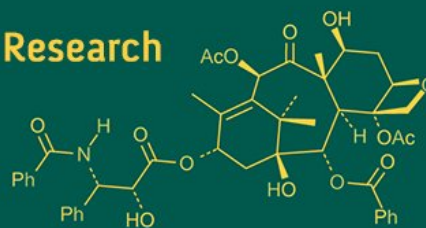


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Molecular detection and seroconversion dynamics of lumpy skin disease virus in natural infected cattle herds

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Abstract

Lumpy Skin Disease (LSD) is a highly infectious, economically devastating poxviral condition in cattle. Because its nodular clinical manifestations mimic other dermatological diseases, definitive laboratory confirmation is critical for effective outbreak management. This study (January-September 2023) evaluated the diagnostic efficacy of different clinical matrices for Lumpy Skin Disease Virus (LSDV) detection in symptomatic cattle. A total of 192 clinical samples—comprising tissue and nodular lavage (n=72), unclotted whole blood (n=78), and nasal swabs (n=42)—were analyzed using conventional PCR. Concurrently, 81 serum samples were screened for anti-LSDV antibodies using a double-antigen ELISA. LSDV was detected in 100% of the tissue samples, whereas no positivity (0%) was observed in the evaluated whole blood or nasal swabs. Furthermore, serological screening identified anti-LSDV antibodies in 31 of the 81 serum samples, establishing a seropositivity rate of 38.27%. These findings highlight the critical importance of selecting appropriate clinical matrices, underscoring the absolute superiority of tissue-based samples over non-invasive matrices for accurate field diagnostics, and illustrating the variability of the immune response based on disease progression.

Keywords: Lumpy skin disease virus, pcr, serosurveillance, field outbreak, elisa

Introduction

Lumpy skin disease (LSD) is caused by the lumpy skin disease virus (LSDV), a member of the genus Capripoxvirus within the family Poxviridae and subfamily Chordopoxvirinae. There is only one serotype of LSDV, the Neethling virus (the prototype strain), which is antigenically closely related to the sheep and goat poxviruses (Guyassa, 2022) [9]. In India, LSD is an emerging disease that was first reported in Odisha in 2019 (Kumar *et al.*, 2021) [13]. This initial outbreak was followed by a devastating second wave in 2022, causing the mortality of approximately 1.5 lakh (150,000) animals nationwide and inflicting severe economic losses. Official reports indicate that approximately 29 lakh cattle were affected, with 1.5 lakh deaths recorded in India during 2022 (DAHD, 2022). The significant economic impact stems from severe emaciation, hide damage, infertility in both males and females, mastitis, a drastic drop in milk production, and abortions (Tuppurainen and Oura, 2012) [21]. The LSD virus spreads among clinically and subclinically infected cattle primarily through insect vectors, including mosquitoes (*Anopheles stephensi*, *Aedes aegypti*, *Culex quinquefasciatus*) and biting midges (*Culicoides nubeculosus*), and less commonly through Ixodid hard ticks like *Rhipicephalus appendiculatus* (Tuppurainen *et al.*, 2013) [22]. Following its initial emergence in West Bengal and Odisha during the summer of 2019, the disease rapidly expanded its geographical footprint across India, spreading to the northeastern, northern, southern, and western regions between 2019 and 2022.

While a preliminary diagnosis of LSD is often based on characteristic nodular skin lesions, accurate diagnostic confirmation is mandatory for implementing effective control measures (EFSA Panel on Animal Health and Welfare, 2015) [4]. Furthermore, mild and subclinical forms of the disease require rapid and reliable laboratory testing. Laboratory diagnosis of LSD encompasses virus isolation in cell cultures, molecular identification techniques such as polymerase chain reaction (PCR)-based assays, detection of viral antigens via fluorescent antibody tests, and the detection of specific antibodies using serological assays (El-Nahas *et*

al., 2011)^[6]. Confirmatory testing is primarily achieved through real-time or conventional PCR assays specific to capripoxviruses (Alemayehu *et al.*, 2013)^[1]. Recent investigations evaluating the efficacy of these diagnostic tools emphasize that the choice of clinical sample drastically affects detection rates; for instance, PCR analysis reveals significantly higher positivity in tissue samples and scabs compared to nasal swabs or whole blood (Gupta *et al.*, 2025)^[8]. Furthermore, combining molecular detection with serological tools like ELISA provides a more comprehensive epidemiological picture of both active infection and prior exposure (Gayal *et al.*, 2022)^[7]. Given these diagnostic variables, this study aimed to investigate the effectiveness of molecular and serological methods, as well as the optimal choice of samples, for the confirmation of LSDV outbreaks in cattle and buffaloes.

Material and Methods

Study area and sample collection

Between September 2022 and October 2023, field officers from the Uttar Pradesh Department of Animal Husbandry collected clinical samples from cattle exhibiting symptoms of Lumpy Skin Disease (LSD) across nine districts of the state (Aligarh, Auraiya, Bahraich, Balrampur, Hamirpur, Hathras, Mahoba, and Mathura Varanasi). A total of 192 of these archived specimens were retrieved from the Department of Veterinary Microbiology repository for the current investigation. The study was approved with approval no. IAEC/24/1/21- 5/03/24 by the institute animal Ethics Committee Uttar Pradesh Pandit Deen dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura UP, India registered with Committee for the purpose of control and Supervision of Experiment on animals (CPCSEA).

Sample processing and DNA extraction

During the defined outbreak period, a total of 273 samples were collected for evaluation, consisting of skin scabs and intranodular saline lavage (n=72), unclotted whole blood (n=78), nasal swabs (n=42), and serum (n=81). Whole blood sample (81) was aseptically collected via jugular venepuncture into EDTA and clot activator vacutainers (Becton-Dickenson). Whole blood samples were initially diluted in PBS at a ratio of 1:1 and later used for DNA extraction. Nasal swabs (n=42) were obtained from affected cattle using sterile swabs. Tubes containing nasal swab samples in PBS were vigorously agitated in a vortex mixer before centrifugation at 3000 x g for 10 min at 4 °C. The clear supernatant fluid was collected and kept at -20 °C for future processing. Nodular tissue samples were homogenized in sterile phosphate buffer saline, subsequently tissue suspension was prepared further centrifuge at 3000 rpm for 10 min and supernatant was collected. Intranodular saline lavage samples were used directly in the DNA extraction procedure. The DNA isolation was carried out using the QIAGEN QIAamp DNA Mini Kit following the manufacturer's instructions. The DNA samples were stored at -20°C till use in amplification.

Molecular detection of LSDV genome in clinical samples by PCR: DNA was amplified utilizing capripoxvirus viral

attachment protein (p32) gene primer EEV-F- 5'-TTTCCTGATTTTCTTACTAT-3' and EEV-R-5'-AAATTATATACGTAAATAAC-3' (Ireland and Binopal, 1998)^[10]. The PCR reactions were prepared in a total volume of 25 µL, consisting of 12.5 µL of 2X EmeraldAmp MAX HS PCR Master Mix (Takara Bio Inc.), 1 µL (10 pmol) of each forward and reverse primer, 5.5 µL of nuclease-free water, and 5 µL of template DNA. The PCR assays were performed in a thermal cycler (Veriti, Applied Biosystems) with the following cycling conditions: an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 1 minute, 50 °C for 30 seconds, and 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes. The PCR product were analysed in 1.5 % agarose gel electrophoresis. The gel was visualized under the gel documentation system

Serological detection to estimate anti- LSDV antibodies in serum samples

Anti-LSDV antibodies in serum samples were detected using the ID Screen Capripox Double Antigen Multi-species ELISA kit (Innovative Diagnostics, France) strictly following the manufacturer's instructions. Briefly, 50 µL of test sera and controls were added to microplate wells pre-loaded with 50 µL of dilution buffer and incubated for 90 minutes at room temperature. Following five washes (300 µL/well), 100 µL of the prepared 1X conjugate was added to each well and incubated for 30 minutes at room temperature. Plates were washed five additional times before the addition of 100 µL of substrate solution. After a 15-minute incubation, the reaction was halted with 100 µL of stop solution. Optical density (OD) was measured at 450 nm. Samples exhibiting a sample-to-positive (S/P) percentage of ≥30% were classified as positive for anti-LSDV antibodies.

Results

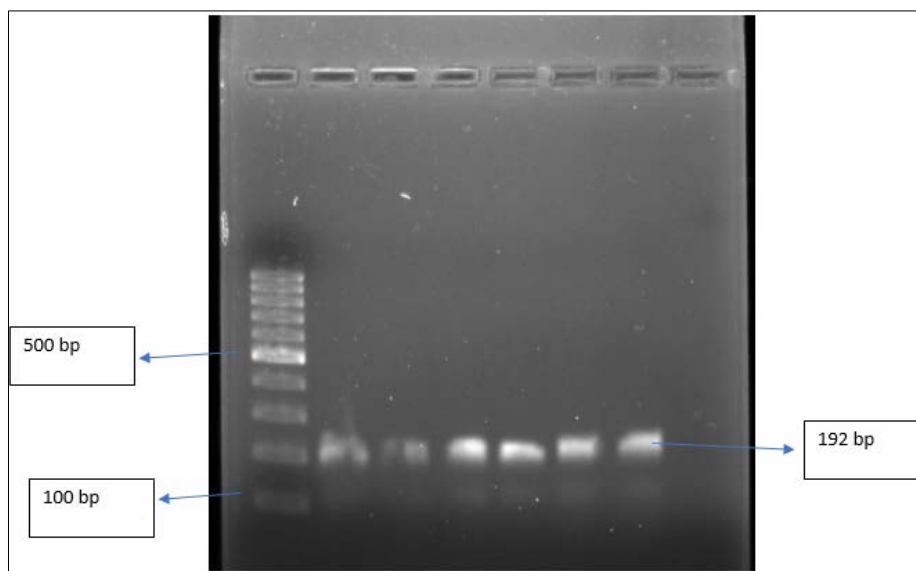
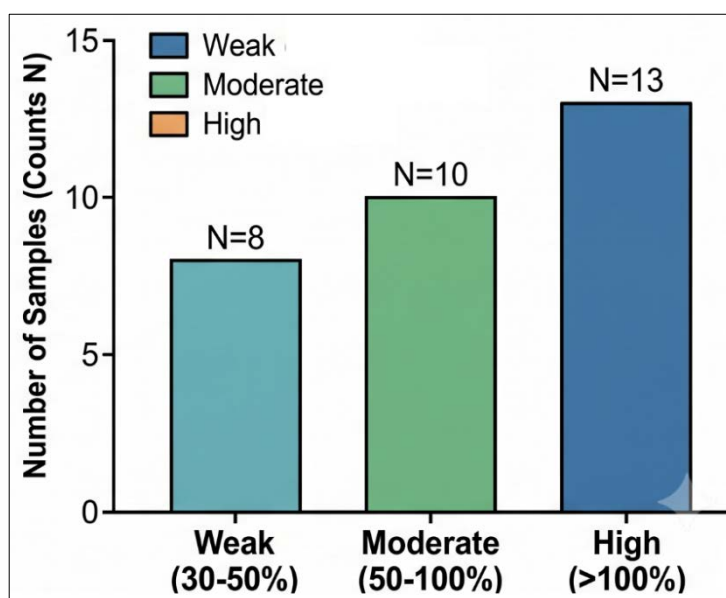
A comprehensive screening was performed on a total of 192 samples, which included tissue or nodular lavage samples (n=72), nasal swabs (n=42), and whole blood specimens (n=78). The detection of Lumpy Skin Disease Virus (LSDV) was achieved by targeting the viral attachment protein gene using the OIE-recommended primers in a conventional polymerase chain reaction (PCR) assay. A positive result was confirmed by the specific amplification of a 192 bp amplicon, as illustrated in Fig. 1

The detection of anti-LSDV antibodies was strictly dependent on the clinical matrix evaluated (Fisher's exact test, $p < 0.0001$). The target was detected in 100% of tissue/nodular lavage samples (72/72; 95% CI: 95.0-100%). In stark contrast, no positivity was observed in any of the evaluated non-invasive matrices, including nasal swabs (0/42; 95% CI: 0.0-8.4%) or whole blood samples (0/78; 95% CI: 0.0-4.7%).

A total of 31 samples met the criteria for positivity (S/P% ≥ 30%). Stratification of these positive sera by antibody titer revealed that the majority of the cohort mounted a robust humoral response. Specifically, 74.2% of the seropositive samples exhibited either high (S/P% > 100%; n=13, 41.9%) or moderate (S/P% 50-100%; n=10, 32.3%) seroconversion. Only a minority of the samples (n=8, 25.8%) fell into the weak seroconversion bracket (S/P% 30-50%) Fig. 2.

Table 1: Details of clinical sample matrices and their geographical origins from LSD-symptomatic animals

District	Tissue/Nodular Lavage	Unclotted Whole blood	Nasal swab	Serum Sample
Mathura	14	6	8	81
Hamirpur	1	1	1	-
Varanasi	16	14	4	-
Auraiya	11	28	6	-
Aligarh	12	7	5	-
Hathras	10	11	7	-
Balrampur	4	5	5	-
Mahoba	1	1	1	-
Bahraich	3	5	5	-
Total	72	78	42	81

**Fig 1:** Agarose gel showing the amplification of Capripoxvirus viral attachment protein (p32) gene. Lane M- 100 bp DNA ladder. Lane 1-6- Postive amplification the 192 bp amplicons Capripoxvirus viral attachment protein (p32) gene.**Fig 2:** A bar chart illustrating the distribution of samples by intensity classification, showing the number of samples (counts N) across three categories: Weak (30-50%, N=8), Moderate (50-100%, N=10), and High (>100%, N=13)

Discussion

Lumpy Skin Disease, by virtue of being a transboundary animal disease, harboured and realised the potential to spread across the globe inflicting substantial losses as well as leading to negative public health outcomes affecting the Indian economy. The present study provides compelling evidence that the successful detection of Lumpy Skin

Disease Virus (LSDV) is profoundly dependent on the clinical matrix selected for evaluation. The absolute detection rate (100%) in tissue and nodular lavage samples, contrasted against the complete absence of positivity (0%) in non-invasive matrices such as whole blood and nasal swabs, underscores a critical diagnostic threshold. This stark dichotomy ($p < 0.0001$) aligns with the established

pathogenesis of LSDV, which exhibits a strong tropism for cutaneous tissues and endothelial cells of the skin (Babiuk *et al.* 2008) [2]. While viremia is a known phase of LSDV infection, it is characteristically transient, typically peaking during the early febrile stage before declining rapidly. The failure to detect the target in whole blood and nasal swabs strongly suggests that these matrices are unreliable for field surveillance unless sampling is strictly timed to the acute, early stages of infection.

These findings emphasize the suitability of scab/nodular lavage over whole blood samples, as the latter are only effective during the early viremic phase of infection. Previous investigations have highlighted the low occurrence of LSDV detection in whole blood and nasal swabs seen in this research (Khoo *et al.*, 2022; Kumar *et al.*, 2021; Li *et al.*, 2022; Sariya *et al.*, 2022) [11, 13, 18]. LSDV in the bloodstream reaches its zenith at 9 days post-infection, following which the virus often vanishes from the blood (Babiuk *et al.*, 2008) [2]. According to Carn and Kitching (1995) [3], the viral concentration during viremia is greater in the nodules than it is in the blood hence, skin nodule biopsy samples are preferred for PCR-based detection in clinically infected animals. According to Parvin *et al.* (2022) [16], LSD virus DNA was detected in 100% of skin lesions. According to Sanganagouda *et al.* (2023) [17], the detection rates were 14.28% in blood samples, 95.04% in skin swabs, and 66.66% in nasal swabs. Previous studies have reported varying detection rates of LSDV in blood samples from suspected animals: 31.81% in India (Sudhakar *et al.*, 2020) [20], 13% in Russia (Sprygin *et al.*, 2018) [19], 40% in Azerbaijan (Zeynalova *et al.*, 2016) [25], and 72.7% in Serbia (Manic *et al.*, 2019). The viremic phase in LSDV-infected animals is relatively short, with blood samples typically testing PCR-positive 4-11 day's post-infection. Zeedan *et al.* (2024) [24] reported positive detection rates of 56.6% from cattle skin biopsies and 11.11% from blood samples using mPCR and RT-qPCR methods. The findings align with previous studies, which report higher detection rates in tissue samples compared to blood and nasal swabs, due to the short viremic phase in infected animals.

The serological data further corroborates the matrix-dependent findings and provides crucial context regarding the infection timeline of the sampled population. Among the seropositive cohort, nearly three-quarters of the animals (74.2%) exhibited a robust humoral response, characterized by moderate to high seroconversion ($S/P^0 \geq 50\%$). The predominance of high-titer antibodies indicates that the majority of these animals had progressed past the acute phase and were well into the post-acute or convalescent stages of infection. This mature IgG response effectively explains the concurrent clearance of the virus from the systemic circulation (resulting in 0% blood positivity), while the virus or viral antigens remained sequestered in the localized cutaneous lesions (resulting in 100% nodular lavage positivity). A previous study by Krešić *et al.* (2020) [12] found that 85.91% of serum samples tested positive for LSDV antibodies, while 14.09% tested negative, based on a total of 291 bovine serum samples. In the study conducted by Walid *et al.* 2010 [23] Antibodies against LSDV were identified in 56% of the clinically infected cows using indirect ELISA (iELISA) using the ID Screen® Capripox Double Antigen Multi-species ELISA kit. Its sensitivity in cattle ranges from 35.5% to 75%. Zeedan *et al.* (2024) [24] reported a 54.8% positive detection rate for LSDV

antibodies in cattle using EP-ELISA. The variable detection rate of LSDV antibodies in symptomatic animals can be attributed to the timing of sample collection. Antibody levels might not have risen to detectable levels if samples were taken too early in the course of infection, before a robust immune response developed, or after the immune response had waned.

These findings carry significant implications for the optimization of diagnostic protocols during active field outbreaks. Routine surveillance often relies on blood collection or nasal swabbing due to their minimally invasive nature and ease of collection. However, our data unequivocally demonstrate that such non-invasive sampling can lead to a high rate of false-negative diagnostic interpretations, particularly in herds where the infection has progressed beyond the initial viremic window. To minimize diagnostic failures and ensure the accurate molecular and serological confirmation of LSDV, veterinary field personnel must prioritize the collection of skin scabs and intranodular lavage.

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