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Original Article

# Hemato-biochemical changes, molecular characterization and phylogenetic analysis of the 2022 Lumpy Skin Disease (LSD) outbreak in Cholistan, Pakistan

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## Abstract

The lumpy skin disease virus (LSDV) is a member of the Capripox genus of the Poxviridae family. It is the causative agent of lumpy skin, a highly contagious disease of cattle, water buffalo, sheep, and goats. In 2022, several outbreaks of LSD were reported in the Cholistan region of Pakistan, which has a large population of livestock living in arid conditions. A total of 230 blood/serum and scab samples were collected from three LSD outbreak locations. Clinically, affected cattle showed acute clinical signs characterized by skin nodules, fever, enlarged lymph nodes, emaciation, and lower leg edema. Hematological findings revealed non-significant changes in red blood cell and white blood cell counts (some animals had leukocytosis while others were leukopenic) whereas, hemoglobin level were significantly low. Platelet count, MPV, PCT**,** P-LRC, and P-LCC were elevated. Granulocytes were significantly low in LSD affected cattle while lymphocyte counts were significantly high. Serological findings revealed elevated protein levels, along with high creatinine and ALT concentrations. Amplification of DNA-dependent RNA polymerase 30 kDa subunit gene (RPO30) confirmed the presence of LSD virus in all suspected samples. Phylogenetic analysis showed that all Pakistani isolates clustered closely with isolates from neighboring countries. The SNPs differences were less than 20 among these isolates, indicating their close resemblance with each other. It can, therefore, be inferred that our LSD strains might be originated from neighboring Asian countries, that were affected by LSD in previous years.

**Keywords**: Lumpy skin disease virus (LSDV), Cholistan, Skin lesions, Hematology, Phylogenetic analysis

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## Introduction

The livestock industry in Pakistan is the largest sub-sector of agricultural production, contributing Rs. 1466 billion in the value addition, a 2.5% increase over the previous years. It also contributes 60.6% of the value addition in agriculture, 11.7% to the national GDP, and 3.1% of all exports, making it a major source of foreign exchange. Approximately eight million families directly work with cattle, and this industry contributes 35–40% of their income (Jamil et al., 2022). Lumpy skin disease (LSD) epidemic has impacted the lives of approximately 05 million farmers, posing a serious challenge to the livestock industry and the individuals associated with it (Ali et al., 2020; Jamil et al., 2022). Lumpy skin disease, commonly known as LSD, is a contagious disease affecting primarily cattle and less commonly water buffalo. The causative agent of this disease is the lumpy skin disease virus (LSDV), which belongs to the family Poxviridae, subfamily Chordopoxvirinae, and genus Capripoxvirus. The disease has several other names like "Neethling viral disease","exanthema nodularis bovis", “Pseudo-urticaria”, and “knopvelsiekte”, but “LSD” is the most commonly used term (Al-Salihi, 2014).

Lumpy skin disease is non-zoonotic but is transboundary and primarily transmitted via vectors. The host range of this disease is believed to be limited, as it has primarily been observed in large ruminants like cattle (*Bos sp.*) and water buffalo (*Bubalus sp.*). Common carriers of the disease include mosquitoes, ticks, and biting flies, such as Culicoides (Lubinga et al., 2013; Tuppurainen et al., 2011). The disease is characterized by high morbidity but low mortality rates, indicating that the disease does not usually result in death in most cases (Abutarbush et al., 2015). The virus (LSDV) causes extensive economic losses due to various factors, including weight loss, abortion, infertility, and drastically reduced milk production and damage the quality of hides in affected animals. Additionally, these infections can cause notable metabolic, immunological, and hematological changes, further adding to the detrimental consequences in the affected animals especially in the desert ecosystem (Hasan and Alsaad, 2018; Hussain et al., 2022; Neamat-Allah, 2015). The disease is characterized by fever, the presence of nodules ranging from 2 to 5 cm in diameter on the skin and mucosal membranes, lesions in the digestive and respiratory systems, as well as swollen superficial lymph nodes. These clinical manifestations are considered hallmark signs of LSD (Salib and Osman, 2011). In affected cattle, extensive pox lesions may develop, not only on the skin but also internally in the organs throughout the body (Babiuk et al., 2008). The disease was first documented in Zambia in 1929, and within the next three years, it rapidly spread to other southern and northern African countries (Namazi and Khodakaram, 2021). The countries including China, India and Bangladesh, which share borders with Pakistan, experienced LSD outbreaks in 2019, according to different published reports (Sudhakar et al., 2020; Ul-Rahman et al., 2022). In Pakistan, a suspected outbreak of lumpy skin disease (LSD) was reported in the cattle population of Sindh province in 2021. Since then, several LSD outbreaks have occurred across the country, notably in Sindh, Punjab, and KPK provinces. LSD has caused more than 7,500 deaths nationwide, with over 190,000 cases reported. However, the recovery rate is encouraging, with over 141,000 animals successfully recovered (Albayrak et al., 2018; Ul-Rahman et al., 2022).

Lumpy skin disease virus (LSDV) is a double-stranded DNA virus that can be easily detected in a variety of clinical specimens from infected cattle. These specimens include skin nodules, ulcerations, semen, blood, and serum. The virus can be detected using a variety of methods, including polymerase chain reaction (PCR), serology, and virus isolation and culturing. The most common method of identifying LSDV is PCR, which is relatively inexpensive, easy to perform, and highly sensitive and specific (Wolff et al., 2022). The current study aimed to determine the hematological and biochemical changes in LSD infected cattle and to confirm the presence of LSD virus in these animals using PCR targeting the RPO30 gene.

## Material and Methods

### Study area

Outbreak of LSD in Pakistan occurred in 2021-22 in Cholistan and surrounding areas. The study, therefore, involved sampling animals suspected of having LSD in this region called the Cholistan Desert, (commonly known as Rohi in the local

dialect), extends 16,000 km² and is located thirty kilometers from Bahawalpur in southern Punjab. It is geographically connected to the Thar Desert, extending into Sindh in Pakistan and India. The Cholistan Desert, consists of three districts Bahawalnagar, Bahawalpur, and Rahim Yar Khan, covering a total area of 6,655,360 acres. Among these districts, Bahawalpur is the largest area of Cholistan, spanning 4,028,217 acres. The temperature in the Cholistan region varies between 6 to 50°C (Muhammad et al., 2013). It stretches 480 km in length and has a width ranging from 32 to 192 km. The human population in Cholistan is approximately 155,000, while the livestock population reaches 1,318,000 (Farooq et al., 2008). The outbreak of LSD was first reported in Pakistan in this region and surrounding areas, having higher population of livestock animals (Fig 1).

**Study population and sample collection**

The livestock population of Cholistan suspected for LSD on the basis of clinical signs and symptoms was chosen and blood/serum samples/skin scabs (n=230) were taken from affected cattle at three different locations. The samples were taken from local healthy animals (n=23) of these areas for comparison purpose, including hematological and serological parameters.

**Hematological parameters**

Blood samples were collected from the heathy, and diseased cattle from the jugular vein under aseptic conditions in vacutainers with and without anticoagulants (EDTA; 1.00mg/ml) (Ahmad et al., 2024). Hematological parameters, including total erythrocyte count (RBC's), hematocrit (HCT) value, hemoglobin (Hb) concentration, mean corpuscular volume (MCV), white blood cell counts (WBC's), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), and platelet hematocrit (PCT) were determined by an automated hematology analyzer (Biobase, BK-3100VET, Shandong, China) at the Central Diagnostic Lab Complex (CDLC), The Islamia University of Bahawalpur, Pakistan.

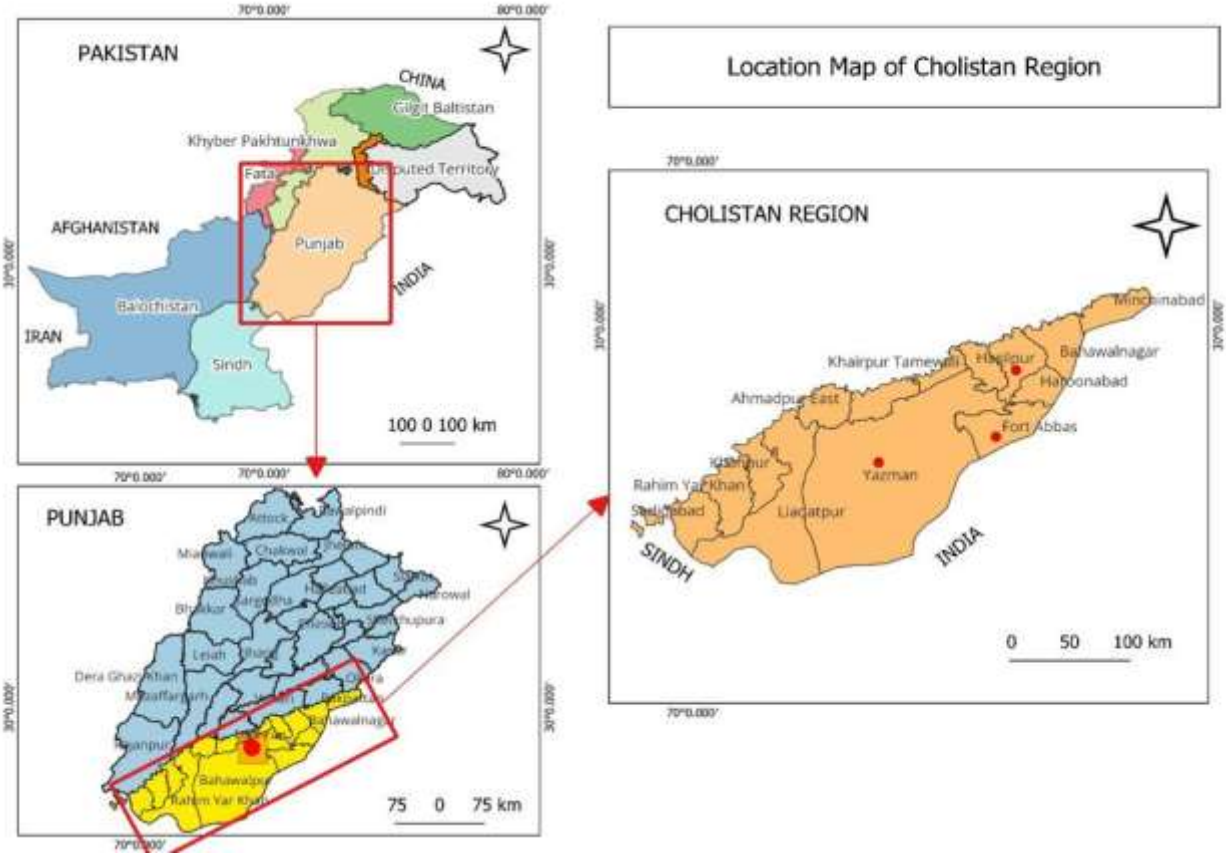


**Figure-1. Map showing the location of Cholistan region in Punjab, Pakistan**

**Serum biochemical parameters**

Serum was separated from the blood samples and stored at -20°C until analysis (Khaliq et al., 2020; Zubair et al., 2022). Serum harvested from blood was used to measure total protein, albumin, urea, creatinine, iron (Fe), calcium (Ca), magnesium (Mg), phosphorus (P), and alanine aminotransferase (ALT).

**Molecular detection via PCR**

The viral DNA was extracted using the QIAamp Mini Kit (Qiagen, USA). Primers were designed targeting the RPO30 gene segment (554 bp in length) (Molini et al., 2018). The primer sequences were forward primer: 5'-CAGCTGTTTGTTTACATTTGATTTTT-3' and reverse primer: 5'-TCGTATAGAAACAAGCCTTTAATAGA-3'. The PCR reaction mixture was 20µL, containing 0.8 µL of each primer, 2.5µL dNTPs, 2µL PCR buffer $(NH_4)_2SO_4$, 2.5µL of $MgCl_2$, 0.8µL Taq polymerase, 1.5µL template DNA and 9.1µL distilled $H_2O$. The amplification conditions were: 01 cycle (denaturation at 95°C for 4 min), 40 cycles (denaturation: 95°C for 30s, annealing: 55°C for 30s, and extension: 72°C for 45s) and 01 cycle (final extension: 72°C for 7 min). After amplification, the 554 bp band was observed on 1% agarose gel stained using ViSafe Red Gel Stain (Vivantis, Malaysia) in Gel Documentation System (Bio-Rad, USA).

**Sequencing and phylogenetic analysis**

The amplified products were sent for sequencing to the commercial sequencing company (Macrogen, Korea). The RPO30 LSDV sequences were multiple-aligned using ClustalW in the BioEdit program. The aligned sequences were then compared with the already reported sequences deposited in GenBank via BLAST software, NCBI (https://www.ncbi.nlm.nih.gov/).

The maximum neighbor-joining method (MEGA v.11) was employed in constructing phylogenetic tree with Bootstrap value set at 1000 replications to estimate confidence in the branching patterns of the trees. RPO30 sequences of GTPV and SPPV were used as the out-groups for phylogenetic analysis.

**Statistical analysis**

All statistical analyses were performed using SPSS 19.0. The data were presented as mean ± standard error. Statistical analysis was conducted using the two-sample t-test to compare the LSDV-infected and healthy groups. A significance level of $P \leq 0.05$ was considered statistically significant (Leech et al., 2007).

## Results

**Clinical signs and lesions in infected cattle**

Clinical signs observed in affected animals include anorexia, high grade fever with salivation, skin nodules frequently on the neck, dorsum of the back, abdomen and legs and in some cases all over the body. Lactating animals experienced high drop in milk production. Snoring sounds were heard in some animals due to nodule formation in the nasal septum.

**Hematological findings**

Hematological findings for naturally infected cattle are given in table 1 along with comparison to healthy ones. The infected cattle exhibited significant increase in the LYM, PLT, PCT, RDW-SD, P-LCR, P-LCC and decrease in MID, GRAN and HGB, and PDW.

**Table 1. Hematological values in LSDV infected and healthy cattle**

| Parameters | Reference Values | Diseased group (n= 230) | Healthy group (n=23) |
|---|---|---|---|
| WBCs($x10^9$/L) | 4.0-12.0 | 7.528 ± 0.281$^a$ | 7.75±0.47$^a$ |
| LYM (%) | 20-60 | 93.507±0.142$^a$ | 43.32±2.33$^b$ |
| MID (%) | 4-12 | 3.796± 0.062$^a$ | 7.20±0.45$^b$ |
| GRAN (%) | 30-70 | 2.780± 0.086$^a$ | 49.72±2.52$^b$ |
| LYM ($10^9$/L) | 1.5-9.6 | 7.030± 0.260$^a$ | 6.13± 0.37$^a$ |
| MID ($10^9$/L) | 0.3-1.6 | 0.289±0.009$^a$ | 0.55±0.07$^b$ |
| GRAN ($10^9$/L) | 2.3-9.1 | 0.236± 0.01$^a$ | 4.79±0.40$^b$ |
| RBCs($x10^{12}$/L) | 5-10 | 5.726±0.133$^a$ | 6.72±0.3$^a$ |
| HGB (g/dL) | 8.7-12.4 | 4.494±0.172$^a$ | 9.90±0.2$^b$ |
| HCT (%) | 22-33 | 25.773± 1.534$^a$ | 27.73±1.37$^a$ |
| MCV (fL) | 38-50 | 43.366± 1.358$^a$ | 42.20±0.71$^a$ |
| MCH (pg) | 14-18 | 17.705±0.559$^a$ | 15.94±0.24$^a$ |
| MCHC (g/dL) | 36-39 | 43.038±1.539$^a$ | 37.47±0.17$^a$ |
| RDW-CV (%) | 11.5-14.5 | 11.666± 0.066$^a$ | 12.53±0.19$^b$ |
| RDW-SD (fL) | 22-50 | 54.548±0.388$^a$ | 40.85±1.89$^b$ |
| PLT($x10^9$/L) | 120-820 | 1539.0±47.869$^a$ | 511.39±38.90$^b$ |
| MPV (fL) | 3.8-7.0 | 8.418±0.048$^a$ | 5.60±0.27$^b$ |
| PCT (%) | 0.15-0.3 | 1.253±0.036$^a$ | 0.210±0.02$^b$ |
| PDW (%) | 15-17 | 5.619±0.241$^a$ | 16.40±0.15$^b$ |
| P-LCR (%) | 22-50 | 59.514±2.167$^a$ | 36.40±1.56$^b$ |
| P-LCC($x10^9$/L) | 15-17 | 1108.92±58.64$^a$ | 16.28±0.16$^b$ |

Note: Values (means ± SE) within the same row having different superscripts are significant ($P< 0.05$)

**Biochemical findings**

The biochemical findings revealed significant association ($P$<0.05) between the mean values of LSD infected versus healthy cattle. Specifically, total protein concentration, ALT, and creatinine were significantly elevated ($P$<0.05) while albumin levels were decreased in diseased cattle No significant differences were observed for other parameters ($P$ > 0.05).)

**Table 2. Biochemical values in LSDV infected and control cattle**

| Parameters | Reference Values | Diseased group (n= 230) | Healthy group (n=23) |
|---|---|---|---|
| Calcium (mg/dL) | 8-10 | 8.4002±0.146[a] | 8.32±0.31[a] |
| Magnesium (mg/dL) | 1.7-2.4 | 2.399±0.044[a] | 2.18±0.10[a] |
| Phosphorus (mg/dL) | 4.9-9 | 4.670±0.129[a] | 6.77±0.43[b] |
| Total protein (g/dL) | 5.5-7.5 | 10.287±0.071[a] | 6.76±0.20[b] |
| Albumin (g/dL) | 2.5-3.8 | 1.677±0.040[a] | 3.20±0.13[b] |
| Urea (mg/dL) | 10-25 | 19.272±0.439[a] | 19.30±1.16[a] |
| Creatinine (mg/dL) | 0.5-2.2 | 2.879±0.141[a] | 1.59±0.12[b] |
| Iron (µg/dL) | 130-250 | 202.76±4.28[a] | 189.52±9.93[a] |
| ALT (IU) | 25-75 | 91.648± 0.808[a] | 50.90±3.60[b] |

Note: Values (means ± SE) within the same row having different superscripts are significant ($P$< 0.05)

**Molecular detection of LSDV**

Gel electrophoresis of amplified PCR product of RPO30 gene showed a 554 bp band, as illustrated in fig 2. The PCR assay therefore, confirmed LSD virus in the representative samples collected from diseased cattle.

**Sequencing and phylogenetic analysis**

Representative samples chosen on the basis of location were sent for sequencing of the amplified RPO30 gene. The raw sequences were quality-checked and edited, and deposited in the GenBank database (PAK00701, PAK00702, PAK00703). The phylogenetic analysis revealed that the Pakistan isolates have made cluster with the isolates from neighboring countries i-e, India and Bangladesh and the SNPs among them were less than 20 (Fig 3).

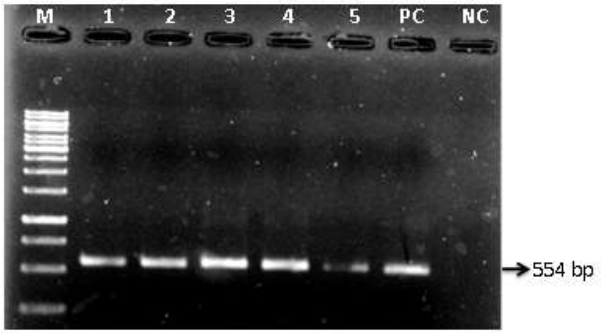


**Figure-2. Agarose gel electrophoresis showing 554 bp amplicon of RPO30 gene. Lane M: 1kb DNA ladder, Lane 1-5: LSDV positive samples, Lane PC: positive control, Lane NC: negative control**

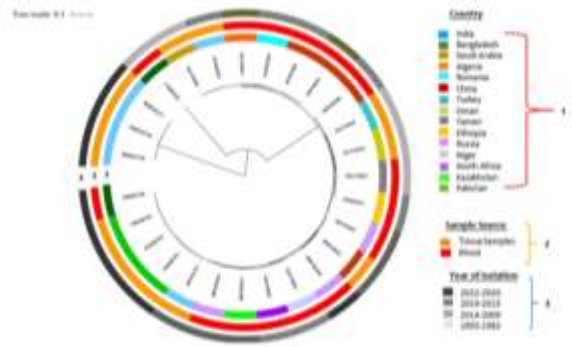

**Figure-3. Phylogenetic tree showing the relationship between LSDV RPO30 gene sequences from Pakistan, with other Capripoxvirus RPO30 gene sequences from GenBank. Numbers indicate the values calculated at 1000 bootstrap replicates.**

## Discussion

Lumpy skin is a transboundary disease of animals, which has significantly impacted global animal trade and also poses food security challenges (Tuppurainen and Oura, 2012). Animals affected by this disease suffers permanent hide damage, decrease milk production, emaciation, infertility problems, abortion in pregnant, and disruption in their trade across borders (Abutarbush et al., 2015; Tuppurainen et al., 2017). In the Cholistan region, this study is the first to document the hematological and serum biochemical manifestations of lumpy skin disease in cattle. The red blood cells count (RBC's) was found to be low in diseased animals. Hemoglobin (HGB) levels fell below than normal and were significantly

low, suggesting severe macrocytic hypochromic anemia in the disease affected group. Viral infections, as seen in LSD infected cases, can lead to hemolytic anemia. Low levels of HGB may also be characterized as inflammation-related, which is brought on by inflammatory cytokines, including TNF, IL-1α, IL-1β, and IF-γ, decreased erythropoietin response in the bone marrow, anorexia, and decreased blood iron levels. Affected animals revealed little variations in WBCs count, meanwhile both Leucocytosis and leuckopenia was observed occasionally in the individual animals, however most of them had values within normal range. Neutrophils count was significantly low, while lymphocytes count was high in all animals. The platelet counts (PLT), mean platelet volumes (MPV), Platelet crits (PCT), platelet large cell ratio (P-LRC), and platelet large cell count (P-LCC) values were significantly higher in infected animals as reported previously (Latimer, 2011; Roland et al., 2014). Increased platelets count, or thrombocytosis, could be a consequence of epinephrine-induced splenic contraction. It could also be triggered by the release of cytokines and is associated with stress, chronic blood loss, inflammation, neoplasia, or iron deficiency (Latimer, 2011; Stokol, 2010).

There are few reports available indicating the serum biochemical values in LSD affected cattle. The assessment of the biochemical indicators during infection phase can help in understanding disease pathogenesis and prognosis. Elevated total protein levels (hyperproteinemia) were observed in cattle with LSDV infection, which might be due to dehydration and/or the inflammatory response (El-Mandrawy and Alam, 2018). A modest significant decrease in serum albumin levels in LSDV-infected cattle was an indication of hepatic insufficiency, and the induction of humoral immune responses against the virus, which was probably caused by enhanced protein catabolism or diminished protein synthesis, as well as hepatic damage. The creatinine concentrations were found higher in diseased animals and is consistent with the reported association between increased creatinine concentration and reduced glomerular filtration rate (Drakesmith and Prentice, 2008; Ul-Rahman et al., 2023). The present study found a significant increase in alanine aminotransferase (ALT) concentrations, which is indicative of muscle injury; a common manifestation of LSDV pathogenesis (Hassan et al., 2011). The other selected serological parameters were having readings close to reference values. Factors apparently responsible for variations in different parameters include; differences in age, breed, and body condition of exposed animals. The timing of sampling, methodology adopted for serum analysis, and individual variations may also play a critical role (Adamu et al., 2013). It is also possible that the viral infection itself affected iron metabolism and regulation in the body (Drakesmith and Prentice, 2008). The hematological and serum biochemical alterations induced by lumpy skin disease may be heterogeneous, with the underlying disease process being the primary driver for these changes. Variations within the same parameter are likely to be driven by the stage of the disease and the severity of the case.

Polymerase chain reaction (PCR) has been successfully applied in this study and other studies for the detection and confirmation of the causative agent of LSD. The specific primers, targeting the DNA-dependent RNA polypeptide RPO30 gene was amplified using DNA of the virus isolated from blood/serum samples of the infected cattle. The 30-kDa RNA polymerase subunit gene (RPO30) is widely used for detecting nucleotide polymorphism in the lumpy skin disease virus (LSDV) genome. Moreover, the RPO30 gene is a suitable target for constructing phylogenetic relationships between members of the Capripoxvirus genus (Yan et al., 2012), including LSDV strains from wild field populations as well as vaccine strains (Gelaye et al., 2015). The partial RPO30 gene sequence (554 bp) was reported for the first time from Cholistan region, Pakistan. Phylogenetic tree revealed that the Pakistani isolates clustered with the isolates from neighboring countries (India and Bangladesh). The SNPs difference among these was less than 20, which indicated their close resemblance with each other. It can therefore be inferred that our LSD strains might be originated from neighboring Asian countries, which were affected by LSD in previous years.

## Conclusion

This study highlights the hematological and biochemical changes associated with LSD in naturally infected cattle. The results provide evidence of LSDV's detrimental effects on various physiological systems and emphasize the need for effective disease management strategies. Moreover,

confirmation of LSD virus was done via PCR targeting the RPO30 gene. Phylogenetic analysis revealed close association of Pakistan LSDV strains with the isolates reported by neighboring countries like India and Bangladesh, where the disease has already been reported in the previous years. These findings can aid in the development of targeted control measures and facilitate the prevention and management of LSD outbreaks in the affected areas.

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### Contribution of Authors

Asghar T, Mohiuddin A: Performed experiments, analyzed the data and prepared draft of the manuscript, approved the final manuscript.
Mohiuddin M: Conceived the idea, designed the experiments and funds acquisition, approved the final manuscript.
Mansoor MK, Habib M, Rizwana H & Abid I: Data interpretation and revision of the manuscript, approved the final manuscript.
Siddique A, Kamal T, Hussain R, Ghori MT, Hameed H, Ehsan M & Shabbir A: Sample collection, data analysis and drafting of the manuscript, approved the final manuscript.

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