 °C for 5 min (1 cycle)	95 °C for 5 min (1 cycle)
Denaturation	95 °C for 30 sec	95 °C for 1 min
Annealing	55 °C for 30 sec	50 °C for 1 min
Extension	72 °C for 1 min (35 cycles)	72°C for 1 min (35 cycles)
Final extension	72 °C for 5 min (1cycle)	72 °C for 5 min (1 cycle)

2.6 Virus isolation

2.6.1 Preparation of primary lamb testicular cell cultures

From a local abattoir in Gannavaram, testes were collected from 3- to 4-month-old lambs and placed in DMEM (Dulbecco's Modified Eagle media) media before being washed seven to ten times in sterile phosphate-buffered saline (PBS) with 100 U/mL Penicillin and 100 mg/ml streptomycin. The testicular tissues were cut into 1- 2 mm pieces and digested with 0.25% pre warmed trypsin at 37 ℃ for 20 min resulting in formation of single cell suspension, and then trypsin activity was stopped by adding 2 ml of foetal bovine serum (FBS). The digested cells were filtered by 200 µm sterilized muslin cloth and the filtrate was centrifuged to remove trypsin residues the cells were re-suspended with DMEM medium along with FBS and centrifugation was repeated. The cell counting was done by trypan blue staining using haemocytometer. Then the cell suspension was distributed in the cell culture flasks (25 cm^2) at the rate of 2 x 10^4 cells/cm3 and make the final volume of 7ml/flask with DMEM medium containing 10% FBS. Incubate the seeded cell culture flasks in CO_2 incubator (5%) until monolayer is formed (Wu *et al*., 2014; Guitian *et al.*, 2019) [28, 9].

2.6.2 Infection of primary lamb testicular cell culture with LSDV field isolate

To adapt LSDV to PLT cells, 500 µl of filtered virus inoculum which was confirmed by PCR using LSDV specific primers was used to infect PLT cells for 2 hrs at 37 ºC in 5% CO_2 tension, followed by washing with PBS to remove the unabsorbed virus. Then the maintenance medium was added and incubated at 37 ºC in 5% CO_2 tension. The infected cell cultures were monitored daily for cytopathic effect. Later the monolayers showing 80% CPE, were taken and stored at -80 ºC for further analysis (Coves-Datson *et al*., 2020; Kumar *et al*., 2021) [5, 12].

3. Results

3.1 Detection of LSDV by Polymerase chain reaction

All the DNA samples were subjected to PCR assay. Out of 36 skin scabs 32 samples (88.89%), 7 out of 21 nasal samples (33.33%) and 1 out of 4 faecal samples (25%) were found positive for capripox generic PCR and produced the amplification with product size 192 bp (Fig. 2). LSDV specific fusion (F) gene was detected in 27 skin scabs (75%), 3 nasal swabs (14.29%) and 1 faecal sample (25%) and produced the amplification with product size 472 bp (Fig. 3). All the blood samples did not react with any primers.

3.2 Phylogenetic analysis

To determine the origin and degree of genetic relatedness between the LSDV strains circulating in other countries and those from Andhra Pradesh, India, the coding region of F gene from 6 field isolates namely SKLM-1S, VZM-7S, VZM-16S, MCP-2S, KSN-4S, and PMR-1S was sequenced and submitted to GenBank with the accession numbers OR423062, OR423063, OR423064, OR423065, OR423066, and OR423067 respectively.

The phylogenetic analysis was carried out by comparing with nucleotide sequences of sheep and goatpox viruses, as well as vaccine and field strains of LSDV retrieved from GenBank. The phylogenetic tree (Fig. 4) generated showed that the isolates from Vizianagaram region were in one node and 100% similar to the Kenya and KSGP 0240 strains. Isolate from Srikakulam was in cluster with isolates from the field outbreaks in Tamil Nadu, and Odisha during 2019. Isolates from Krishna and Machilipatnam were one node and clustered separately as well as Palamaner isolate which also clusters separately. All the vaccine strains were grouped together in one node.

3.3 Virus Isolation

3.3.1 Isolation of LSDV in primary lamb testicular cell cultures

For the virus isolation, one of the scab samples, KSN4-S, which tested positive for the LSDV specific F gene by PCR assay, was employed. Confluent monolayers of PLT cells were infected using the virus that was obtained from the scab tissue. It did not show any CPE in the first passage until five days post infection. Therefore, the supernatant from these infected cells was utilised to infect fresh PLT cells (first blind passage) after they had been freeze-thawed three times and centrifuged at 2000 rpm for 20 min at 4°C. Now PLT cells began to exhibit cell rounding, cell aggregation, shrinking of cells after 72 hrs PI and clear CPE was evident after 96 hrs PI (Fig 5).

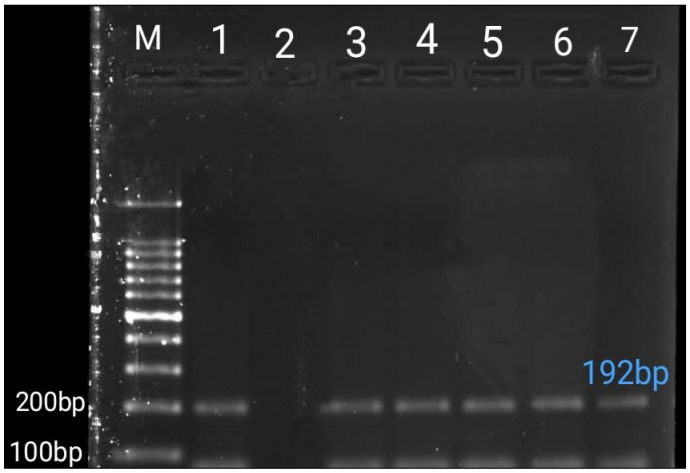

Fig 2: Gel image of PCR targeting p32 gene. Lane M- DNA ladder (100 bp); lane 1-positive control (192bp); lane 2-negative control; lane 3-7 Samples positive for p32 gene (192bp)

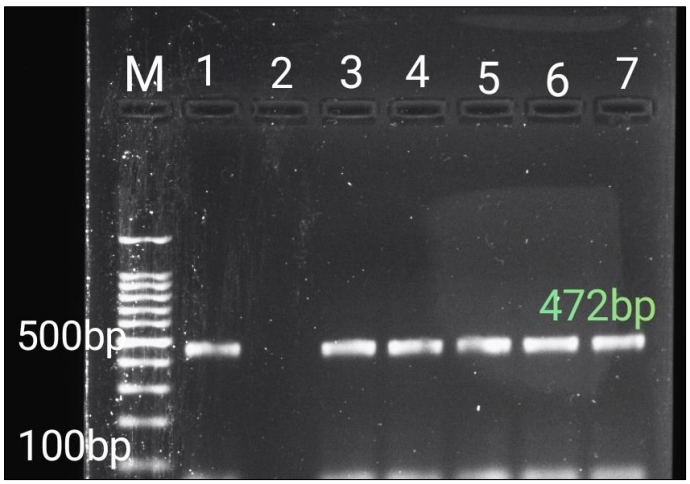

Fig 3: Gel image of PCR targeting F gene. Lane M-DNA ladder (100bp); lane 1-Positive control (472bp); lane 2-Negative control; lane 3-7 Samples positive for LSDV F gene (472bp)

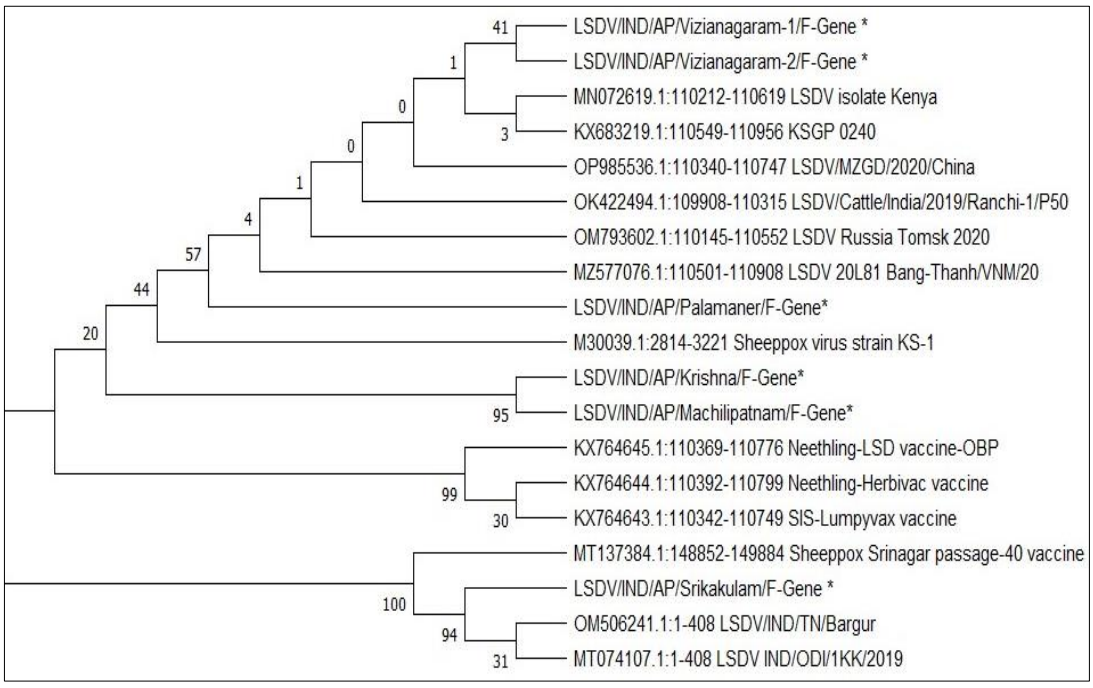

Fig 4: Phylogenetic tree based on partial fusion gene sequences by Maximum Likelihood Method [1]

[1] The sequences obtained through this investigation are identified by (*) labels. Other LSDV, SPPV, and GTPV sequences used in this analysis were obtained from GenBank.

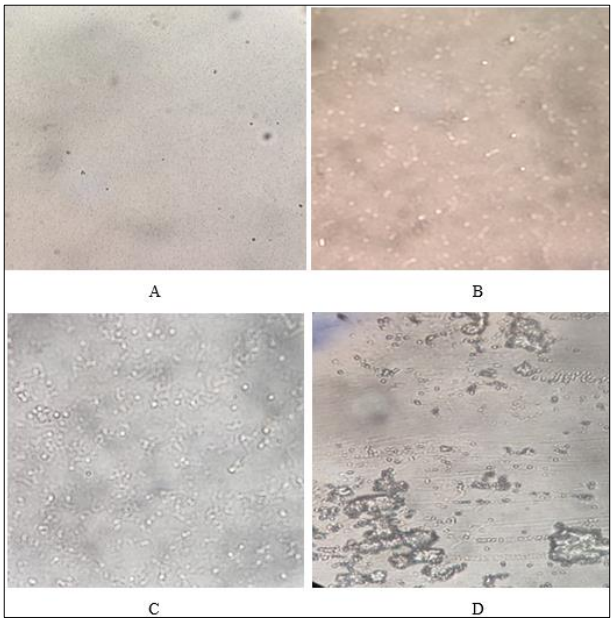

Fig 5: (A-D): Cytopathic changes in LSDV infected PLT monolayers at different time intervals, a) Uninfected confluent monolayer, b) Rounding of cells at 72 h PI, c) Cell aggregation and cell rounding at 96 h PI, d) Disintegration of monolayer and cell clumps floating in the media at 120 h PI

3.3.2 Confirmation of LSDV isolated from PLT cell culture lysates

The DNA was extracted from the infected and uninfected cell culture monolayers (negative control) and subjected to PCR assay targeting F gene. The positive amplification of 472 bp was obtained from the infected cell culture, while no amplification was observed from the uninfected cell culture i.e., negative control (Fig 6). This showed that the LSDV field isolate had successfully adapted and replicated in PLT cell culture.

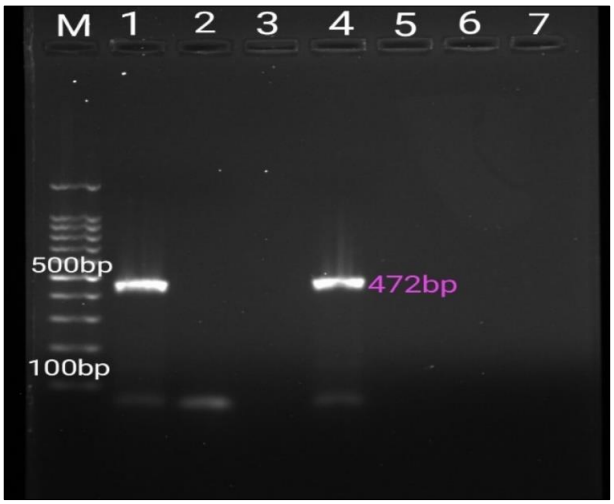

Fig 6: Gel image of PCR confirming LSDV isolated from PLT cell cultures by amplifying F gene. Lane M-DNA ladder (100bp); lane 1-Positive control (472bp); lane 2-Negative control; lane 4-LSDV isolate (KSN-4) positive for F gene (472bp).

4. Discussion

In the late 20th and early 21st centuries, LSD continued to spread within the Africa and reached the Middle East, becoming a significant concern for livestock industries in affected countries due to the potential for trade restrictions and economic losses. The spread of lumpy skin disease to areas outside of Africa and the Middle East raised concerns about its potential to become a global threat (Saegerman *et al*., 2019; Sprygin *et al*., 2019) [20, 22]. The first case of lumpy skin disease in India was reported in the state of Odisha in 2019 (Sudhakar *et al*., 2019) [23]. Subsequent outbreaks occurred in different parts of the country. Due to its detrimental effects on animal welfare, financial losses to the livestock sector, trade restrictions, potential for zoonotic transmission, fast expansion across areas, and possible repercussions on food security, it is crucial to diagnose and control this illness to protect animal health, livelihoods, and international trade.

In the current investigation, skin scabs, blood samples, nasal swabs, and faecal samples were collected from clinically infected cattle and used for molecular detection by performing PCR assay. This illness has been diagnosed globally using PCR over the past 10 years (Elsheikh *et al*., 2023) [6]. Thus, PCR was highlighted as the most effective method for diagnosing LSD and was advised as a rapid and reliable means to detect LSDV in emergency scenarios.

Preliminary detection was done by amplification of capripoxvirus specific p32 gene 192bp which was suggested by OIE (OIE, 2021) [18]. In accordance with earlier findings (Sudhakar *et al*., 2019; Nayakvadi *et al*., 202; Sethi *et al*., 2022) [23, 15, 21], amplification of F gene 472 bp was used for the LSDV specific detection. Skin scabs, nasal samples, and faecal samples were the greatest sources for virus detection when compared to other sources like blood and milk because of the tissue tropism of LSDV, which was detected by the PCR assay in these samples (Tuppurainen *et al*., 2005; Babiuk *et al*., 2008) [26, 3]. Presence of virus in skin wounds also indicated that virus may spread throughout the cattle population by direct contact with the virus shed from skin wounds or bodily secretion, both of which were noted in prior researches (Alexandr *et al*., 2020; Manjoor *et al*., 2023) [1, 14].

Primary cells of lamb testis were used for the isolation of LSDV (Binepal *et al*., 2001; Babiuk *et al*., 2007; OIE, 2017) [4, 2, 16]. These PLT cells displayed the characteristic CPE of cell rounding, cell aggregation, and shrinkage in the first blind passage alone, indicating that they are the most sensitive for LSDV isolation, which is consistent with the earlier findings by Kumar *et al*. (2021) [12]. MDBK cells are frequently used to propagate LSDV *in vitro* at high titres (Fay *et al*., 2020) [7]. However primary cells are more sensitive for viral isolation than MDBK cells which also suggested by previous studies (Salnikov *et al*., 2018; Kumar *et al*., 2021) [19, 12].

In this study phylogenetic analysis was done based on fusion gene to know the genetic relatedness of the virus. According to the F gene's topology, some of the LSDV strains from our study in Andhra Pradesh, India noticeably clustered together with LSDV strains reported from Kenya, South Africa, China, Russia and some strains from the previous LSD outbreak in 2019 in India. But some strains were clustered separately. According to the blast analysis, understudied LSDV strains had 98% to 99% of their genetic similarities with other LSDV vaccine strains and 100% of their genomic similarities with strains from Kenya, Chaina, Russia, Bangladesh, and India. The results of past investigations (Sudhakar *et al*., 2019; Gupta *et al*., 2020; Sethi *et al*., 2021) [23, 10, 21] were in agreement with this. The previous study by Tulman *et al*. (2001) [25], that all capripox viruses are genetically linked and originated from a single ancestral lineage was corroborated by this genetic relatedness (98%–99%) between LSDV strains reported from different geographic regions and vaccine

strains. Although the origin and spread of LSD in India are unknown, it may be linked to the illegal movement of animals across the borders or migration of vectors from neighbouring countries. LSD outbreaks have recently been reported in nearby countries like China, Bangladesh, Pakistan, and Iran (Giasuddin *et al*., 2019; Lu *et al*., 2021) [8, 13].

5. Conclusion

The current investigation provides molecular characterization of LSDV in Andhra Pradesh region of India and isolation of LSDV in PLT cells. The extent of India's LSD pandemic and its impacted areas are still not fully investigated. Given the implications of lumpy skin disease for the economy, public health, and society as a whole, it is essential to look into outbreaks across a wide geographic range, taking into account all provinces nationwide, with the potential for significant genome sequencing to illustrate the molecular epidemiology of LSDV in the country and implement a well-planned vaccination programme against the virus.

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