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Research Article

Molecular detection and phylogenetic analysis of lumpy skin disease virus from clinically infected cattle of Andhra Pradesh

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Abstract

Lumpy skin disease (LSD) is a re-emerging transboundary disease with limited host range and is currently restricted to cattle and water buffaloes. OIE makes it as a notifiable disease because of its potential for rapid spread and significant economic losses worldwide. Therefore, it is crucial to detect and characterize LSD in animals in order to lessen the financial burden on farmers. In the present investigation, a total of 69 samples (34 skin scabs, 17 nasal swabs and 18 blood samples) from 46 clinically infected cattle in different regions of Andhra Pradesh were collected. All the samples were tested by Polymerase chain reaction (PCR) targeting p32 and A33R genes. A total of 88.24% (30/34) skin scabs and 17.65% (3/17) nasal swabs were found positive for both p32 and A33R genes by PCR. The complete coding region of A33R gene from three field isolates representing different regions of Andhra Pradesh was sequenced, and the phylogenetic analysis showed 100% similarity with isolates from Turkey, Serbia, Russia and Kazakhstan. This new technique of amplifying LSDV A33R genome for the detection of LSDV in clinical samples is equivalent to the OIE-recommended PCR technique that amplifies the p32 gene.

Keywords: Andhra Pradesh, LSDV, PCR, Phylogenetic analysis

Highlights

- PCR is a quick and reliable way to detect LSDV from clinical samples.
- A33R based PCR assay was also used for the detection of LSDV from clinical samples in comparison with the p32 based PCR assay, which was suggested by OIE, as both of them produced similar results.
- Phylogenetic analysis reveals nucleotide sequences of the field isolates in this study were clustered with other LSDV sequences from Turkey, Kazakhstan, Russia and Serbia.

INTRODUCTION

Lumpy skin disease is a vector-borne pox disease of cattle and Asian water buffaloes. Manifestation of skin nodules is the defining feature of this illness. The disease, which was formerly restricted to Africa and the Middle East, has expanded to the Balkans, the Caucasus, and the southern Russian Federation since 2015. In India, the first case was recorded in 2019 from Odisha (Sudhakar *et al*., 2022).

LSDV is classified under the genus *Capripoxvirus*, subfamily *Chordopoxvirinae*, and family *Poxviridae* (Fenner *et al*., 1987). Arthropods serve as mechanical vectors and play a major role in spreading the LSD virus. Rare transmissions happen when cattle are in direct contact and drink or eat contaminated food (Ali *et al*., 2012). Incidence is more common during the moist, warm conditions of summer and fall, which supports arthropod reproduction in low lying agro-climate zones and along water courses (Ahmed *et al*., 2020). Secretions, including blood, saliva, semen, nasal discharge, lachrymal discharge, milk, and skin lesions of the infected animals, contain the virus (Babiuk *et al*., 2008). LSD has a morbidity range of 50% to 100%, and the mortality rate is typically between 1% - 5% with occasional higher rates (Kumar *et al*., 2021).

LSD outbreaks result in significant economic losses for the afflicted nations, but poor, small-scale, and backyard farmers are the hardest hurt. The illness has a significant negative influence on milk production, animal body condition and overall productivity. Abortion, infertility, and harm to hides are all consequences. The tentative diagnosis can be made by recognisable clinical symptoms (OIE, 2021). Amplification and detection of p32 gene by PCR is the most commonly used diagnostic assay. However, the p32 gene is not suitable for phylogenetic analysis to record the relationship with other viruses circulated in different parts of the globe. In this study, we developed

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a PCR technique for LSDV detection through the detection of A33R gene, an immunodominant gene that codes for envelope proteins. The complete coding region of A33R gene of field isolates representing different regions of Andhra Pradesh was sequenced, and the phylogenetic analysis was done.

MATERIALS AND METHODS

Area of study: The study was carried out in different districts of Andhra Pradesh from August 2022 to April 2023 (Fig. 1), where the weather is hot and humid, providing suitable conditions for developing vector-borne diseases.

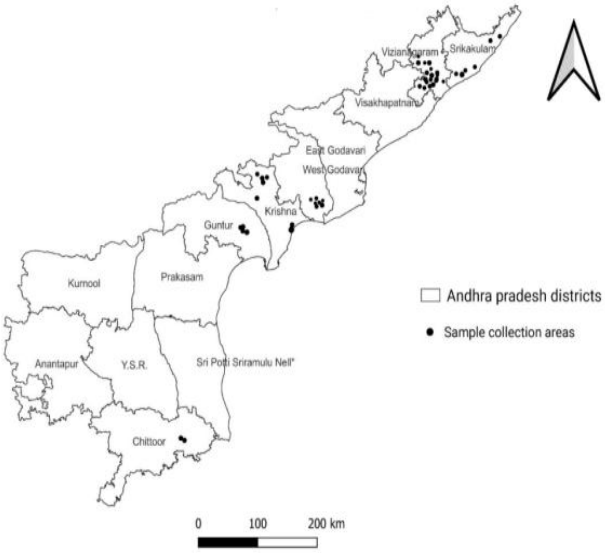


Fig. 1. Map of Andhra Pradesh showing the sample collection areas. This map is created using QGIS software

Sample collection: A total of 34 skin scabs, 17 nasal swabs and 18 blood samples were collected from 46 clinically infected cattle in Andhra Pradesh, showing symptoms of fever, skin nodules, and enlarged lymph nodes. The skin scabs were collected in sterile 50% glycerol saline, nasal swabs in sterile phosphate buffer saline (PBS) (pH 7.4) and whole blood with EDTA anticoagulant. The samples were collected aseptically, placed in an ice box and transferred to the Department of Veterinary Microbiology, NTR College of Veterinary Science (NTRCVSc), Gannavaram. The skin scabs and nasal samples were stored at −80°C and blood at 4°C for further analysis.

Sample processing and viral DNA extraction: About 10-20 mg of scab tissues were homogenised in mortar and pestle using 1 mL PBS, centrifuged @ 1000 g for 10 min, and the supernatant was collected. This is used for viral DNA extraction using GeNei™ TRIsoln reagent according to the manufacturer's instructions. Similarly, viral DNA was extracted from nasal swabs and blood according to the manufacturer's instructions. The DNA was eluted in 50 µL Tris-EDTA buffer (concentration of 10 mM Tris and 1 mM EDTA with pH adjusted to 8.0 by HCL), and the concentration of DNA was measured with Nanodrop spectrophotometer 200C (Thermo Scientific, USA). The DNA samples (Optical density ratio of 1.8 to 2) were stored at −80°C until further use. The viral DNA extracted from the Goat pox vaccine was used as positive control.

Amplification of LSDV p32 and A33R gene by PCR: All the DNA samples were first tested for detection of p32 gene using OIE recommended primers (Table 1). For PCR, 12.5 µL of 2X GoTaq Green master mix (GoTaq® DNA polymerase in 2X Green GoTaq® reaction buffer of pH 8.5, 400 µM of each dNTPs and 3 mM $MgCl_2$) (Promega, USA), 1.25 µL of each forward and reverse primers (20 pmol), 150 ng of DNA with final volume of 25 µL was prepared using nuclease free water. The assay was carried out in Proflex PCR systems, Applied biosystems, under standardized cycling conditions (Table 2).

For the detection of immunodominant A33R gene, primers and cycling conditions used were mentioned in Table 1 and 2, respectively. The PCR products were subjected to gel electrophoresis in 1.5% agarose, visualized under UV illumination and documented using BIO-RAD gel documentation system.

Table 1. 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)
A33R	F:5′-GGTCGACATGTTAGTTGATATTCCAAAGAGT-3′ R:5′-CCGGGATCCTTAAAAAAAAGATCTTACACAG TAATAGC-3′	588bp	(Le Goff *et al*., 2009; Jaferin *et al*., 2022)

Table 2. Standardized thermal cycling conditions used in this study

Steps	Standardized thermal cyclical conditions	
	p32	A33R
Initial denaturation	94°C for 5 min (1 cycle)	94°C for 5 min (1 cycle)
Denaturation	95°C for 30 sec	94°C for 45 sec
Annealing	55°C for 30 sec	57.7°C for 45 sec
Extension	72°C for 1 min (35 cycles)	72°C for 45 sec (35 cycles)
Final extension	72°C for 5 min (1 cycle)	72°C for 10 min (1 cycle)

Nucleotide sequencing and phylogenetic analysis: The PCR products of three field isolates, namely VZM-7S, KSN-4S, and TNK-2S positive for A33R gene representing different regions of Andhra Pradesh were sequenced by Barcode Biosciences Private Limited, Bangalore, Karnataka, India. The nucleotide sequences were edited by aligning with the reference sequences of LSDV, sheeppox virus (SPPV), goatpox virus (GTPV) and other vaccine recombinant strains retrieved from GenBank using Codon Code Aligner software.

The Phylogenetic analysis was carried out further in MEGA 11 using ClustalW, and a maximum likelihood tree was constructed with a bootstrap value of 1000 replicates. These three isolates' nucleotide sequences have been submitted to GenBank.

RESULTS

Detection of LSDV by PCR: A total of 88.24% (30/34) skin swabs and 17.65% (3/17) nasal swabs were found to be positive for p32 (192 bp, Fig. 2) and A33R genes (588bp, Fig. 3). None of the blood samples were found positive by PCR assays. The details of PCR results are mentioned in Table 3.

The agreement between the two PCR assays was evaluated by determining Cohen's kappa coefficient (k) which is shown in Table 4. We found that the two assays have a coefficient of 1.0, which denotes a perfect agreement.

Table 3. Details of PCR results by targeting p32 and A33R gene[1]

SI. No	Sample ID	Type of Sample	p32	A33R
1	SKLM-1S	Skin scab	✔	✔
2	SKLM-2S	Skin scab	✔	✔
3	SKLM-3S	Skin scab	✔	✔
4	SKLM-4S	Skin scab	✔	✔
5	SKLM-5S	Skin scab	✔	✔
	SKLM-5N	Nasal swab	✔	✔
	SKLM-5B	Blood	×	×
6	SKLM-6S	Skin scab	✔	✔
7	VZM-1S	Skin scab	✔	✔
8	VZM-2S	Skin scab	✔	✔
9	VZM-3S	Skin scab	×	×
10	VZM-4B	Blood	×	×
11	VZM-5B	Blood	×	×
12	VZM-6B	Blood	×	×
13	VZM-7S	Skin scab	✔	✔
	VZM-7B	Blood	×	×
14	VZM-8S	Skin scab	✔	✔
	VZM-8N	Nasal swab	×	×
	VZM-8B	Blood	×	×
15	VZM-9N	Nasal swab	×	×
	VZM-9B	Blood	×	×
16	VZM-10B	Blood	×	×

Cont. Table 3.

Table 3., Cont. ...

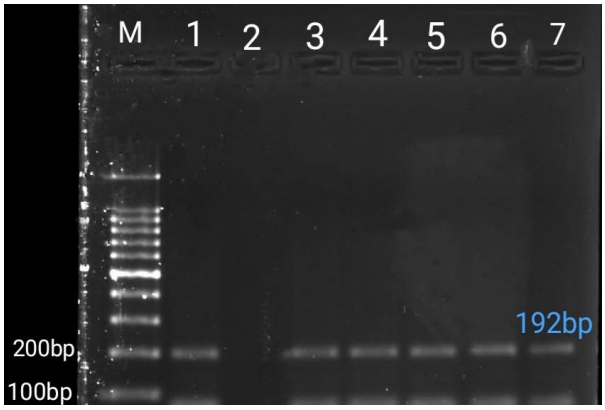
SI. No	Sample ID	Type of Sample	p32	A33R
17	VZM-11N	Nasal swab	×	×
	VZM-11B	Blood	×	×
18	VZM-12B	Blood	×	×
19	VZM-13S	Skin scab	×	×
	VZM-13B	Blood	×	×
20	VZM-14B	Blood	×	×
21	VZM-15N	Nasal swab	×	×
22	VZM-16S	Skin scab	✔	✔
	VZM-16N	Nasal swab	✔	✔
	VZM-16B	Blood	×	×
23	VZM-17S	Skin scab	✔	✔
	VZM-17N	Nasal swab	×	×
24	VZM-18S	Skin scab	✔	✔
	VZM-18N	Nasal swab	×	×
25	TNK-1N	Nasal swab	×	×
26	TNK-2S	Skin scab	✔	✔
	TNK-2N	Nasal swab	✔	✔
27	TNK-3S	Skin scab	✔	✔
	TNK-3N	Nasal swab	×	×
28	TNK-4S	Skin scab	✔	✔
	TNK-4N	Nasal swab	×	×
29	TNK-5N	Nasal swab	×	×
30	TNK-6S	Skin scab	✔	✔
	TNK-6N	Nasal swab	×	×
31	KSN-1S	Skin scab	✔	✔
	KSN-1N	Nasal swab	×	×
	KSN-1B	Blood	×	×
32	KSN-2S	Skin scab	✔	✔
33	KSN-3S	Skin scab	✔	✔
	KSN-3B	Blood	×	×
34	KSN-4S	Skin scab	✔	✔
35	KSN-5S	Skin scab	✔	✔
	KSN-5B	Blood	×	×
36	KSN-6S	Skin scab	✔	✔
	KSN-6B	Blood	×	×
37	KSN-7B	Blood	×	×
	KSN-7N	Nasal swab	×	×
38	KSN-8S	Skin scab	×	×
39	GTR-1S	Skin scab	×	×
40	GTR-2S	Skin scab	✔	✔
	GTR-2N	Nasal swab	×	×
41	GTR-3S	Skin scab	✔	✔
42	GTR-4S	Skin scab	✔	✔
43	MCP-1S	Skin scab	✔	✔
44	MCP-2S	Skin scab	✔	✔
45	PMR-1S	Skin scab	✔	✔
46	PMR-2S	Skin scab	✔	✔

[1] ✔ Position: Positive PCR confirmation; ×: Negative PCR confirmation

Table 4. Determination of Cohen's kappa coefficient (k)[2]

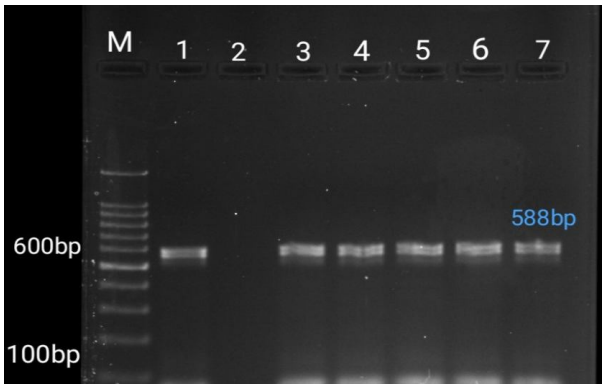
PCR assay		p32 gene		
		Positive	Negative	Total
A33R	Positive	33	0	33
	Negative	0	36	36
	Total	33	36	69

[2]Cohen's kappa coefficient (k) = P_0-P_e/1-P_e = 1-0.502/1-0.502 = 1 (Perfect agreement)
P0: Observed agreement, Pe: Expected agreement



[Lane M: DNA ladder (100 bp); lane 1: Positive control (192bp); lane 2: Negative control; lane 3-7: Samples positive for LSDV p32 gene (192bp)]

Fig. 2. Gel image of PCR targeting p32 gene



[Lane M: DNA ladder (100bp); lane 1: Positive control(588bp); lane 2: Negative control; lane 3-7: Samples positive for LSDV A33R gene (588bp)]

Fig. 3. Gel image of PCR targeting A33R gene

Phylogenetic analysis: To ascertain the origin and degree of genetic relatedness between the LSDV strains from Andhra Pradesh, India, and the strains circulating in other nations, the complete coding region of the A33R gene from three field isolates (KSN-4S, VZM-7S, and TNK-2S) was sequenced. The nucleotide sequences of these three field isolates, VZM-7S, KSN-4S, and TNK-2S, were submitted to GenBank under the accession numbers OQ858567, OQ858568, OQ868794, respectively.

A phylogenetic tree (Fig. 4) was constructed in which our field isolates nucleotide sequences were found to be in a cluster with LSDV isolates from Turkey (Pendik), Serbia, Russia, and Kazakhstan. However, vaccine strains were clustered together separately. The goatpox and sheeppox strains also clustered separately and were far from the field strains.

DISCUSSION

The livelihood of rural people is severely hampered by diseases that impact livestock output and population. LSD is one of the economically significant diseases, and it was detected in cattle for the first time from Odisha, India in 2019 (Sudhakar *et al*., 2022). As this disease causes greater economic losses to farmers worldwide, proper diagnosis is essential. In the current study, skin scabs, nasal swabs and blood samples taken from the clinically infected cattle were used for molecular detection by performing PCR assay. Our findings are in agreement with earlier studies (Khalefa *et al*., 2015; Allam *et al*., 2020; Gupta *et al*., 2022), which highlighted the importance of using PCR as a quick and reliable way to detect LSDV in emergency situations and recommended it as the best method for diagnosis of LSD.

Primary detection was done by amplification of capripoxvirus specific p32 gene 192bp, which was suggested by OIE (OIE, 2021). Later, the samples were subjected to A33R gene amplification, as suggested by Jaferin *et al*. (2022). Both the PCR assays produced similar results, and the agreement between these two assays has a Cohen's kappa coefficient of 1.0, which denoted a perfect agreement. Hence, A33R-based PCR assay's sensitivity was 100% compared to p32-based PCR assay. This finding is in agreement with the previous study (Jaferin *et al*., 2022). Skin scabs were the greatest sources for virus detection when compared to nasal and blood samples because of the virus tissue tropism, which was evident from current study results, and this is in agreement with earlier studies (Tuppurainen *et al*., 2005; Babiuk *et al*., 2008).

Furthermore, to know the genetic relatedness of the virus, the sequencing data was employed for phylogenetic analysis by comparing with different LSD field strains, goatpox and sheeppox strains, as well as vaccine strains retrieved from GenBank. The phylogenetic analysis of A33R gene revealed that LSDV isolates from the current study were clustered with isolates from Turkey, Kazakhstan, Russia, and Serbia. This is in agreement with the study by Srinivas *et al*.

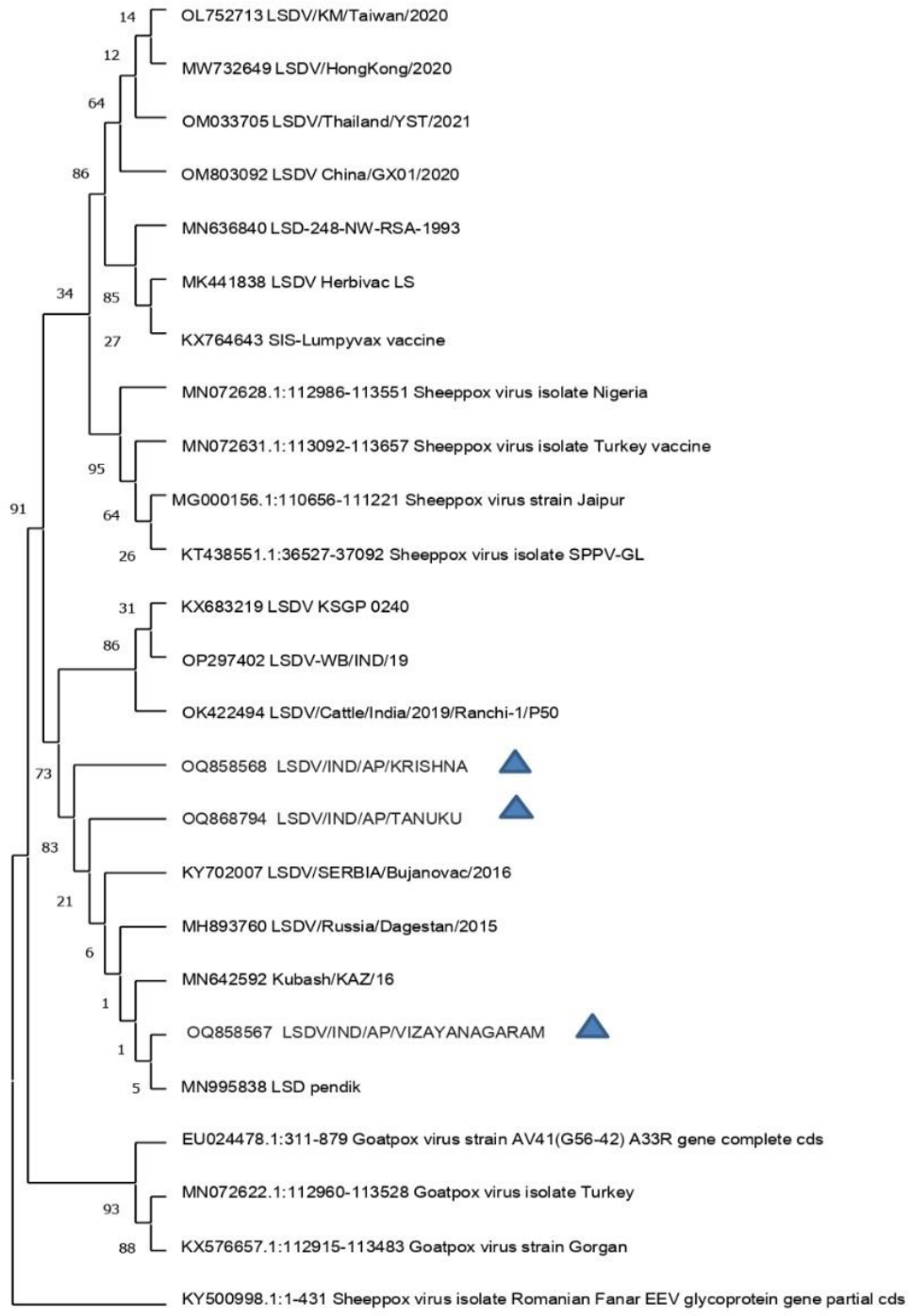


Fig. 4. Evolutionary analysis of A33R gene by Maximum Likelihood Method[1]

[1]The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-2053.99) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. This analysis involved 25 nucleotide sequences. There were total of 589 positions in the final dataset. Evolutionary analyses were conducted in MEGA11(Tamura *et al*., 2021).

(2023), which said that LSDV strains circulating in India were similar to those in Turkey, Kazakhstan, Kenya, Russia, Serbia, Nigeria and other Indian LSDV strains. According to blast analysis, present study isolates had 99.8-100% genetic identity with newly emerged vaccine-like LSDV recombinants linked to the field outbreaks in Russia and Kazakhstanthus indicating a shared exotic source of introduction, which is in accordance with earlier findings (Sprygin *et al*., 2018; Amirgazin and Ragatova, 2020). Contrarily, current study isolates were not clustered with Neethling NI-2490/1958, Kenya/ 1958, and KSGP O-240 strains that were connected to the 2019 LSD epidemics in Bangladesh and India (Badhy *et al*., 2021; Gupta *et al*., 2022; Sudhakar *et al*., 2022).

Although we don't have any hard proof of how these strains got to India, our findings in this study points to an exotic source of introduction. This is because, before the first LSD outbreaks in 2019, India had never immunised cattle against LSD; instead, only an emergency immunisation with the GTPV strain (Uttarkashi strain) was performed as a prophylactic step. Arthropods are the primary mechanical carriers of this virus, but live animal movement can also disseminate it across regions and continents (Sprygin *et al*., 2019). So, there is a chance that LSD could enter India through the informal and illegal transborder trade of live cattle and buffalo.

The current investigation provides molecular detection and phylogenetic analysis of LSDV in Andhra Pradesh region of India. Through this, we found that A33R-based PCR assay was also used for the detection of LSDV from clinical samples in comparison with the p32 based PCR assay, which was suggested by OIE, as both of them produced similar results. In the present study, phylogenetic analysis was done based on limited information. Hence, whole genome sequencing is to be carried out to determine the lineage. As the LSD-impacted areas across the country are not thoroughly investigated, there is a need for continuous monitoring and molecular characterisation of the circulating LSDV, which aids in developing disease management strategies, provides information for the development of targeted vaccines for the diagnosis and control of LSD in India as well as avoiding the financial losses caused by this condition in the dairy industry.

Conflicts of interest: The authors declare no conflict of interest. The funders had no role in the design of the study, the collection, analysis, or interpretation of data, the writing of the manuscript, or the decision to publish the results.

Author's contributions: RPRN: Contributed to the study conception, design and methodology; SKG: Sample collection, molecular experiments, data analysis, data interpretation and manuscript drafting; SAR: Contributed to the review and editing. All authors have read and agreed to the final version of the manuscript.

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