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Isolation and histopathological analysis of Lumpy skin disease virus in embryonated chicken eggs

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ABSTRACT

Lumpy skin disease (LSD) has emerged as a major transboundary viral disease of cattle in recent years, causing substantial economic losses. The present work focused on isolation and molecular characterization of LSD virus (LSDV) field strains from Andhra Pradesh using embryonated chicken eggs (ECE) as an experimental host system. Skin scab samples collected from clinically suspected cattle were first examined using Capripoxvirus-specific PCR. The positive samples were inoculated into the chorioallantoic membrane (CAM) of 10-day-old ECEs to study the virus's growth pattern. After several passages, infected CAMs showed congestion, haemorrhage, thickening, and discrete white pock-like foci. Histopathological examination revealed degeneration of epithelial cells, vascular congestion, haemorrhage, and eosinophilic intracytoplasmic inclusions typical of Poxvirus infections. The LSDV presence in CAM was confirmed by PCR targeting the F gene. Sequencing and phylogenetic analysis demonstrated that Andhra Pradesh isolates clustered with previously reported isolates from Odisha, Tamil Nadu, and West Bengal. The study concludes that isolation of LSDV in ECE is a practical, economical diagnostic method, though adaptation is variable due to strain heterogeneity.

Keywords: Embryonated chicken eggs, Histopathology, Lumpy skin disease virus, PCR, Phylogenetic analysis

Lumpy skin disease (LSD) is a highly infectious condition primarily affecting cattle and occasionally buffaloes and wild ruminants such as antelopes. The disease is caused by the lumpy skin disease virus(LSDV), a member of the genus *Capripoxvirus* and *Poxviridae* family, subfamily *Chordopoxvirinae* (Gupta *et al*. 2020). The virions are brick-shaped or ovoid, enveloped, and contain a linear double-stranded DNA genome closely related to the sheeppox and goatpox viruses. Like other poxviruses, replication occurs in the cytoplasm of host cells, forming distinct perinuclear viral factories (Maclachlan and Dubovi, 2010).

Transmission of LSDV occurs mainly through blood-feeding arthropods such as mosquitoes, ticks, and biting flies, which act as mechanical vectors (Liang *et al*. 2022). Infected cattle shed the virus through saliva, nasal secretions, blood, semen, milk, and lesions (Babiuk *et al*. 2008). The disease exhibits high morbidity, ranging between 50–100%, but mortality is typically low (1–5%), though higher rates have been noted in naïve herds (Kumar *et al*. 2021). Clinically, it manifests with fever, enlarged lymph nodes, nasal discharge, and multiple firm papules or *nodules* (0.5–5 cm) over the skin, including the head, limbs, udder, and mucous membranes (Tageldin *et al*. 2014; Sudhakar *et al*. 2020).

The disease poses serious concerns for animal health, trade, and livelihoods due to its production losses and potential for rapid spread. Although LSD outbreaks have been confirmed in several Indian states since 2019, data from Andhra Pradesh remains limited. Hence, the current study aimed to isolate and molecularly characterize LSDV from local field cases of Andhra Pradesh to aid in understanding viral diversity and support diagnostic and preventive strategies.

MATERIALS AND METHODS

Ethical considerations: Prior permission was sought and obtained from the university to conduct this research, ensuring that it aligns with established ethical standards and guidelines. The investigation necessitated the collection of skin scabs from cattle suspected of LSD. Following established procedures for such sample collection, skin

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scabs were obtained without the use of anaesthesia.

Sample collection: Two skin scab samples, designated VZM-1S and KSN-4S, were obtained from cattle in Vizianagaram and Krishna districts of Andhra Pradesh. The animals exhibited fever, skin nodules, lymphadenopathy, anorexia, and nasal discharge, consistent with LSD infection.

Sample processing and DNA extraction: Approximately 10–20 mg of each scab was homogenized in sterile phosphate-buffered saline (PBS, 1x) using a mortar and pestle. The homogenate was centrifuged at 1000 rpm for 10 minutes, and the supernatant was used for DNA extraction. Genomic DNA was isolated using GeNei™ TRIsoln reagent as per manufacturer's protocol and eluted in 50 µL of TE buffer. DNA concentration and purity were determined with a Nanodrop 200C spectrophotometer (Thermo Scientific, USA). Samples with A260/A280 ratios between 1.8–2.0 were stored at –80°C until use. Viral DNA from a Goatpox vaccine served as a positive control.

Amplification of Capripox specific p32 gene: Detection of the *p32* gene of Capripoxvirus was carried out using OIE-recommended primers (F: 5′-TCCGAGCTCTTTCCTGATTTTTCTTACTAT-3′, R: 5′-TATGGTACCTAAATTATATACGTAAATAAC-3′), generating a 192 bp amplicon (Ireland & Binepal, 1998; Tuppurainen *et al*., 2005; OIE, 2021). PCR was performed in a 25 µL reaction volume containing GoTaq® Green Master Mix, primers, template DNA, and nuclease-free water. Cycling conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 1 min), with a final extension at 72°C for 5 min. Amplified products were resolved in 1.5% agarose gel and visualized under UV light using a BIO-RAD gel documentation system.

*Virus isolation in embryonated chicken eggs***:** Samples positive for the *p32* gene were processed for virus isolation. Scabs were triturated in PBS with sterile sand to make a 10% suspension, treated with antibiotic-antimycotic solution (1X Liquid w/v penicillin@ 10,000 U, streptomycin@ 10mg, amphotericin B @ 25µg), centrifuged at 2000 rpm for 20 min, and filtered through a 0.22 µm membrane. The filtrates were stored at –80°C and used for inoculation.

Nine- to eleven-day-old specific-pathogen-free embryonated chicken eggs (Srinivasa Hatcheries Ltd., Andhra Pradesh) were disinfected with 70% ethanol. Using a sterile syringe, 0.1 mL of filtered suspension was inoculated on the chorioallantoic membrane (CAM) (Schmidt, 1964). Eggs were candled at 24-hour intervals; embryos dying within the first 24 hours were discarded as non-specific deaths. The CAMs were harvested from embryos that died between 24–120 hours post-inoculation and stored at –80°C for subsequent analysis.

Confirmation of Isolated Virus: DNA extracted from infected CAMs was subjected to PCR using LSDV F gene-specific primers (F: 5′-ACTAGTGGATCCATGGACAGAGCTTTATCA-3′, R: 5′-GCTGCAGGAATTCTCATAGTGTTGTACTTCG-3′) targeting a 472 bp fragment (Sudhakar *et al*. 2019; Gupta *et al*. 2022; Sethi *et al*. 2022). Amplification was performed with cycling parameters: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. The products were analyzed via agarose gel electrophoresis.

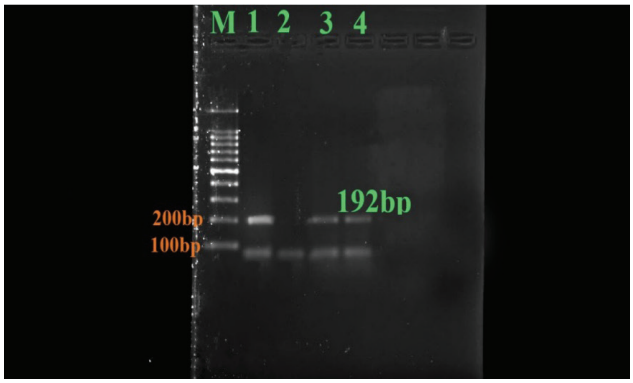
Histopathological Examination: CAMs showing lesions were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax at 56–60°C. Thin sections (4–5 µm) were stained with hematoxylin and eosin (H&E) and examined microscopically for characteristic lesions.

Nucleotide Sequencing and Phylogenetic Analysis: PCR products of the F gene from positive isolates were sequenced commercially (Barcode Biosciences Pvt. Ltd., Bangalore). Sequence editing and alignment were done using Codon Code Aligner v10.0, and BLAST searches were conducted in NCBI for homology comparison. Phylogenetic trees were constructed in MEGA 11 using the Maximum Likelihood method with 1000 bootstrap replicates (Tamura *et al*., 2021). The sequences were deposited in GenBank.

RESULTS AND DISCUSSION

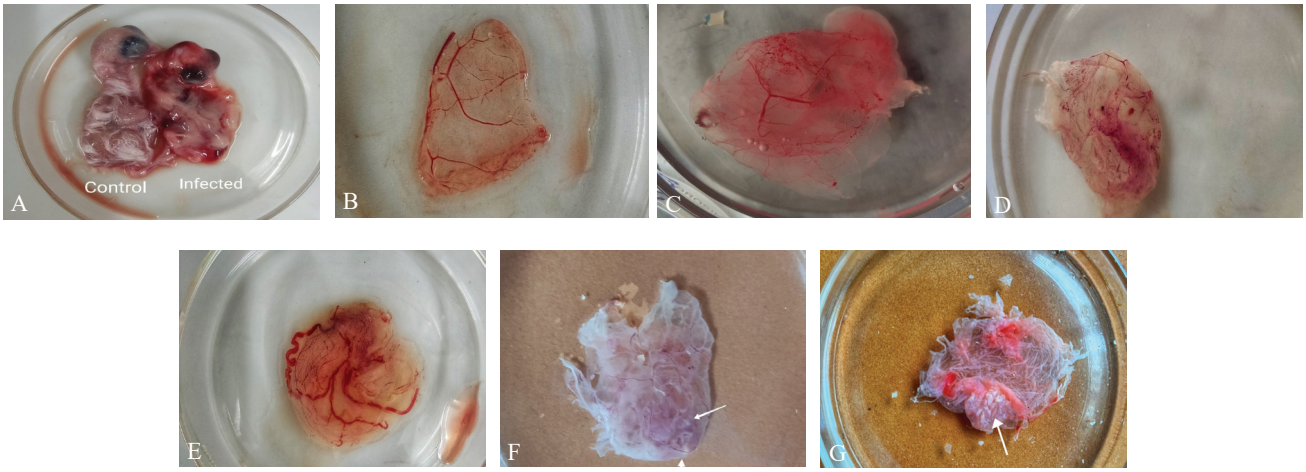
Detection of LSDV by PCR: Both DNA samples tested positive for the Capripoxvirus *p32* gene, yielding a 192 bp amplicon (Fig. 1). This confirmed the presence of Capripoxvirus DNA in the collected scabs.

Virus isolation: Isolation of LSDV in ECE: Two skin scab viral suspensions (VZM-1S and KSN-4S), which were confirmed as *Capripoxvirus* by PCR assay targeting the *p32* gene, were propagated through the CAM route in embryonated chicken eggs up to 5th passage (Fig. 2). The mortality of chicken embryos was observed after 96 h at each passage level. Both the isolates produced the characteristic pock lesions on CAM of ECE after fourth passage and became clear after fifth passage. The changes



Lane M: DNA ladder (100bp)
Lane 1: Positive control -Goatpox Vaccine (192bp)
Lane 2: Negative control
Lane 3: Positive sample (KSN-4S)
Lane 4: Positive sample (VZM-1S)

Fig. 1 Amplification of Capripox specific p32 gene (192bp).



A. Infected embryo showing haemorrhages and stunted growth; B. Uninfected CAM (control); C. 1st passage CAM showing pin point haemorrhages and congestion
D. 2nd passage CAM showing haemorrhages, edema, and thickness
E. 3rd passage CAM showing haemorrhages, edema, thickness and occlusion of blood in blood vessels
F. 4th passage CAM showing haemorrhages, and micro pock lesions
G. 5th passage CAM showing haemorrhages, and visible pock lesions

Fig.2 (A-G) Growth behaviour of LSDV on embryonated chicken eggs

on the CAM observed were listed in Tables 1 and 2. Since the isolates originated from the same geographical area, the consistency in their embryonic development patterns indicated less obvious differences in the growth of viruses on CAM. These pathological findings are in agreement with the observations made by Prakash *et al*. (1995), Kamal *et al*. (2016), Abdallah *et al.* (2018), El-Ansary*et al*. (2022) and Elsheikh *et al*. (2023).

Confirmation of LSDV isolated from Embryonated chicken eggs: PCR amplification targeting the F gene yielded a 472 bp product from infected CAMs, whereas the uninfected controls showed no amplification (Fig. 3). This confirmed successful replication of LSDV within the embryonated eggs.

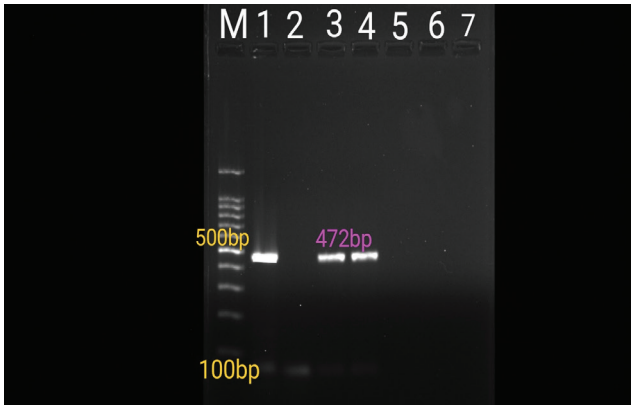
Histopathological findings on the CAM of LSDV-infected embryonated chicken eggs: In the present study, growth pattern LSDV was isolated in ECE. The LSDV was adapted to CAM ectodermal cells starting from the first passage level, with macroscopic findings of pin point

Table 1. Pathological changes of LSD field isolates in embryonated chicken eggs at different passage level

Passage no.	Changes at each passage level
1	Embryo mortality at 96 h PI, no major changes on CAM, except for pin point haemorrhages and congestion.
2	Embryo mortality at 96 h PI, haemorrhages on embryo, edema and haemorrhages on CAM.
3	Embryo mortality at 96 h PI, haemorrhages on embryo, thickness, edema and haemorrhages on CAM along with occlusion of blood in blood vessels.
4	Embryo mortality at 96 h PI, haemorrhages on embryo, thickness, edema and haemorrhages on CAM along with micro pock lesions on CAM.
5	Embryo mortality at 96 h PI, haemorrhages on embryo, thickness, edema and haemorrhages on CAM along with visible pock lesions on CAM.

Table 2. Observations of LSD virus growth and pathogenicity on the CAM

S. No	Character	Passage No.				
		1	2	3	4	5
1.	Growth on CAM	++	+++	+++	++++	++++
2.	Pocks					
	a. Size of pocks	–	–	–	2 mm	2-3 mm
	b. Colour	–	–	–	White	White
	c. Shape of pocks	–	–	–	Round	Round
	d. Haemorrhages on pocks	–	–	–	–	–
3.	Lethality of chicken embryo	+	+	+	+	+
4.	Haemorrhages on chicken embryo	+	+	+	+	+



Lane M: DNA ladder (100bp)
Lane 1 : Positive control(472bp)
Lane 2 : Negative control
Lane 3 : LSDV positive isolate (KSN-4S)
Lane 4: LSDV positive isolate (VZM-1S)

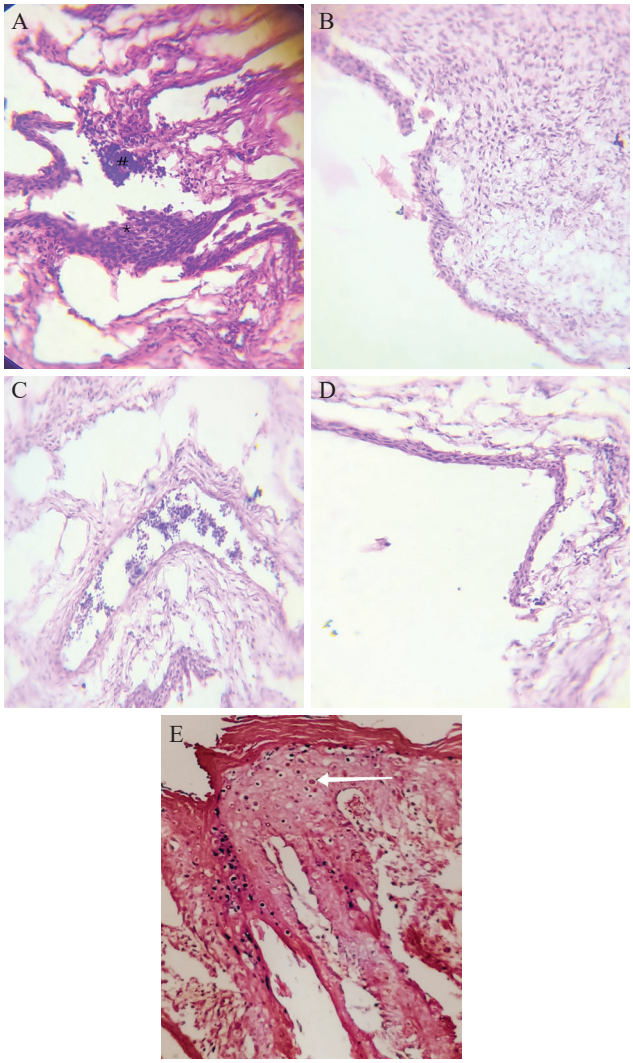
Fig. 3 PCR assay confirming LSDV isolated from chicken embryos by amplifying F gene (472bp)

haemorrhages and congestion. The lesion in the infected CAM was noticeable with thickening of the membrane over the inoculation site. The viraemic stage was marked by haemorrhage. Furthermore, at the 4th and 5th passage levels, the virus displayed virulence by generating necrotic pock lesions of 2-3 mm in diameter, which is characteristic of pox virus. The findings were presented in Fig. 4 (A-E). The present study results were supported by Wallace *et al*. (1994) and Elsheikh *et al*. (2023). On the contrary, Adlakha *et al*. (1971) and Bandyopadhyay *et al.* (1984) made observations that chicken embryos were not suitable for the growth of Capripoxviruses in their experiments, although Prakash et al. (1995) had clarified that the reason for the virus failure to grow in chicken embryos might be strain variations.

Phylogenetic analysis: The partial F gene sequences from the two isolates, namely VZM-1S & KSN-4S were sequenced to find out the degree of genetic relatedness among LSDV strains found in various nations and those originating in Andhra Pradesh, India and submitted to the GenBank with the accession numbers OR906274 and OR906275 respectively. Blast analysis showed 100% similarity with Kenya, Odisha, Tamil Nadu, Karnataka and China isolates from GenBank.

A phylogenetic tree (Fig. 5) was constructed, which showed that the present study isolates from Vizianagaram & Krishna were in one node and clustered with isolates from Odisha, Tamil Nadu and West Bengal. This finding is in agreement with Bodlapati *et al*. (2023). All the vaccine strains were clustered together in one node. Current study phylogenetic analysis outcome suggests that, circulating field isolates were similar to the isolates that enter India during 2019 outbreak (Sudhakar *et al*. 2019; Gupta *et al*. 2020; Sethi *et al*. 2021).

This study confirmed the successful isolation of LSDV from field cases in Andhra Pradesh using



A: Image of LSDV infected CAM showing haemorrhage(#) and thickening of the membrane(*) (H&E×400); B: Image of LSDV infected CAM showing its thickening over inoculation site; (H&E×400); C: Image showing congestion of blood vessel (H&E×400); D: Vacuolar degeneration of epithelial cells in the LSDV infected CAM (H&E×400); E: LSDV inoculated CAM showing eosinophilic, intra-cytoplasmic inclusions in epithelial cells (H&E×400).

Fig. 4 (A-E) Histopathological changes in the CAM of embryonated chicken eggs infected with LSDV

embryonated chicken eggs. The combination of PCR and histopathological evaluation proved reliable for confirming infection. Although adaptation of LSDV in ECEs required multiple passages, the method is simple, efficient, and low-cost diagnostic tool. Future investigations using complete genome sequencing may provide a deeper insights into the molecular evolution and epidemiology of LSDV circulating in India.

Authorship contributions: Saikumar G: sample collection, molecular analysis, data interpretation and manuscript drafting; Ramani Pushpa RN: study conception, design and methodology; Supriya AR: review and editing; CH. Sudha Rani Chowdary: pathological examination. All

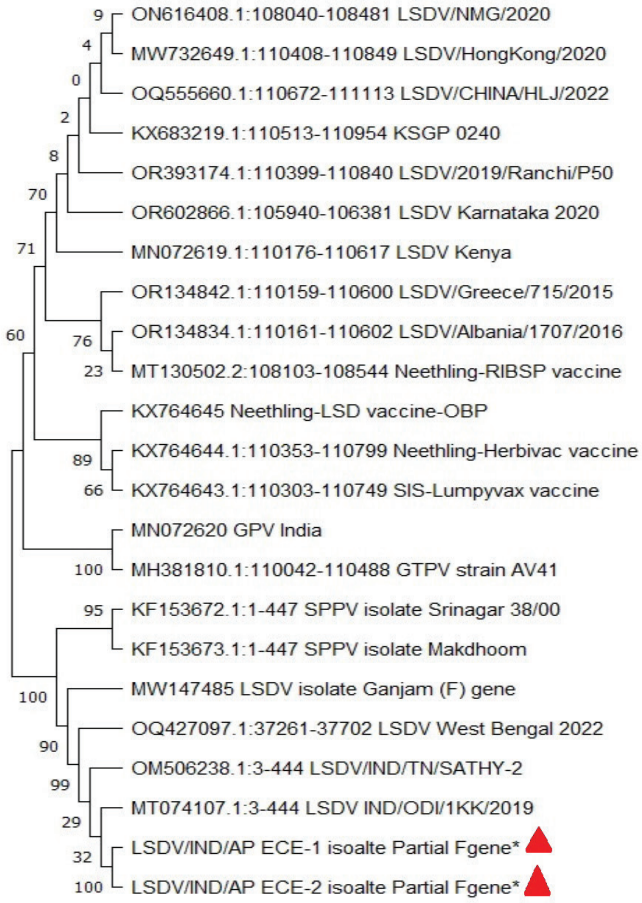


Fig. 5 Phylogenetic tree based on partial fusion gene sequences by Maximum Likelihood method
(Note: The sequences obtained through this investigation are identified by ( ) labels. Other LSDV, SPPV, and GTPV sequences used in this analysis were obtained from GenBank.)

authors have read and agreed to the final version of the manuscript.

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