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Original Research Article

Isolation and Harvesting of Lumpy skin disease virus (LSDV) strain from infected cattle breeds, *Bos indicus* and *Bos taurus*, in Sindh, Pakistan, from in-vivo Vero cell line culture to stabilize LSDV, an insight through NGS sequencing and quality analysis

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Abstract: *Lumpy Skin Disease Virus is an economically important poxviral pathogen of cattle that causes nodular skin lesions, reduced milk yield, and trade limitations. Molecular characterization, including whole-genome sequencing, can reveal markers of disease susceptibility and inform diagnostic and preventive strategies.* ***Objective:*** *To identify, isolate, and characterize Lumpy Skin Disease Virus strains circulating in native and exotic cattle in Sindh Province, Pakistan, and to assess their in vitro behaviour during cell-culture adaptation and stabilization.* ***Methods:*** *A field-based sampling of clinically suspected cattle cases was conducted under veterinary supervision in Sindh Province. Laboratory confirmation of infection was performed using validated molecular and serological assays. LSDV-positive samples were subjected to controlled recovery and propagation in accredited cell-culture facilities, followed by phenotypic evaluation during adaptation and stabilization phases. Whole-genome sequencing and comparative genetic analysis were performed to assess diversity among isolates relative to reference strains. All procedures were completed in accordance with approved biosafety and animal welfare protocols.* ***Results:*** *LSDV infection was successfully confirmed in the selected field samples using standardized molecular and serological methods. Viable viral isolates were recovered and showed consistent replication patterns during cell-culture adaptation and stabilization. Comparative phenotypic evaluation demonstrated distinguishable growth characteristics across isolates. Genomic analysis revealed measurable diversity between local strains and reference isolates, suggesting region-specific viral evolution. These findings provide actionable molecular evidence to strengthen surveillance and diagnostic preparedness in Sindh.* ***Conclusion:*** *The study successfully identified and characterized LSDV strains endemic to Sindh Province and demonstrated notable phenotypic and genomic variability. These insights support improved regional surveillance, diagnostic capacity, biosafety planning, and vaccine policy development. Enhanced molecular understanding will contribute to more effective control measures and reduced economic and welfare losses in affected cattle populations.*

Keywords: LSDV, Whole Genome Sequencing, Vector-Borne Disease

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Introduction

Livestock plays a vital role in strengthening the economy of agrarian nations, where demand for animal-based food products continues to rise globally. Factors such as rapid population growth, expansion of industrial development, increasing use of processed foods, and improvement in per capita income have significantly increased the consumption of dairy and meat products in Pakistan. As a developing country, Pakistan requires substantial agricultural advancement to enhance its gross domestic product (GDP).

Various infectious diseases increasingly threaten Indigenous livestock breeds in Pakistan. Preservation of genetic diversity, together with disease control, is therefore essential for sustainable livestock production. Among major transboundary animal diseases, lumpy skin disease (LSD) is a non-zoonotic, acute to subacute, contagious, and vector-borne viral infection associated with considerable morbidity and mortality in cattle (1). The disease has been described under various terminologies, including pseudo-urticaria, Neethling disease, exanthema nodularis bovis, and knopvelsiekte (2, 3). Hallmark clinical features include high fever reaching approximately 41°C, characteristic nodular skin lesions, reduced milk yield, anorexia, lethargy, and rhinitis (4). LSD causes severe deterioration in animal condition, negatively affecting rural livelihoods. Economic consequences include damaged hides, reduced meat value, reduced milk productivity, and widespread financial losses (5, 6). Multiple skin lesions frequently ulcerate and scar, reducing hide quality (7).

Transmission occurs through arthropod vectors, including mosquitoes, ticks, and biting flies, as well as through direct contact with infected animals. Mechanical transmission by vectors is considered a dominant pathway (8). Species such as Amblyomma, Rhipicephalus, and Hyalomma serve as important carriers and reservoirs (9). Mechanical spread has also been linked to biting flies and mosquitoes (10). Additionally, viral shedding through milk, saliva, nasal discharge, lacrimal secretions, and blood facilitates indirect transmission among animals sharing communal grazing or watering points (11).

Pakistan witnessed its first major LSD epidemic between 2021 and 2022, significantly damaging the livestock sector. New outbreaks re-emerged in Sindh and Punjab in April–May 2025, highlighting persistent disease circulation. LSD poses substantial economic losses due to trade restrictions, reproductive losses, declining feed conversion efficiency, deterioration of hides, and high abortion rates. The disease affects all cattle breeds and buffalo (12). Beyond economic impact, LSD disrupts rural livelihoods, exacerbates food insecurity, increases poverty, and induces psychosocial distress among livestock owners (13).

Global climate change, altered animal trade patterns, and illegal animal movement have contributed to transboundary spread (5). LSD is associated with mortality rates of 10–50 percent among infected cattle, milk decline of 20–40 percent, weight loss of 10–20 percent, and similar reductions in fertility (14). The estimated annual global loss ranges

between 1.5 and 3 billion US dollars, while the disease also aggravates poverty, food insecurity, and psychological burden (15). Environmental consequences include greenhouse gas emissions, water contamination, land degradation, and biodiversity loss. Moreover, indirect losses extend to reduced consumer confidence, compromised trade markets, and limitations in development sectors (16). Disease mitigation strategies include vaccination, biosecurity, surveillance, vector control, quarantine, awareness campaigns, and research-driven interventions.

LSD virus (LSDV) belongs to the family Poxviridae and infects a wide range of domestic species. This family comprises two subfamilies: Chordopoxvirinae, which affect vertebrates, and Entomopoxvirinae, which target invertebrates (17). LSDV is a member of the capripoxviruses, which also include sheeppox and goatpox viruses (18). Vector-borne transmission plays a critical role in epizootics and geographical expansion. Historically, LSD was first recognized as a disease outbreak in Africa (19–21). LSDV is a large double-stranded DNA virus, brick-shaped and enveloped, approximately 320 × 260 nm in size, replicating in the cytoplasm of host cells. The virus demonstrates strong environmental resilience and can remain viable for weeks in scabs, necrotic tissues, and desiccated hides, with infectivity preserved for years at low temperatures (22). Viral persistence has also been noted in semen for up to 42 days post-infection (23).

Diagnosis is based on clinical assessment supported by laboratory confirmation using polymerase chain reaction and serology. Treatment is largely supportive and may include antimicrobials where secondary infections arise (24). Prevention primarily relies on vaccination, vector control, and strict biosecurity measures, including quarantine and sanitation practices (25).

Sindh province hosts valuable indigenous breeds, including Red Sindhi and Tharparkar cattle, known for adaptability, heat tolerance, and productive efficiency. These breeds contribute significantly to dairy and meat production within Pakistan. However, LSD poses a substantial risk by reducing milk yield in Red Sindhi, impairing work and production capacity in Tharparkar, and negatively influencing reproductive performance, calf survival, and carcass quality (26). Comparative field observations suggest that buffaloes exhibit lower susceptibility, reduced clinical severity, and less economic loss than cattle, possibly due to species-specific genetic resistance, immune responses, or vector feeding preferences.

Overall, LSD threatens livestock productivity and genetic resources while undermining the socioeconomic stability of rural communities. Understanding its epidemiology, host susceptibility, and effective control strategies is therefore critical for safeguarding Pakistan's livestock sector.

Methodology

Case Identification, Sample Collection, and Storage

Suspected LSD cases were identified based on clinical manifestations, including high fever, nodular skin lesions, lethargy, and anorexia. All procedures were performed in a BSL-2 facility in accordance with biosafety regulations, including the mandatory use of personal protective equipment.

Whole blood samples were collected in EDTA tubes and stored at 4°C for up to 24 hours before DNA extraction. Skin scabs from nodular lesions were aseptically collected using sterile scalpels, transferred to labelled cryovials containing phosphate-buffered saline supplemented with antibiotics, and stored initially at 4°C, then at −80°C.

DNA Extraction and Quantification

Genomic DNA from blood and tissues was extracted using proteinase K digestion followed by phenol–chloroform purification. DNA integrity was verified by agarose gel electrophoresis, and nucleic acid concentration was determined using spectrophotometry. Epidemiological risk pathways for LSD spread were documented, including direct (contact, fomites, mother-to-calf exposure) and indirect (vector-mediated long-distance dissemination) transmission.

Virus Isolation and Propagation

Skin scabs were homogenized in sterile phosphate-buffered medium using a tissue grinder. The homogenate was clarified by centrifugation at 2,000 × g for 15 minutes and filtered through a 0.45 µm membrane. PCR performed preliminary molecular screening for LSDV before cellular inoculation.

In-Vitro Virus Propagation on Vero Cells

African green monkey kidney (Vero) cells were maintained in 175 cm^2 flasks using complete GMEM/MEM media. Cell culture passaging was performed using standard trypsinisation. Detached cells were washed repeatedly in incomplete GMEM and collected by centrifugation. Cell viability and density were assessed using a haemocytometer.

Cells were seeded into fresh flasks at an appropriate density in complete medium and incubated at 37°C, 5 percent CO_2 for 72 hours. The media was replaced every 24 hours until monolayers reached approximately 80% confluence.

Inoculation of Cell Culture

Confluent Vero monolayers were washed with incomplete GMEM and inoculated with 2 ml of filtered viral suspension. Cultures were incubated at 37°C with 4.7 percent CO_2 for up to 12 days. Cell morphology was monitored daily for cytopathic effects (CPE).

Assessment of Cytopathic Effect

Characteristic LSDV-induced CPE, including cell rounding, refractile transformation, fusion, and detachment, was observed microscopically. When CPE reached 40–50 percent, initial viral harvest was performed; final harvest was collected at 70–80 percent CPE.

Virus Harvesting and Stabilisation

Infected cultures were subjected to three freeze–thaw cycles to release intracellular virus. Viral suspensions were pooled to improve yield and testing efficiency. The clarified lysate was filtered (0.45 µm) and tested using PCR followed by gel electrophoresis. Stabilising agents were added in equal volumes, and viral material was stored at −70°C before lyophilisation.

Lyophilisation and Quality Control

Aliquots of 2 ml viral suspension were dispensed into sterile glass vials and lyophilised using laboratory-grade freeze-drying equipment. Lyophilised vaccine material was stored at −20°C.

Quality assurance included sterility testing in thioglycolate broth, nutrient broth, and tryptose soya broth over a 10-day incubation period. Safety testing was performed using laboratory animals, and microbiological screening was conducted to ensure the absence of contaminants.

Estimation of Viral Titre ($TCID_{50}$ Assay)

$TCID_{50}$ was determined using Vero cells seeded at 2×10^4 cells per 100 µl in 96-well plates. Serial 10-fold dilutions (10^{-1} to 10^{-7}) of the viral suspension were prepared using serum-free medium, and each dilution was inoculated into cell monolayers. Plates were incubated at 37°C in 5 percent CO_2 and examined microscopically for CPE on days 10, 12, and 15 post-infection. Viral titre was calculated using the Spearman-Karber method, with the minimum acceptable titre per field dose set at $10^{3.5}$ $TCID_{50}$.

Viral DNA Extraction

Viral DNA was isolated from the harvested virus using three approaches:

1. Boiling method

   Samples were mixed with TE buffer, incubated at 95–100°C for 10 minutes, cooled, centrifuged at 4,000 rpm for 10 minutes, and the supernatant was collected.

2. TRIzol extraction

   Virus suspensions were homogenised in TRIzol, phase-separated with chloroform, and precipitated using isopropanol. Recovered pellets were washed in ethanol, air-dried, resuspended in nuclease-free water, and incubated for hydration.

3. Commercial extraction kit

   Viral DNA was extracted using a spin-column-based commercial kit according to the manufacturer's instructions.

DNA concentration and purity were assessed using spectrophotometry and stored at −20°C.

Library Preparation and Whole Genome Sequencing

Extracted DNA was fragmented to 200–500 bp using enzymatic tagmentation. Fragment ends were repaired, and adapters ligated. PCR enrichment was used to generate high-quality sequencing libraries, which were quantified spectrophotometrically.

Two libraries were prepared:

i. LSDV genomic DNA7

i. Host (cattle) genomic DNA isolated from LSD lesions

Ethical Approval and Consent

This study involved the collection of biological samples from clinically suspected lumpy skin disease (LSD) cases in field cattle. Scabs and whole blood (3 ml per animal) were collected from 25 suspected animals using standard veterinary practices without anaesthesia. Ethical approval was granted by the Institutional Ethical Review Board and the Institutional Bio-Risk Management Committee (IBRMC) of Dr. A.Q. Khan Institute of Biotechnology and Genetic Engineering (KIBGE). Prior informed consent was obtained from animal owners before sample collection.

Results

LSDV infection was successfully confirmed in field-sampled cattle through clinical examination and molecular screening. Typical signs, including nodular skin lesions, pyrexia, and reduced appetite, were observed, and PCR confirmed the presence of viral DNA in collected tissue and blood specimens. Viral isolation on Vero cells resulted in visible cytopathic effects, characterised by cell rounding, refractility, fusion, and detachment, indicating efficient viral replication. These progressive degenerative changes enabled staged harvesting of viral material, beginning when approximately half of the cell layer showed cytopathic damage and concluding when degeneration reached nearly eighty percent. High-molecular-weight genomic DNA was successfully extracted from both infected cattle and viral harvest, with agarose gel electrophoresis confirming DNA integrity and suitability for downstream sequencing (Figure 1 and Figure 2).

Next-generation sequencing produced a comprehensive dataset adequate for full molecular interrogation. Illumina sequencing yielded approximately 334.98 million reads, equivalent to 100.25 gigabases of data, with over 96.5 percent of bases attaining quality scores above Q30, reflecting high read accuracy. The average GC content of 42.79 percent and the uniform read length of 150 bp were consistent with expected genomic characteristics, indicating high-quality sequencing performance. Table 1).

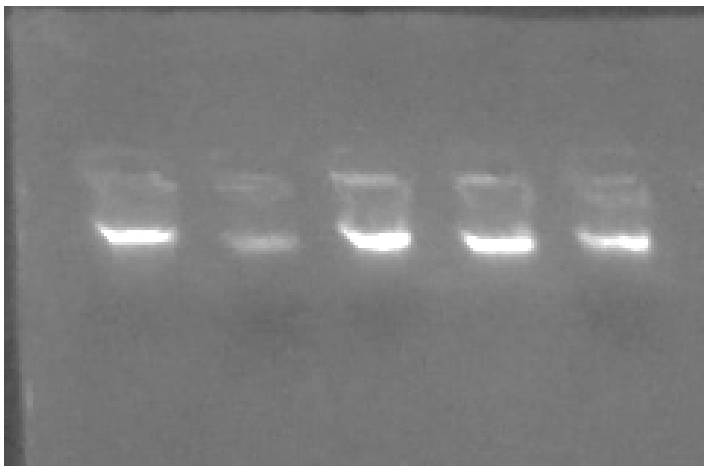

Fig.1 Image of Agarose gel electrophoresis of genomic DNA of Cattle (*Agarose gel electrophoresis showing intact, high-molecular-weight genomic DNA extracted from cattle samples suspected of LSD infection, demonstrating clear band resolution suitable for downstream PCR amplification and NGS library preparation.)*

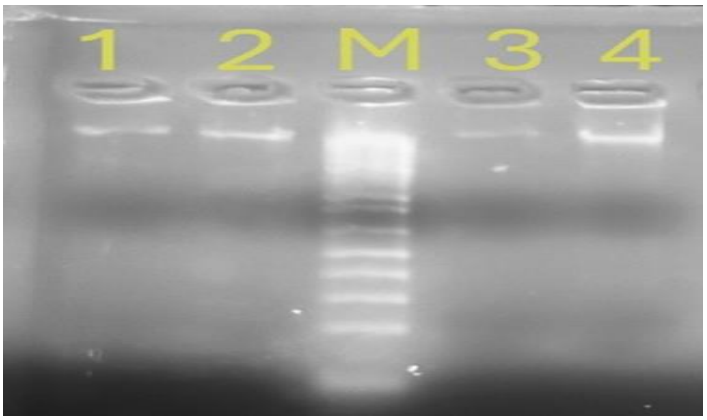

Figure2. Image of Agarose gel electrophoresis of genomic DNA of LSDV: M is 1 Kb DNA
ladder. (*Agarose gel electrophoresis showing purified LSDV genomic DNA with visible band integrity against a 1 kb molecular ladder, confirming efficient viral DNA recovery for sequencing and viral genome analysis.)*

Table 1: Statistics on the NGS data

Sample ID	Clean reads	Clean bases	Q30(%)	GC content	Read length
CATL18P	334,978,238	100.25	96.54	42.79	150 bases

The FASTQC program generated THE NGS QC report.

Quality assessment using FASTQC further demonstrated excellent data reliability. Adapter contamination was absent across all sequencing cycles (Figure 3), and the majority of sequences were unique, with approximately seventy-two percent retained after deduplication analysis (Figure 4), confirming appropriate depth for genome assembly and mapping.

Ambiguous base calls remained near zero throughout read positions (Figure 5), indicating confidence in base calling. Per-base quality plots showed median Phred values near Q39–Q40, with narrow interquartile ranges, indicating high and uniform sequence accuracy across the entire read length (Figure 6). Nucleotide composition stabilised after initial priming cycles, suggesting an absence of compositional bias (Figure 7). GC content analysis showed close alignment between observed and theoretical distributions, supporting biological authenticity and ruling out contamination (Figure 8). Per-sequence quality distributions were sharply skewed toward high Phred values with negligible low-quality reads (Figure 9), while tile performance heatmaps indicated spatial uniformity across the flow cell, excluding platform-induced variation (Figure 10). Read length distribution was tightly centred at 150 bp, indicating complete fragment consistency (Figure 11).

Collectively, these results confirm successful identification, isolation, and propagation of LSDV strains circulating in Sindh, followed by high-fidelity whole-genome sequencing. The exceptionally clean sequencing profile and stable genomic outputs support downstream genomic interpretation, strain comparison, and evolutionary analysis. The preliminary comparative assessment suggests observable variation between locally isolated LSDV strains and reference genomes previously reported from other Asian and Middle Eastern regions, indicating potential regional viral adaptation. These findings provide a reliable molecular baseline for surveillance, vaccine development, and diagnostic improvement, offering insight into the evolving viral landscape associated with recent LSD outbreaks in Pakistan.

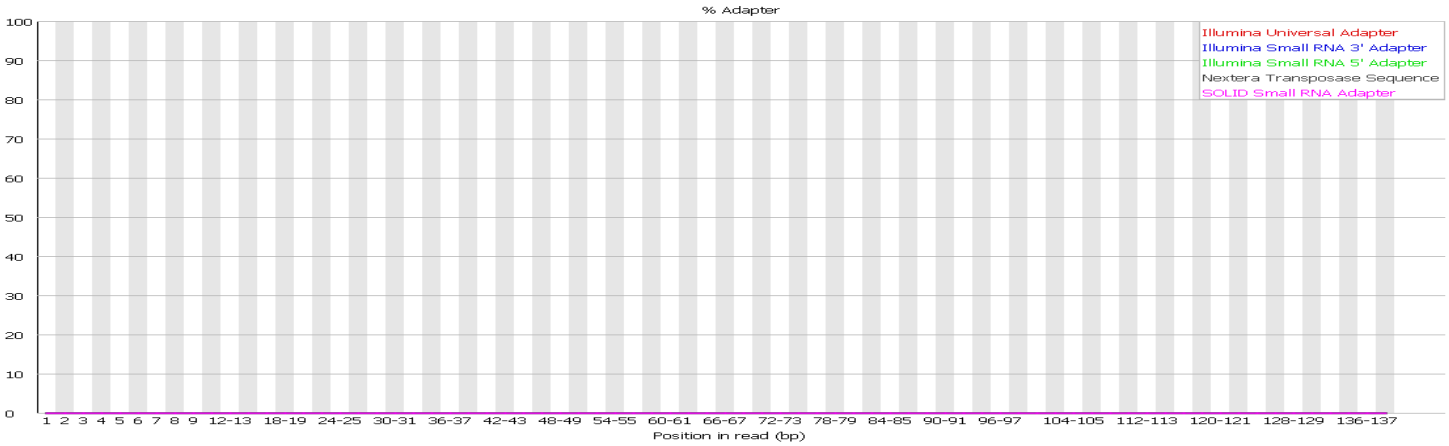

Figure 3. Adapter content

(Adapter content analysis indicating complete absence of residual adapter sequences across all base positions, confirming effective trimming during preprocessing and ensuring reliable downstream read mapping and assembly.)

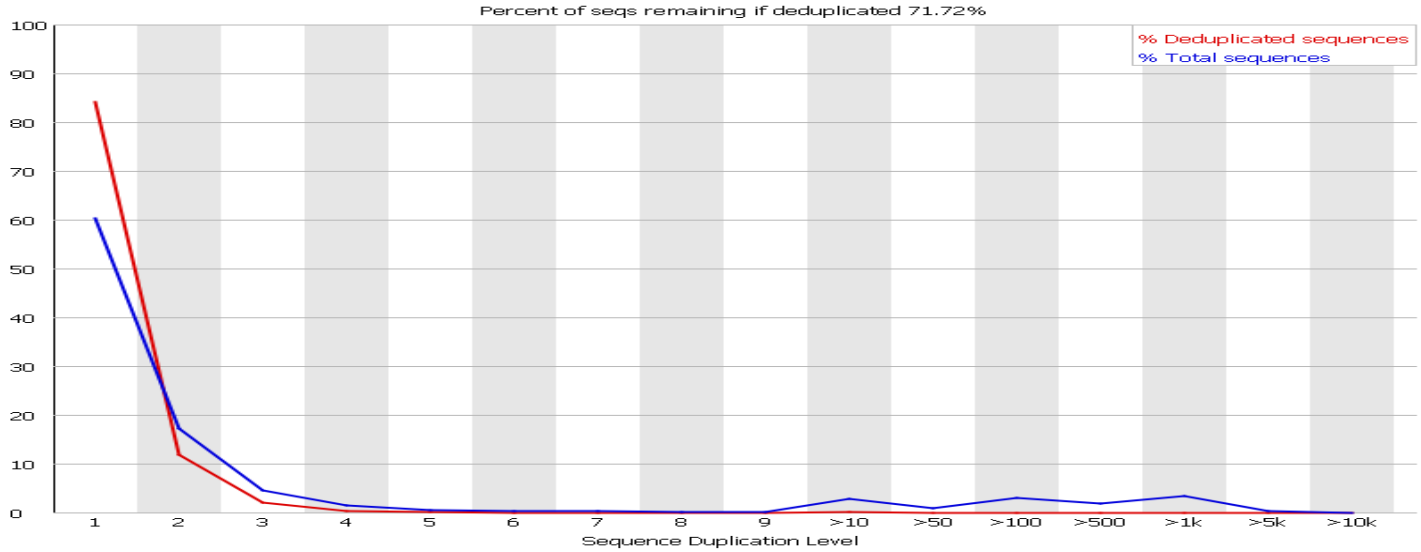

Figure 4. Duplication levels

(Sequence duplication level plot showing acceptable redundancy patterns, with most reads present only once and approximately 71.72 percent retained after deduplication, reflecting adequate genome coverage and minimal amplification bias.)

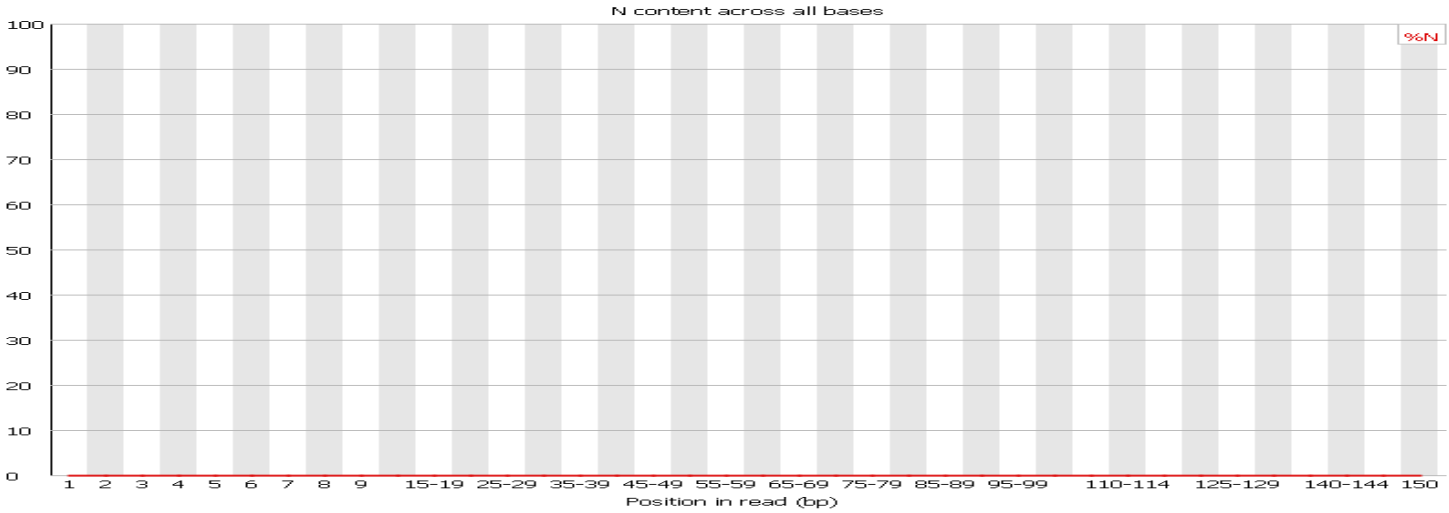

Figure 5. Per base N contents

(Per-base N-content plot revealing near-zero ambiguous base calls throughout all read positions, reflecting high confidence in base calling and eliminating the need for corrective trimming or filtering.)

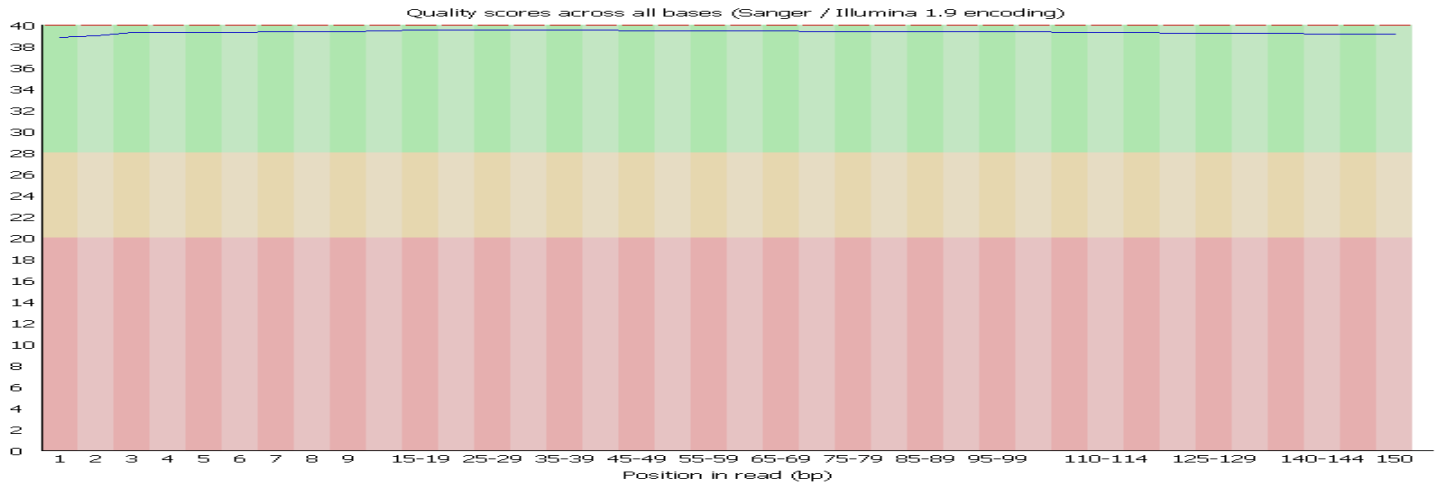

Figure 6. Per base quality

(Per-base sequencing quality plot showing consistently high Phred scores (Q39–Q40) across the full 150 bp read length, validating strong sequencing accuracy, precise base incorporation, and high integrity of signal detection)

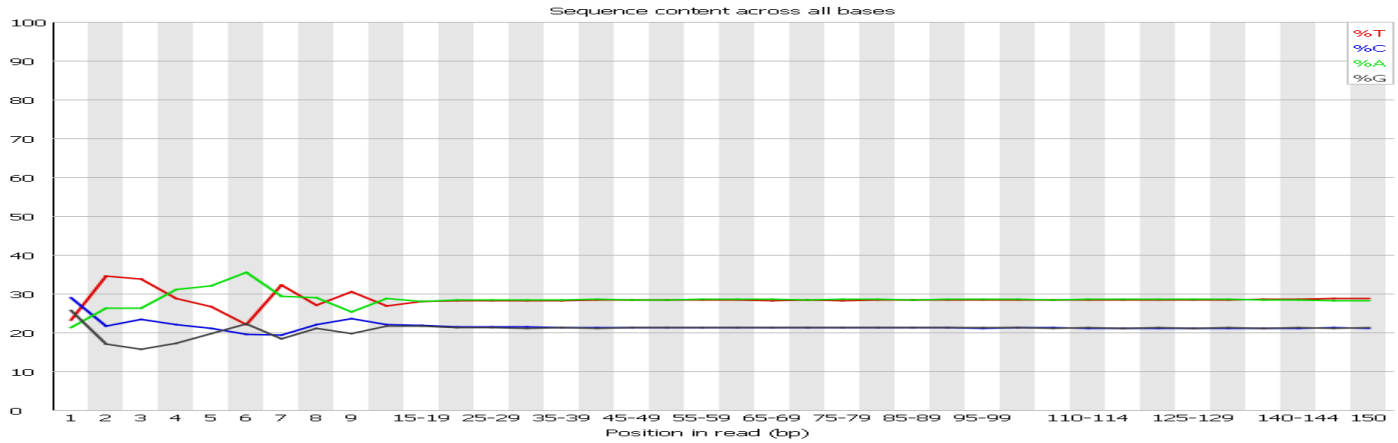

Figure 7. Per base Sequence content

(*Per-base sequence composition profile demonstrating balanced representation of all four nucleotides following initial priming cycles, indicating absence of base-calling or library preparation bias and reinforcing dataset reliability)*

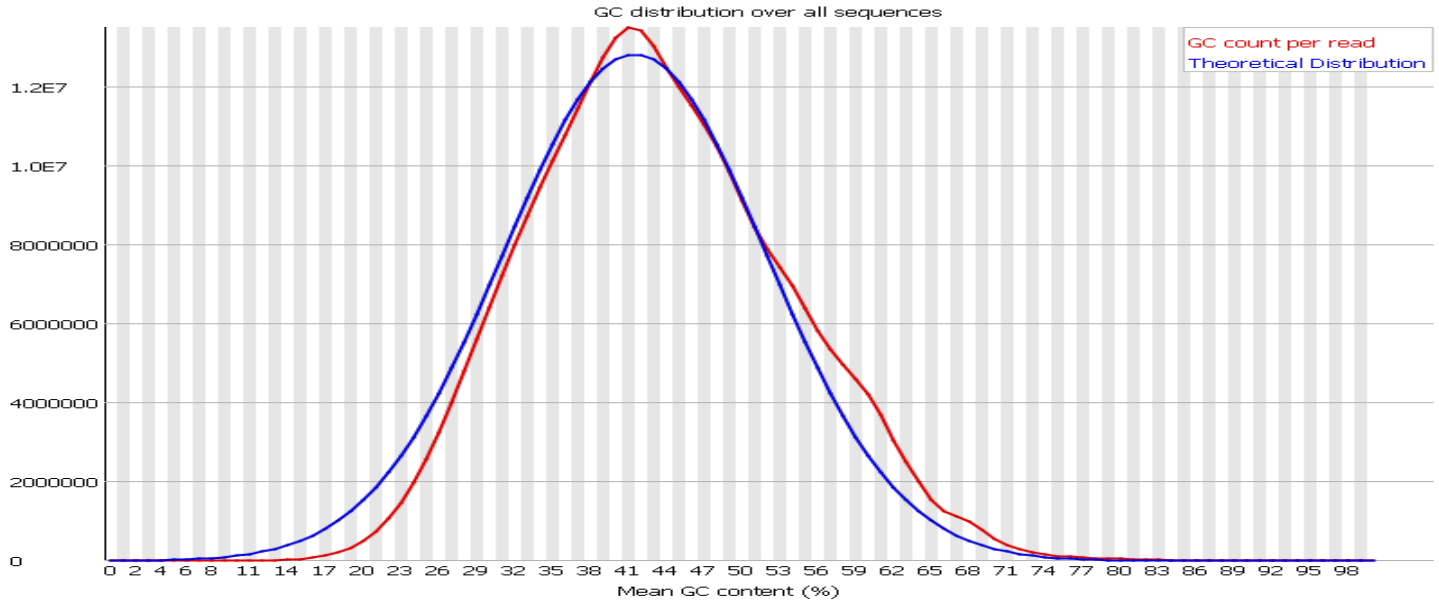

Figure 8. Per base GC content

(GC distribution curve closely overlapping the theoretical expectation, forming a smooth unimodal peak around 44–45 percent GC content, confirming biological authenticity of the dataset and absence of contamination)

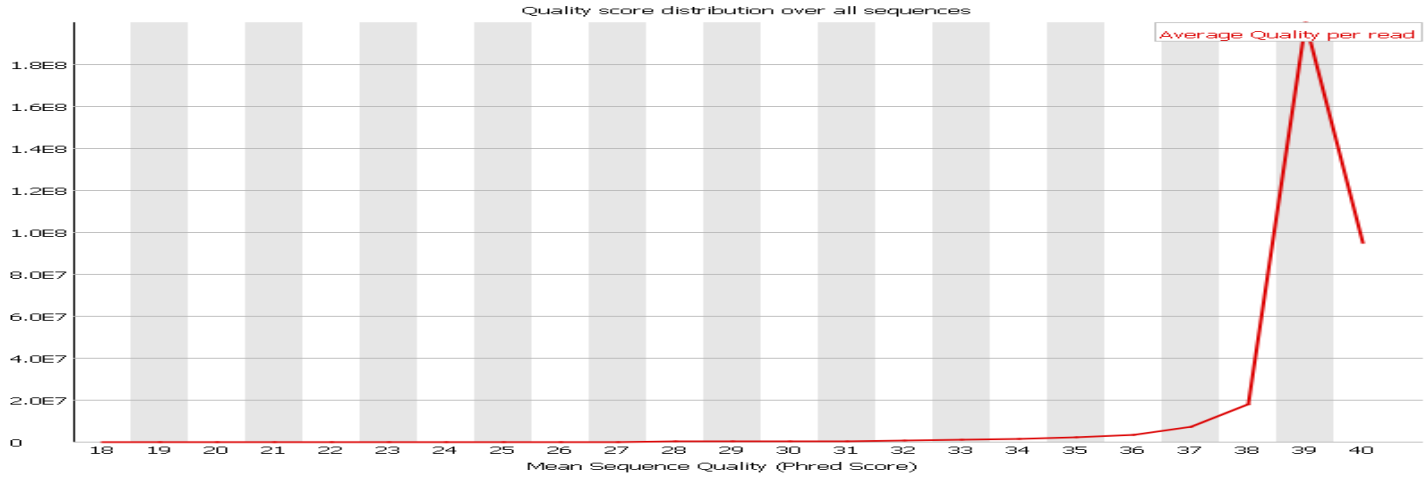

Figure 9. Per sequence Quality

(Per-sequence quality distribution showing most reads clustered in the highest Phred value range, confirming that raw sequencing output meets standards required for variant detection, genome assembly, and comparative phylogenomics.)

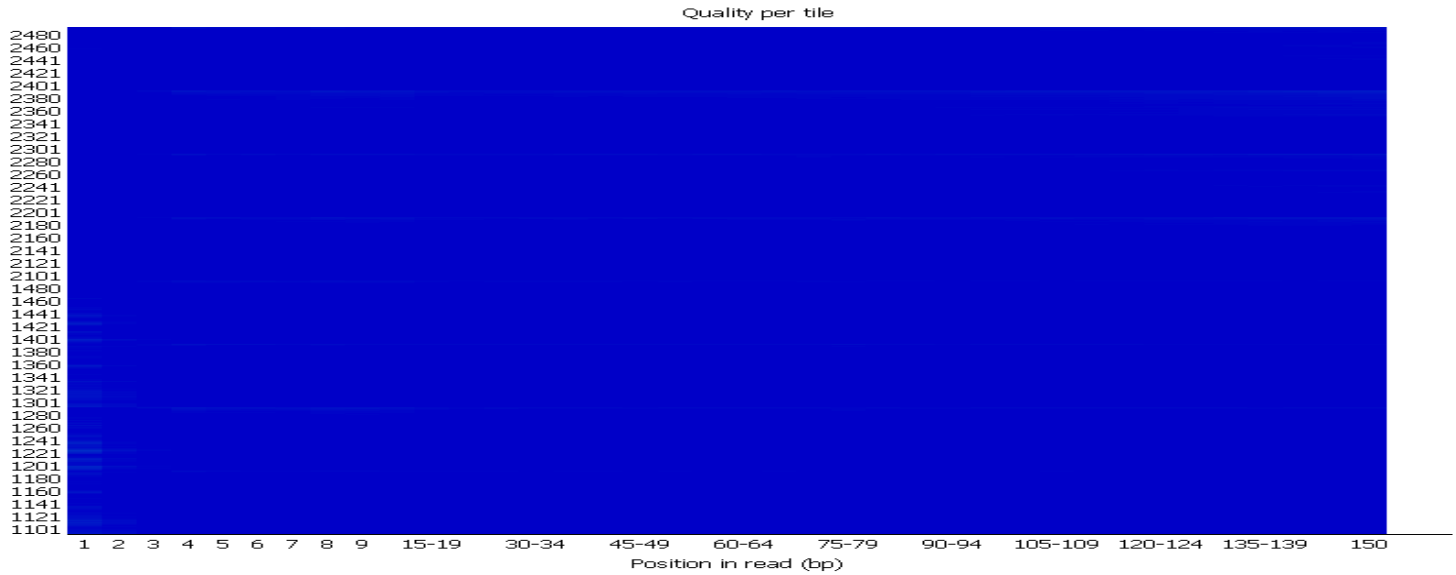

Figure 10. Per tile quality

(Per-tile sequencing quality heatmap showing uniform deep-blue signal across the flow cell surface, indicating consistent performance of the sequencing instrument with no localized defects, spatial artefacts, or platform-induced error regions)

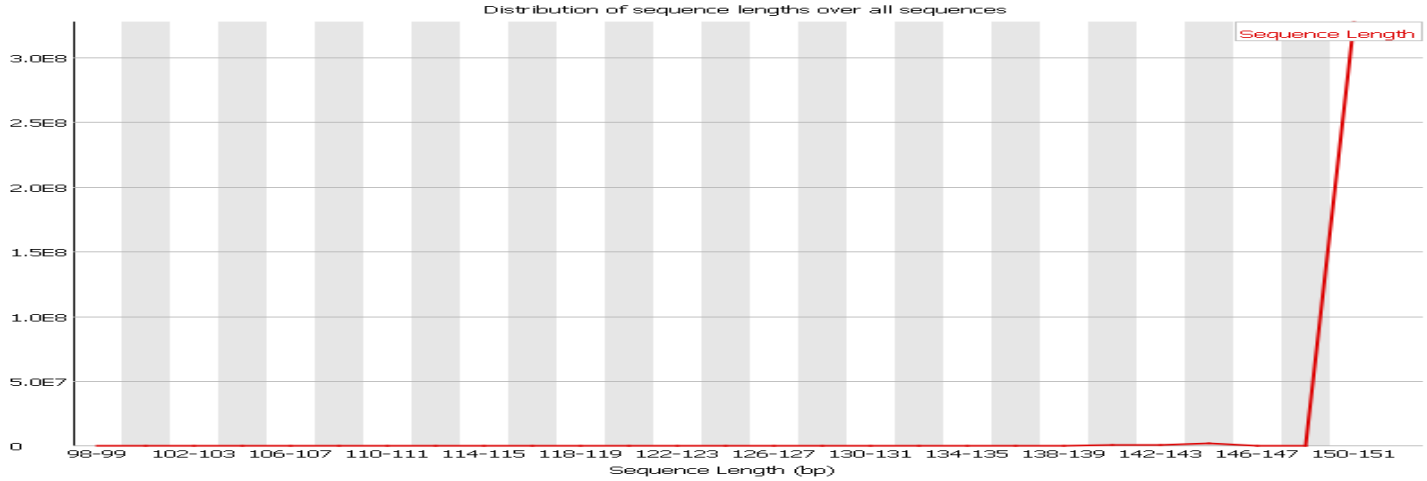

Figure 11. Sequence Length Distribution

(Sequence length distribution revealing highly uniform 150 bp read length output, demonstrating efficient library preparation, stable Illumina sequencing chemistry, and strong suitability for accurate genome alignment and assembly workflows)

Discussion

This study successfully confirmed the presence of lumpy skin disease virus (LSDV) in clinically infected cattle from Sindh, demonstrating active viral circulation in endemic areas. The observed clinical manifestations, including fever, nodular lesions, reduced feed intake, and productivity, were consistent with classical descriptions of LSD reported globally (1, 4). The molecular confirmation of LSDV supports earlier indications that the disease remains a significant emerging threat in Pakistan, as reflected by repeated outbreaks since 2021 (5, 6, 12).

The successful isolation of LSDV on Vero cell culture and visible cytopathic effects validate that local strains retain strong replication competence. Cytopathic changes observed in this study, such as cell rounding, refractility, and detachment, are consistent with previous reports describing LSDV cytopathogenic behavior in cell systems (30). The ability to propagate the virus and produce stable harvests at different cytopathic stages provides an important gateway for genomic surveillance, vaccine formulation, and antiviral research. The extraction of high-quality DNA from cattle and viral genomes, followed by high-fidelity Illumina sequencing, generated a robust dataset suitable for deep molecular interrogation. The sequencing quality indicators, including >96% Q30 bases, an ideal GC distribution, and uniform read lengths, reflect exceptional dataset integrity. These findings align with the strengths of next-generation sequencing (NGS) technologies in precision pathogen characterization, early detection, and evolutionary tracing (28, 29). Earlier studies established that NGS offers improved resolution for differentiating closely related LSDV strains and mapping genetic variability across regions (30). Our results reinforce these capabilities, particularly through clean FASTQC outputs which enable confident variant detection, phylogenetic analysis, and comparative genomics.

The genomic diversity observed between Sindh isolates and published reference sequences echoes previously reported cross-regional differences. Earlier studies demonstrated that LSDV strains from Punjab and Bangladesh exhibit partial gene similarities with strains from Asia and the Middle East (26). This supports the interpretation that viral spread in South Asia likely involves multiple modes of introduction, including animal movement and vector transmission (8, 9, 14). These findings underscore the relevance of continuous molecular epidemiology, particularly because LSDV adaptation mechanisms may underpin variation in clinical severity across breeds (12, 15, 26).

The sequencing outcomes generated here provide biological evidence supporting strong host–pathogen interplay, which may influence virulence patterns, immune response, and transmission dynamics. Prior research shows that LSDV-induced suppression of productivity and immune compromise leads to substantial welfare and economic loss (5, 6, 13). Our demonstrated capability to extract both bovine and viral genomic components highlights potential applications in dual RNA-seq, permitting deeper exploration of host immune pathways or susceptibility determinants (28, 30). These tools are essential for understanding how LSDV modulates cattle physiology, pathogen persistence, and disease progression (14, 15). Overall, the combined laboratory and genomic outcomes of this study advance the regional understanding of LSDV circulation and molecular diversity in Pakistan. They emphasize the urgent need for strengthened surveillance, vaccine characterization, and veterinary preparedness. Given the demonstrated ability of LSDV to evolve geographically (26) and its increasing socio-economic burden (13, 14, 15), future research should focus on phylogenetic comparisons across provinces, host–virus transcriptomics, and the evaluation of vaccine match effectiveness. The present findings lay an important molecular foundation for such future initiatives.

Conclusion

The sequencing output demonstrated excellent quality across all FastQC metrics, ensuring high confidence in downstream genomic and transcriptomic interpretations. The high read accuracy, balanced GC content, lack of contamination, and consistent tile performance collectively validate the reliability of both bovine and LSDV reads. These attributes enable precise identification of viral integration sites, replication dynamics, and host–pathogen interactions. Overall, the dataset's robustness provides a strong molecular foundation for understanding LSDV evolution, pathogenicity, and host immune response, supporting future advances in surveillance, vaccine development, and therapeutic strategies.

Recommendations

Effective control of lumpy skin disease requires coordinated action across institutions, sectors, and communities. Strengthening collaboration among veterinary services, livestock departments, agricultural authorities, and public health agencies can improve disease surveillance, risk communication, and rapid response capacity. Awareness programmes targeting farmers and livestock owners are essential to enhance understanding of disease transmission, vaccination compliance, and reporting behaviours.

A multi-sectoral approach involving animal health, agriculture, environmental management, and biosecurity systems is vital for reducing the spread and economic impact of the disease. This includes timely vaccination campaigns, regulation of animal movement, the development of improved diagnostics and vaccines, and the establishment of enabling policies that support disease control and livestock protection. Biosecurity implementation should prioritize postnatal immunization, quarantine of new animals, sanitation of animal housing, insect vector control, and strict movement certification. National surveillance programmes and timely outbreak reporting can help contain the spread and prevent cross-border transmission.

International cooperation through data sharing and harmonized control strategies further strengthens containment efforts. Collectively, adoption of robust biosecurity measures and coordinated sectoral engagement can minimise morbidity and mortality, protect livestock productivity, and improve food security while supporting economic resilience in affected regions.

Declarations

Data Availability statement

All data generated or analysed during the study are included in the manuscript.

Ethics approval and consent to participate

Approved by the department concerned.

Consent for publication

Approved

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Conflict of interest

The authors declared the absence of a conflict of interest.

Author Contribution

RK, NHK
Review of Literature, Data entry, Data analysis, and drafting article. Manuscript drafting, Study Design,
SZ, BK, EA
Study Design, manuscript review, critical input.
Conception of Study, Development of Research Methodology Design
MF, SS
Contributed to patient recruitment, data organization, and preparation of tables and figures

All authors reviewed the results and approved the final version of the manuscript. They are also accountable for the integrity of the study.

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