

Molecular Detection and Phylogenetic Analysis of Lumpy Skin Disease Virus from Cattle Population of Punjab, Pakistan

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ABSTRACT

Lumpy skin disease (LSD) is a viral endemic disease, caused by a virus (LSDV) within the *Capripoxvirus* genus of the Poxviridae family. Clinical manifestations of the disease include fever, reduced milk production, and the appearance of skin nodules. The disease is primarily transmitted through insects, such as flies, mosquitoes, and ticks. This study is focused on detection and characterization of LSDV from Pakistan. Suspected samples were collected from Faisalabad, Jhang, Bahawalpur and Bhakkar during the summer 2022. DNA was extracted and presence of the virus was confirmed by PCR using *P32* and *RPO30* gene specific primers. Overall, LSDV was detected in 32.6% (212/650) of samples and it was present in all surveyed cities, with prevalence ranging from 25% to 36.8%. Significant geographic heterogeneity was observed across regions. Prevalence of the virus was highest in samples from diseased cattle, followed by slaughter houses, while detection rates were lower in dairy farms. The nucleotide sequences of the PCR products were determined through Sanger Sequencing method and analyzed by BLAST tools on NCBI/ GenBank. Phylogenetic analysis was performed using MEGA 6 software. It revealed that LSDV strains of Pakistan have a close genetic relationship with their corresponding circulating strains of neighboring countries, such as Kazakhstan, Iraq, Turkey and India. This study has provided insights into the epidemiological aspects of LSDV outbreaks in Pakistan. It was concluded that LSDV remained widely circulating in Punjab Pakistan, with distinct epidemiological patterns. Considering the possibilities of genetic evolution, there is a need for continuous surveillance and optimized vaccination.

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Authors' Contribution

AB: Sample collection and analysis. MN, MM: Data analysis. MN, MSS, MM: Interpretation of the results. SS: Sample collection. HMA: Study designed and sample analysis. MSS: Supervised study and drafted the manuscript. MH: Conceived the study, resources. MY: Statistical analysis. MIC: Interpretation of results. MH, MIC: Reviewed the manuscript.

Key words

Cattle, Lumpy skin disease, PCR, Phylogenetic analysis

INTRODUCTION

Lumpy skin disease (LSD) is an infectious viral condition that can cross borders, causing significant economic losses, particularly in cattle. The disease is caused by the LSD virus (LSDV), which belongs to the genus *Capripoxvirus* within the family Poxviridae (Bishnoi *et al.*, 2024; Luqman *et al.*, 2024). LSDV is

a double-stranded DNA virus with a genome size of approximately 150 kb. It features an inverted terminal repeat, 2.4 kilobase pairs in length, on either side of the core coding region. The virus is thought to have around 156 potential genes. Notably, the *Capripoxvirus* genomes are highly conserved, with over 95% genetic similarity between LSDV, sheep poxvirus (SPPV), and goat poxvirus (GPV). The other members of this genus include GTPV and SPPV (Nayakvadi *et al.*, 2021).

LSD manifests in several clinical symptoms, including fever, decreased appetite, nasal discharge, excessive drooling and the formation of skin nodules, which range from 2 to 7 cm in diameter (Abdullah *et al.*, 2015). The skin nodules typically form on the neck, legs, tail, and back shortly after the onset of fever. In severe cases, the disease can be fatal. Post-mortem lesions may include coagulative necrosis in subcutaneous muscles, vasculitis, and complications such as keratitis, diarrhea,

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lameness, pneumonia, mastitis, and myiasis (Adamu *et al.*, 2024). Skin lesions are characterized by thickened layers, damage to spinous cells, and the formation of small, fluid-filled sacs. The primary natural hosts of LSDV are cattle, buffalo, and water buffaloes (Geletu *et al.*, 2024).

LSD was first reported in Zambia in 1929. Until 1989, it remained endemic to the region, after which it spread to the Middle East, Southeast Europe, the Balkans, and the Caucasus. More recently, in 2021, the disease was reported in Asia (Selim *et al.*, 2021). In Pakistan, the LSDV outbreak that began in late 2022 has rapidly spread across Sindh and neighboring regions, including Punjab, Khyber Pakhtunkhwa (KPK), Kashmir, and Balochistan. As of the latest reports, over 20,000 animals in Sindh have been infected, with Karachi being the hardest-hit area (Lamien *et al.*, 2011). Across the country, there have been over 190,000 recorded LSDV cases, resulting in the deaths of 7,500 animals. However, there has been a remarkable recovery rate, with over 141,000 animals successfully treated (Alkhamis and Vander Waal, 2016).

The disease is primarily spread by blood-feeding arthropods, such as mosquitoes, ticks, and flies, which transmit the virus through their bites. Given its potential for rapid cross-border spread and significant impact on cattle production, LSDV is classified as a notifiable disease by the World Organization for Animal Health (WOAH) (Sudhakar *et al.*, 2024).

LSDV must be differentiated from several other conditions, including ringworm, streptotrichosis (*Dermatophilus congolensis* infection), hypodermosis (caused by *Hypoderma* bovis), photosensitization, bovine papular stomatitis, foot and mouth disease, and bovine viral diarrhea 1. The main strategy for preventing LSDV is vaccination, with live, attenuated LSDV vaccines being widely used. The Neethling field strain of LSDV, developed in the 1950s and 1960s by the Onderstepoort Veterinary Research Institute (South Africa), is the basis for current vaccines (Ronchi *et al.*, 2024). The virus was attenuated through serial passage in primary lamb kidney cells and chorioallantoic membranes (CAMs) of embryonated eggs (Philips *et al.*, 2024).

To effectively control and eradicate LSDV, it is crucial to understand its molecular epidemiology. Control measures include limiting cattle movement, vaccinating

animals, implementing sanitary practices, and controlling arthropod populations (Haider *et al.*, 2024). However, successful eradication requires collaboration among farmers, veterinarians, livestock traders, herders, and other stakeholders. Awareness campaigns targeting these groups are essential to prevent further outbreaks (Butt, 2025).

The aim of this study was to investigate genetic composition and phylogenetic relationships of LSDV strains circulating in Pakistan. Through this research, insights into the nature of the infection, its transmission dynamics, and the extent of its spread will be gained. Additionally, assessing the effectiveness of current control measures and monitoring the ongoing spread of LSDV will be essential for informing future mitigation strategies.

MATERIALS AND METHODS

Collection of suspected samples

Investigations on possible LSDV epidemics in cattle herds of different breeds, genders and ages from Bahawalpur, Bhakkar, Jhang and Faisalabad were conducted from July-September 2022. Based on the clinical symptoms of fever, lacrimation and skin nodules, 650 skin biopsy samples were collected, immediately submerged in phosphate buffer saline (PBS) and transported on ice in insulated cool boxes. Information regarding the clinical signs of suspected LSD-affected animals was recorded and evaluated by veterinarians. These samples were stored in Lab at -80°C till analysis.

Viral DNA extraction

The clinical skin biopsy samples (quantity 0.5 g) were processed and homogenized in 500 µL PBS buffer. Further, DNA was extracted by adopting phenol-chloroform method which was developed by Macharia *et al.* (2024).

Detection of viral DNA by PCR

The presence of the virus was confirmed by PCR. Specific primers were used for amplification of viral DNA fragments. In this study, two different sets of primers were used; details are shown in Table I. One set of primers were used to amplify 192 bp fragment of P32 gene (Ren *et al.*, 2024). The second set was used to amplify 554 bp fragment of *RPO30* gene (Bishnoi *et al.*, 2024).

Table I. Details of PCR primers showing primer sequences and expected PCR products.

Name of primer	Primer sequence 5'→3'	Expected PCR product	Reference
P32	F= TCCGAGCTCTTTCCTGATTTTCTT ACTAT R= TATGGTACCTAAATTATATACGTA AATAAC	192 bp	Bishnoi <i>et. al.</i> , 2024
RPO30	F= CAGCTGTTTGTTTACATTTGATTTT T R= TCGTATAGAAACAAGCCTTTAATA GA	554 bp	Lamien <i>et. al.</i> , 2011

Nucleotide sequence analysis

PCR products were purified using gel extraction kit method. Commercial sequencing service was used for determination of nucleotide sequences of PCR products. Seven representative positive samples were sent for nucleotide sequencing to LGC Genomics GmbH. (Germany) for Sanger sequencing service.

Phylogenetic analysis

The nucleotide sequences of LSDV from cattle were utilized for the construction of a phylogenetic tree (Sharma *et al.*, 2024) and comparison was done with isolates of different countries, such as India ON964525, OP297402, OQ588787, OK422494, OM362837, OQ943597, OQ030221, OQ943586, OQ030220, OQ943603, OQ030223, OQ943602, OQ030224; Bangladesh OP668129, OP668128; Nigeria OK318001; Kazakhstan MW0306121, MN642592, ON010590, MT130502; Israel KX894508; Greece KY829023; Russia MH893760; Balkan KY702007; USA AF409137; South Africa MW656253; Belgium MT643825; Pendik MN995838; Iraq MN871851; Nigeria OQ094251, OQ094257, OQ094258; Namibia MW115949; Austria GU119943; Pakistan OR393299, OR393300, OR393301, OR393302, OR393303, OR393304 and OR393305.

Statistical analysis

Phylogenetic analysis was performed by aligning nucleotide sequences using Clustal W. A phylogenetic tree was generated using the Neighbor-Joining method based on the Kimura two-parameter model. The robustness of the inferred phylogeny was evaluated by bootstrap analysis with 1,000 replicates and bootstrap support values were indicated at the nodes. All analyses were carried out using MEGA 11 software.

RESULTS

LSDV outbreaks were observed in different areas of Bahawalpur, Bhakkar, Faisalabad and Jhang during summer 2022. Clinical signs were observed in cattle which include fever, swelling of limbs, drop in milk production, loss of appetite, nasal secretion, presence of skin nodules on head, neck and other body parts. Animals at early stage of disease also had pus in wounds.

The overall PCR-based prevalence of LSD in cattle in Bahawalpur (36.8%), Bhakkar (35%), Jhang (34%), Gojra (30%) and Faisalabad (25%) districts was 32.6% (Table II). Among different districts, Bahawalpur had the highest prevalence it was also observed that the crossbred had higher prevalence as compare to local breed.

Presence of the virus was confirmed by PCR using

specific primer sets targeting p32 and RPO30 genes. Viral DNA of all samples were extracted and used as template for amplification of 192bp and 554bp PCR products representing *p32* and *RPO30* genes, respectively (Table II).

Table II. PCR positive samples detected in suspected herds of animals from different districts.

Name of district	No. of cattle examined	PCR positive	% prevalence
Bahawalpur	160	59	36.8
Bhakkar	120	42	35
Jhang	150	51	34
Gojra	100	30	30
Faisalabad	120	30	25
Total samples	650	212	32.6

Multiple sequence alignment showed a high degree of similarity between our sequences and those of regional LSDV isolates. Phylogenetic analysis revealed that our isolates are closely related to LSDV strains previously reported in India, Kazakhstan, Turkey and Iraq (Fig. 1). Nucleotide sequences of numerous LSDV strain were obtained from the NCBI GenBank database for comparison.

Nucleotide sequences of local isolates were submitted to GenBank and got accession numbers for seven isolates of LSDV (OR393299, OR393300, OR393301, OR393302, OR393303, OR393304 and OR393305). The maximum likelihood method-based phylogenetic tree of LSDV (*RPO30* gene) revealed five major clusters. Cluster-I contained isolates from India, Bangladesh, Nigeria, Kazakhstan, Israel and Greece. Cluster-II consisted of isolates from Russia, Iraq, Balkan, USA, Belgium and South Africa. Cluster-III comprised of isolates from Kazakhstan, Pendik and Iraq. Four isolates from Pakistan (OR393299, OR393300, OR393302, and OR393303) were in cluster-IV. Whereas, the isolates collected from Nigeria, Namibia, Austria, India and three isolates of Pakistan (OR393301, OR393304 and OR393305) were in Cluster-V. The isolates belonging to India, Nigeria, Austria and Kazakhstan were closer to current isolates followed by Bangladesh, Greece, Israel and Russian isolates. Isolates from India, Pendik, Iraq and Kazakhstan have higher similarity with the sequences of the current isolates (Fig. 1). Our new sequences (this study) are indicated with yellow color circle while closely related sequences are shown with red triangles. The phylogenetic analysis revealed that our isolates are novel and closely related to corresponding isolates of India, Pendik, Kazakhstan, Iraq, Bangladesh, Nigeria and Namibia.

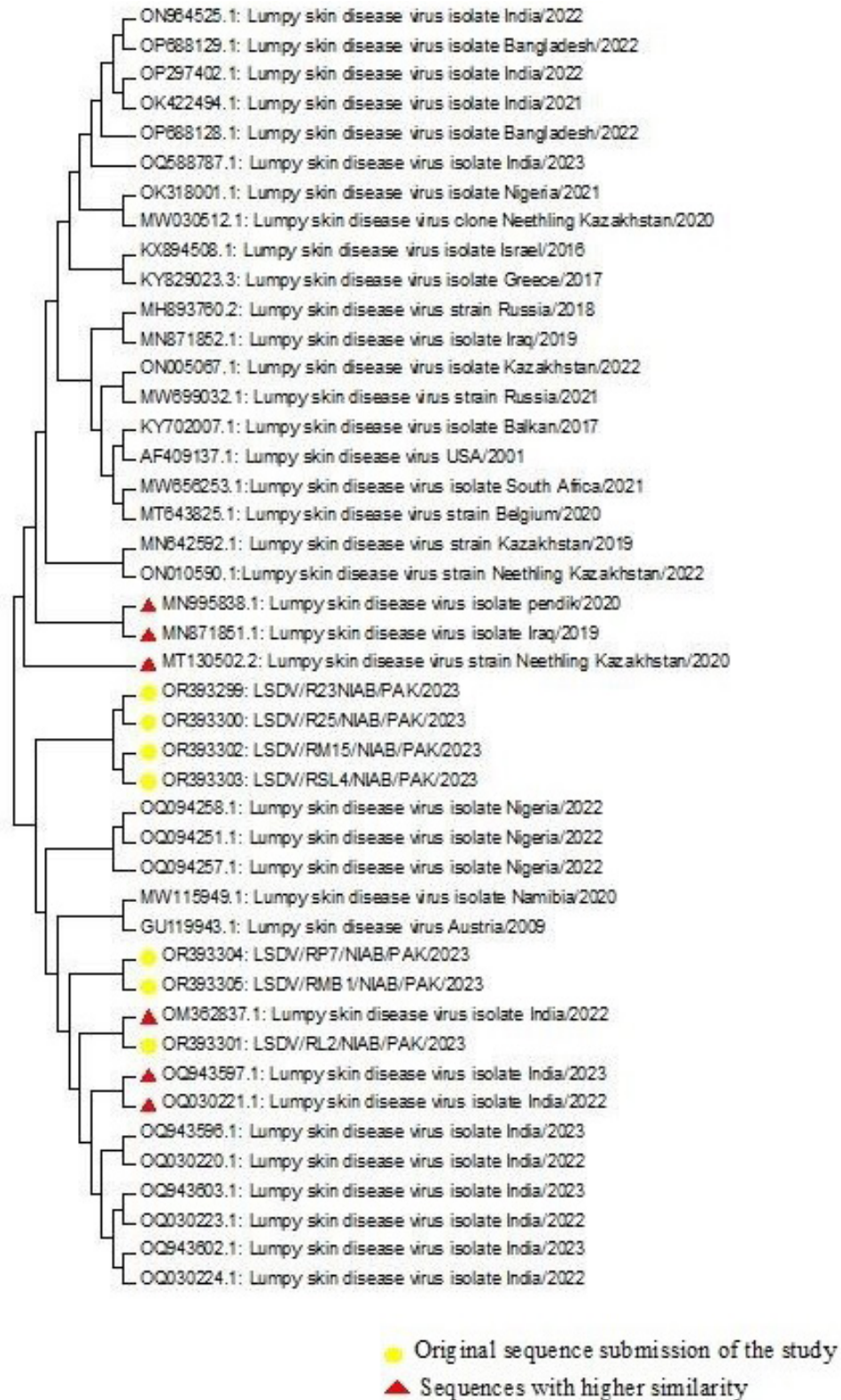


Fig. 1. Phylogenetic tree showing evolutionary relationships of different LSD virus isolates.

The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood is shown. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The analysis involved 44 nucleotide sequences. Evolutionary analyses were conducted in MEGA6. The phylogenetic tree was constructed on the basis of LSDV isolates from NCBI. The original samples sequences LSDV/R23 (accession no. OR393299), LSDV/25 (accession no. OR393300), LSDV/M15 (accession no. OR393302), LSDV/SL4 (accession no. OR393303) are closely related with LSDV strains of Pendik (Turkey) reported in 2020 accession no. MN995838.1 Iraq reported in 2019, accession no. 871851.1, and Kazakhstan reported in 2020 MT130502.2. The original samples sequenced LSDV/RP7 (Accession no. OR393304), LSDV/MB1 (Accession no. OR393305) are closely related with LSDV strains of India reported in 2022 and 2023 Accession no. OM382837.1, OQ030221.1, OQ943597, respectively.

DISCUSSION

Cattle and Buffaloes are susceptible to lumpy skin disease. This disease can be identified by its characteristic signs and symptoms e.g. eruptions of nodules on the skin and other body parts. This is being caused by a Capripoxvirus. The scaly skin ailment spreads via cutaneous lesions. The LSDV outbreak was started in Pakistan late 2021, had quickly spread all over the Country causing economic losses (Adamu *et al.*, 2024). The disease's spread could be caused by the animal movement across districts and due to open cattle markets in a number of places/ districts. LSD often appears in the summer due to the warm weather which is suitable for the insect vectors, which are essential in the transmission of LSDV (Geletu *et al.*, 2024; Tesfaye *et al.*, 2024).

This study used a specific PCR test, phylogenetic analysis, and sequences of LSDV. Our findings are in-line with earlier studies e.g. LSDV cases were confirmed in an field outbreak (Selim *et al.*, 2021). They collected samples from different districts of Punjab including; Bhakkar, Bahawalpur, Faisalabad and Jhang. LSDV suspected cases were confirmed by PCR using specific primers (LSD-F/ LSD-R) targeting the *P32* gene of virus, our results are in line with previous studies (Bishnoi *et al.*, 2024). Total 650 suspected animals were examined and samples were collected, DNA was extracted by phenol-chloroform method and 212 samples were found positive by PCR.

Positive samples were subjected to PCR using a

second set of PCR primers targeting RNA Polymerase Subunit (*RPO30*) gene which has been used by various researchers for viral characterization and phylogenetic analysis (Lamien *et al.*, 2011; Jabbar *et al.*, 2025). Positive samples from different regions/ districts were selected for determining their nucleotide sequence analysis based on quality of PCR products. Similar findings were also reported by Selim *et al.* (2021) and Lamien *et al.* (2011). The DNA-dependent RNA polymerase component encoded by the *RPO30* gene is necessary for viral replication.

Phylogenetic analysis is a systematic approach used to investigate the evolutionary relationships among organisms. Phylogenetic trees and their branches illustrate the genetic connections and evolutionary trajectories of various microbial isolates. These trees provide insights into the interactions among different viral strains and the genetic changes that have occurred over time. Through such analysis, we can differentiate between closely related strains of LSDV and those that are more genetically distant.

During this study, phylogenetic analysis was done targeting *RPO30* gene. The nucleotide sequence of local isolates showed close relationship with the corresponding viral strains of neighboring countries. The evolutionary history was inferred by using the Maximum Likelihood approach. MEGA X was used for evolutionary analysis. BLAST analysis revealed that the nucleotide sequences of Pakistani isolates had high homology with LSDV sequences from GenBank. The phylogenetic analysis of gene *RPO30* revealed that LSDV from Pakistan is more closely related to the strains of LSDV from India, Iraq, Turkey, and Kazakhstan. LSDV was earlier reported from Turkey, Iraq, Kazakhstan and India during 2013, 2018, 2016 and 2019, respectively (Alkhamis and VanderWaal, 2016).

These findings suggest that Pakistan may be exposed to the virus from these neighboring countries. Movements of animals across country boundaries without sufficient monitoring and control may be the cause of spread of disease. This finding increases the likelihood that the virus may have come from these countries to Pakistan. Infected animals crossing international borders having ticks/ insects can easily spread the disease in susceptible animals. Therefore, it has been observed that the disease is trans-boundary in nature as already described by OIE. Pakistan's efforts to prevent sickness and develop vaccinations may be hampered by the genetic diversity among the sequenced LSDV strains (Haider *et al.*, 2024). Results of this study will help to formulate measures for prevention and control of LSDV.

Phylogenetic analysis also revealed that all the LSDV strains were identical and clustered with similar field strains of the virus. This study confirmed the LSDV outbreaks in

Pakistan. Further studies on whole-genome sequencing of the virus is required to shed light on Pakistan's LSDV's genetic epidemiology.

CONCLUSION

Molecular characterization and phylogenetic analysis of Lumpy Skin Disease Virus (LSDV) revealed a close genetic relationship between LSDV strains circulating in Pakistan and those reported in neighboring countries, highlighting the transboundary nature of the disease. Given the highly contagious nature of LSDV, prompt isolation of infected animals, strict biosecurity measures and regulation of animal movement between regions are essential to limit virus transmission and minimize its economic impact. Future studies should focus on continuous molecular surveillance, whole-genome sequencing and monitoring of viral evolution to detect emerging variants and better understand transmission dynamics. Furthermore, strengthening regional collaboration and evaluating the effectiveness of current vaccination and control strategies will contribute to the development of more effective disease prevention and eradication programs.

DECLARATIONS

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IRB approval

The use of animals was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Sciences Division, NIAB, Faisalabad (Approval No. NIAB/2022-005).

Ethical statement

This study involved cattle and did not include human participants. All procedures were conducted in accordance with national and international guidelines for the ethical care and use of animals.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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