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Sequence based Characterization of Lumpy Skin Disease Virus from Punjab, Pakistan

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LSDV, Whole genome sequencing, *GPCR, RP030 P32*, Phylogenetic analysis

ABSTRACT

Lumpy skin disease virus (LSDV) is an acute or subacute, highly infectious vector-borne disease with significant morbidity and mortality rates. A massive epidemic of LSDV occurred in Pakistan during 2021-2022, causing devastating effects on the socio-economic status of the country's livestock sector. This study aims to investigate the genetic diversity and conduct phylogenetic analysis of the GPCR RP030 and P32 genes, along with the genome sequence (WGS) of LSDV. Out of 385 tissue samples examined, 296 tested positive for LSDV. WGS analysis identified 155 open reading frames within the genome, while phylogenetic analysis revealed that the isolated strain clustered within clade 1.2, which includes strains from India, Bangladesh, Eurasian, and African countries. The strain exhibited maximum homology of 99.94% with the Neethling strain NI-2490 (NC003027) from Kenya, and 99.93% with strains from Bangladesh (OP688129), India (OR393169, OR393175, OR393171), and Russia. The *GPCR, RP030* and *P32* genes clustered within clade 2.2, with clade 2.2 being the sister clade of clade 1.2. Homology analysis indicated that these genes share 99 to 100% homology with strains from Russia, India, Nepal, Bangladesh, and Kenya. This study emphasizes the global dissemination and genetic diversity of LSDV strains, underscoring the importance of collaborative surveillance and vaccine development efforts to control LSD outbreaks effectively. The findings suggest that the currently employed vaccines providing an effective protection and highlights the need for continuous monitoring for adopting control strategies and local production of effective vaccines.

INTRODUCTION

Lumpy skin disease (LSD), a vector-borne illness, poses a serious threat to the cattle population by the World Organization of Animal Health (OIE). LSD is an extremely infectious disease that has spread across the borders and affected areas of Asia, the Middle East, and Africa is caused by the LSD virus (Mercier *et al.*, 2018; Suwankitwat *et al.*, 2024). Different episodes of epidemics in provinces of Pakistan (including KPK, Punjab, and Sindh) in late 2021 were reported for the first time from Sindh (Khatri *et al.*, 2023; Ul-Rahman *et al.*, 2022, 2023).

LSD has a significant negative effect impact on the economy by limiting international commerce, including high rates of morbidity, reduction in milk production, lower feed conversion ratios (FCR), skin damage and hides, and abortion (Moudgil *et al.*, 2023). Nationwide 190000 cases with mortality of 7500 animals were reported in Outspread of LSD from Sindh to other provinces by animal movement in 2022 (Khatri *et al*., 2023). This poses severe problems regarding socio-economic stability in a country like Pakistan whose major portion of the economy is agriculture and depends heavily on the agricultural sector (livestock), and LSD causes a severe threat to national food security.

There is evidence of the disastrous consequences of LSD on rural communities of underdeveloped countries such as Pakistan (Khatri *et al*., 2023). Cattle and buffaloes

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are important for beef industry and in particular, are an important socio-economic assets for maintaining food security (Ahmad, 2013). The latest epidemic in Sindh province, which is the first known wave of LSD in Pakistan and is characterized by clinical indicators including skin nodules, pyrexia, and reduced production, highlights how serious the situation is (Khatri *et al*., 2023). Strategies to control this novel LSD outbreak include vaccination and stopping animal movements. Certainly, there was a need to visualize the virus's origin and mode of transmission, necessitating a thorough examination of the LSD sequence.

This study aims to commence a thorough examination of LSD outbreaks in Pakistan by sequencing the genome of LSDV strains through phylogenetic analysis, providing full sequencing data that may be useful for future research. Specifically, study strive for the identification of the genetic diversity and phylogenetic relationships of LSDV strains prevalent in Pakistan and to compare these strains with those from other regions. Remarkably, LSDV strains from Pakistan have a clear grouping pattern with those from Asia, the Middle East, and other African nations, according to the sequence analysis for the G protein-coupled chemokine receptor-like protein (GPCR) partial gene (Manzoor *et al.*, 2023). Because of Pakistan's proximity to areas where LSD is endemic and to cross-border livestock migrations, this complication emphasizes how urgent it is to look into potential introduction channels. This study attempts to offer insights essential for developing focused and efficient vaccination programs by separating field-prevalent strains.

MATERIALS AND METHODS

Sample collection

A total of 385 tissue samples were collected. Samples were transported by maintaining a cold chain (Sudhakar *et al.*, 2022), to the Institute of Microbiology, UVAS, and stored at -20 ºC till further processing. Samples were collected across the Punjab Province of Pakistan from dairy cattle and buffalo herds in the areas where LSD had been reported. The selection criteria for tissue samples included clinical symptoms of disease such as fever, presence of nodules on skin, enlarged lymph nodes, lacrimation indicative of LSD, and representation from diverse geographic locations within Punjab Province (Table 1)**.** Tissue was triturated, ground, and centrifuged at 3000rpm for 10 min and the supernatant was filtered with 0.45 µm syringe filter.

Virus identification and confirmation

The viral genome was extracted by a commercially available viral nucleic acid kit (Geneaid Viral Nucleic Acid Extraction Kit ll), and used for virus confirmation by PCR for immunogenic gene (*GPCR*) of length 1134 by using its specific primers (forward primer 5'-ATGAATTATACTCTTAGYACAGTTAG-3' reverse primer 5'-TTATCCAATGCTAATACTACCAG-3') and PCR conditions were optimized with the help of already cited information in literature (Kumar *et al.*, 2023). With PCR condition at Step 1 initial denaturation 95ºC/5 min of single cycle, Step 2 for 40 cycles at 94 ºC/30 sec, 55 ºC/30 sec, 72 ºC/60sec, Step 3 and 4 of single cycle 72 ºC/10 min and 4 ºC/infinite.

Virus propagation

Vero and Madin-Darby bovine kidney (MDBK) cells were used to propagate LSDV according to the OIE protocol. MDBK cells grown in Dulbecco's Modified Eagle Medium(DMEM), and fetal calf serum(10%). 25 cm^2 tissue culture flask used to cultivate monolayer cells, and 1ml of tissue filtrate with amphotericin B 2.5 µg/ml, streptomycin 100 µg/ml, and penicillin 100 µg/ml. were added and allowed for virus adsorbed for a period of two h at 37°C, 10 ml of cell culture medium was added and the flask was placed in an incubator and checked for cytopathic effect on the 10^{th} day, Cells were freeze-thawed and centrifuged and the supernatant was used to inoculate in fresh cells till 4^{th} passage when the virus was confirmed in the supernatant by PCR of *GPCR* gene. All steps were performed under strict biosafety to avid contamination.

Virus genome extraction

A commercially available viral nucleic acid kit (Geneaid Viral Nucleic acid Extraction Kit ll), was used for DNA extraction according to the manufacturer's recommended protocol**.**

Whole genome sequencing (WGS)

Representative sample was selected and genomic DNA was sent for WGS by local vender ABO Scientific (Flat# 05, 1st Floor (Civil College Building), Main Street, Gulshan-e-Saeed-02, Chakri Road, Rawalpindi) and which prepared libraries for Illumina and Oxford Nano-pore according to the manufacturer's instructions (Illumina, USA). After the quality assessment conducted through FastQC and trimmed to maintain quality of sequence at 30QC, the paired-end reads underwent merging via FLASH version 1.2.11. The amalgamated reads were subsequently subjected to alignment against the NCBI non-redundant (nr) protein database, utilizing DIAMOND version 0.8.33 and Kraken version 1.1. Post alignment, reads attributed to viral entities were isolated. The resulting viral sequences were then assembled utilizing SPAdes version 3.9.1. and genome was annotated by Prokka on Galaxy (Version 1.14.6+galaxy1).

Phylogenetic analysis

WGS of isolate and individual gene RP030 (606 nt), *GPCR* (1146 nt), and *p32* (969 nt) and used to construct the phylogenetic tree with genome sequence-wise aligned with the reference genomes of different clusters reported in Breman *et al*. (2023). Alignment done by ClustalW and MAFFT in BioEdit and Unipro Ugene for genes and WGS, respectively. The phylogenetic tree was constructed using a maximum likelihood tree, with bootstrap value of 1000 in MEGA XI and the tree was saved in PNG format for inclusion in the study.

Quality control measures

Throughout each step of the experimental procedures, quality control measures were implemented. These included validation of PCR conditions, use of control samples in virus propagation, and verification of sequencing quality using FastQC.

RESULTS

Whole genome analysis

A total of 296 out of 385 samples tested positive for the *GPCR* gene using PCR analysis (Table 1). Subsequently, these positive samples were cultured in Vero cell lines to propagate the virus, and genomic material was extracted for whole genome sequencing (WGS).

Table I: LSDV positive samples collected from catle and confirmed by *GPCR* within Punjab Province.

District	Sample collected	Positive	Percentage
Bahawalpur	42	33	78.5%
Lahore	42	35	83.3%
Multan	42	26	62%
Dera Ghazi Khan	42	32	76%
Gujranwala	42	35	83.3%
Sahiwal	42	36	85.7%
Sargodha	42	30	71.4%
Rawalpindi	42	28	66.6%
Faisalabad	49	41	83.6%
Total	385	296	76.6%

The genome of the LSD viral isolate consists of 150,787 nucleotides (nt) with Adenine (A) 56761, Thymine (T) 54964, Guanine (G) 19885 and Cytosine(C). The evaluated Phred quality scores of sequence was (Q-score) of 30, and GC content of sequence was 25.9%. Annotation using Prokka revealed 155 Open Reading Frames (ORFs). Notably, our analysis identified one additional ORF compared to the reference sequences available on the NCBI nucleotide database, specifically at the 4th ORF position (Fig. 1 generated using Proksee). (https://proksee.ca/).

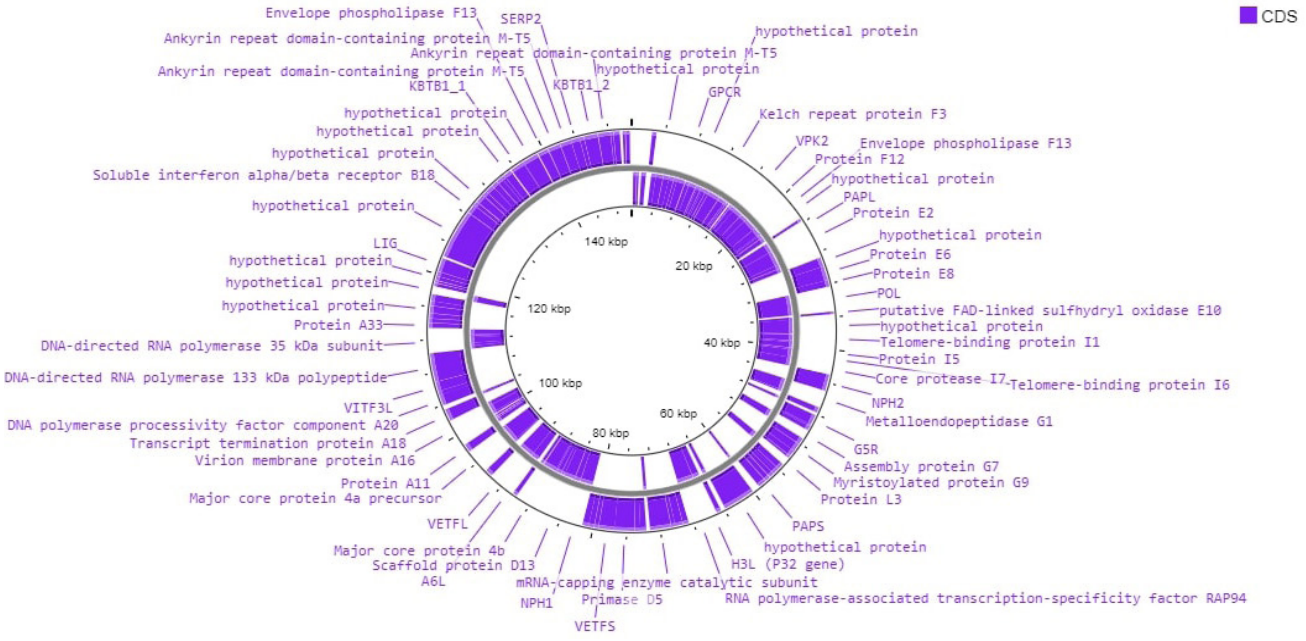

Fig. 1. Annotation of LSD by Prokka and visualized in Proksee. The circular diagram represents the annotated genome of LSDV. The outermost ring indicates coding sequences (CDS) in purple. Key genes and hypothetical proteins are labeled around the periphery of the circle. The innermost rings display various features of the genome, including the positions and density of the CDS.

The nucleotide blast analysis of the whole genome identified the closest relative of the isolate as LSDV NI-2490 (NC_003027.1), showing a high similarity of 99.94% with 16 nucleotide gaps and 98 nucleotide differences. Sequences with over 99.90% identity were identified in strains from Bangladesh, Serbia, India, Russia, and Kenya.

The *GPCR* gene (1146 nt) exhibited 100% similarity with strains associated with outbreaks in Pakistan, as well as in Kenya, India, Russia, Bangladesh, Myanmar, Nepal, and Morocco. The RNA polymerase subunit 30 kD (*RP030*) gene (606 nt) showed complete identity (100%) with sequences from isolates in India, Bangladesh, Morocco, Kenya, Nepal, and Myanmar. Similarly, the envelope protein (*P32*) gene (969 nt) demonstrated 100% homology with isolates from Turkey, India, Kazakhstan, Kenya, Russia, Greece, Serbia, Bangladesh, North Macedonia, Albania, China, Morocco, Bulgaria, and South Africa. These results highlight *RP030* as the most variable gene within LSDV and underscore its potential utility for evolutionary and epidemiological studies of the virus.

Phylogenetic analysis

In this study, we performed phylogenetic analysis using the WGS of the LSDV isolate, along with sequences of the *GPCR* (1146 bp), *P32* (969 bp), and *RP030* (606 bp) genes, in comparison to reference datasets from distinct clusters reported by Breman *et al*. (2023) and Manzoor *et al*. (2023).

Pairwise sequence comparison and phylogenetic analysis of the LSDV isolate's WGS are illustrated in Figure 2a. The sequence clustered with Clade 1.2 and exhibited the highest homology, with 99.94% similarity to the Neethling strain NI-2490 (NC003027) from Kenya, and 99.93% similarity to strains from Bangladesh (OP688129), India (OR393169, OR393175, OR393171), and Russia (**Table** II).

Table II: Data of strains used in phylogenetic analysis of whole genome and their percentage homology with field strain.

Accession Number	Strain Name	Country	Year	Clade	% homology
KX764644.1	Neethling-Herbiac vaccine	South Africa	1999	Clade 1.1	98.91%
KX764645.1	Neethling-LSD vaccine-OBP	South Africa	1988	Clade 1.1	98.91%
MG972412.1	Cro2016	Croatia	2016	Clade 1.1	98.91%
MH646674.1	LSDV/Russia/Saratov/2017	Russia	2017	Clade 2.1	99.10%
MH893760.2	LSDV/Russia/Dagestan/2015	Russia	2015	Clade 1.2.1	99.90%
MK441838.1	Strain Herbvac LS batch 008	South Africa	2019	Clade 1.1	98.91%
MN072619.1	Kenya	Kenya	2011	Clade 1.2.2	99.93%
MN642592.1	Kubash/KAZ/16	Kazakhstan	2016	Clade 1.2.1	99.91%
MT134042.1	LSDV/Russia/Udmurtiya/2019	Russia	2019	Clade 2.2	99.44%
MT643825.1	210LSD-249/BUL/16	Bulgaria	2016	Clade 1.2.1	99.91%
MT992618.1	KZ-Kostanay-2018	Kazakhstan	2018	Clade 2.3	99.38%
MW355944.1	China/GD01/2020	China	2020	Clade 2.5	99.22%
MW435866.1	SA-Neethling	South Africa	1959	Clade 1.1	98.91%
MW732649.1	LSDV/HongKong/2020	Hong Kong	2020	Clade 2.5	99.21%
MW883897.1	LSDV/Cattle/India/2019/Ranchi-1	India	2019	Clade 1.2.2	99.90%
MZ577073.1	20L42 Quyet-Thang/VNM/20	Vietnam	2020	Clade 2.5	99.22%
MZ577074.1	20L43 Ly-Quoc/VNM/20	Vietnam	2020	Clade 2.5	99.22%
MZ577075.1	20L70 Dinh-To/VNM/20	Vietnam	2020	Clade 2.5	99.22%
MZ577076.1	20L81 Bang-Thanh/VNM/20	Vietnam	2020	Clade 2.5	99.22%
OL542833.1	LSDV/Russia/Tyumen/2019	Russia	2019	Clade 2.4	99.32%
OM530217.1	LSDV/Russia/Saratov/2019	Russia	2019	Clade 2.1	99.25%
OM793602.1	LSDV Russia Tomsk 2020	Russia	2020	Clade 2.5	99.34%
OM793603.1	Russia Khabarovsk 2020	Russia	2020	Clade 2.5	99.34%
OM793608.1	Neethling-WC RSA 1957	South Africa	1957	Clade 1.1	98.92%
OM793609.1	Vaccine LW-1959 1988	South Africa	1988	Clade 1.1	98.91%

The phylogenetic analysis of the *GPCR* gene is displayed in Figure 2b, showing the isolate clustering within Clade 1.2. Pairwise sequence comparison (PASC) revealed 100% homology of the study isolate's *GPCR* gene with isolates from Bangladesh (2020: OM273507; 2021: OM273509, OM674668), Nepal (2020: OL689596, OL689600, OL689601), and India (2019: MW452650, MW452643, MW452639), with above 99% homology observed with isolates from Taiwan, Vietnam, and China (2020-2021), and above 98% homology with isolates from various countries including Egypt, Burkina Faso, South Africa, Iran, India, Greece, Sudan, Russia, Kazakhstan, Ethiopia, and Turkey. Additionally, the LSDV isolate showed above 96% homology with strains of Goat pox and Sheep pox used in the phylogenetic analysis details for sequence shown in Table III.

Table III: Data of strains used in phylogenetic analysis of *GPCR* gene and their percentage homology with field strain.

Accession Number	Strain Name	Country	Year	Clade	Per. Homology
AF409137.1	NW-LW isolate Neethling Warmbaths LW	South Africa	1957	Clade 1.2	98.95%
FJ869355.1	Goatpox virus Bangladesh/86 GTPV10	Bangladesh	1986	Clade 1.1	96.46%
FJ869382.1	Sheeppox virus Turkey/98 Corum SPPV19	Turkey	1998	Clade 1.1	96.28%
KX683219.1	KSGP 0240	Kenya	1974	Clade 1.2	100.00%
KX764643.1	SIS-Lumpyvax vaccine	South Africa	1999	Clade 1.1	97.99%
KX764644.1	Neethling-Herbiac vaccine	South Africa	1957	Clade 1.1	97.99%
KY829023.3	Evros/GR/15	Greece	2015	Clade 1.2	98.95%
MH646674.1	LSDV/Russia/Saratov/2017	Russia	2017	Clade 2.1	98.17%
MK441838.1	Herbivac LS	South Africa	2011	Clade 1.1	97.99%
MN072619.1	Kenya	Kenya	1958	Clade 1.2	100.00%
MN636843.1	LSD-148-GP-RSA-1997	South Africa	1997	Clade 1.1	97.91%
MN995838.1	pendik	Turkey	2014	Clade 1.2	98.95%
MT007950.1	Namibia 2016 9F	Namibia	2016	Clade 1.2	97.91%
MT130502.2	Neethling-RIBSP vaccine	Kazakhstan	2018	Clade 1.2	98.95%
MT643825.1	210LSD-249/BUL/16	Bulgaria	2016	Clade 1.2	98.95%
MT992618.1	KZ-Kostanay-2018	Kazakhstan	2018	Clade 2.3	99.48%
MW355944.1	China/GD01/2020	China	2020	Clade 2.5	99.13%
MW435866.1	SA-Neethling	South Africa	1959	Clade 1.1	97.99%
MW732649.1	LSDV/HongKong/2020	Hong Kong	2020	Clade 2.5	99.13%
MZ577073.1	20L42 Quyet-Thang/VNM/20	Vietnam	2020	Clade 2.5	99.13%
MZ577075.1	1.2070 Dinh-To/NVM/20	Vietnam	2020	Clade 2.5	99.13%
MZ577076.1	20L81 Bang-Thanh/VNM/20	Vietnam	2020	Clade 2.5	99.13%
OL542833.1	LSDV/Russia/Tyumen/2019	Russia	2019	Clade 2.4	98.52%
OM033705.1	LSDV/Thailand/YST/2021	Thailand	2021	Clade 2.5	99.13%
OM530217.1	LSDV/Russia/Saratov/2019	Russia	2019	Clade 2.1	98.17%
ON010590.1	Neethling-RIBSP/7C	Kazakhstan	2019	Clade 1.2	98.95%
ON152411.1	LSDV/72/PrachuapKhiriKhan/Thailand/2021	Thailand	2021	Clade 2.5	99.13%
ON400507.1	208/PVRNTVU/2020	India	2020	Clade 1.2	100.00%
ON616408.1	LSDV/NMG/2020	Mongolia	2020	Clade 2.5	99.13%
OP297402.1	LSDV-WB/IND/19	India	2019	Clade 1.2	100.00%
OP508345.1	China/Xinjiang/Cattle/Aug-2019	China	2019	Clade 2.5	99.13%
OP688129.1	V395.1	Bangladesh	2021	Clade 1.2	100.00%

The phylogenetic analysis of the *RP030* gene is shown in Figure 2c, placing the *RP030* gene sequence within Clade 2.2 of the whole genome clade system. The study isolate's *RP030* gene sequence falls within Clade 1.2, merging with Clade 2.2 as a sister clade. PASC analysis revealed the highest homology (above 99%) of the *RP030* sequence with isolates from Kazakhstan, South Africa, Russia, Greece, Sudan, Thailand, Vietnam, China, and Taiwan, while showing 100% identity with isolates from Pakistan, Kenya, India, Bangladesh, and Nepal during outbreaks from 2020 to 2023. The *RP030* gene also demonstrated 98.5% identity with Goat pox virus (MN072624, MN072623) and 97.23% homology with Sheep pox virus (KF495220) details for sequence (Table IV).

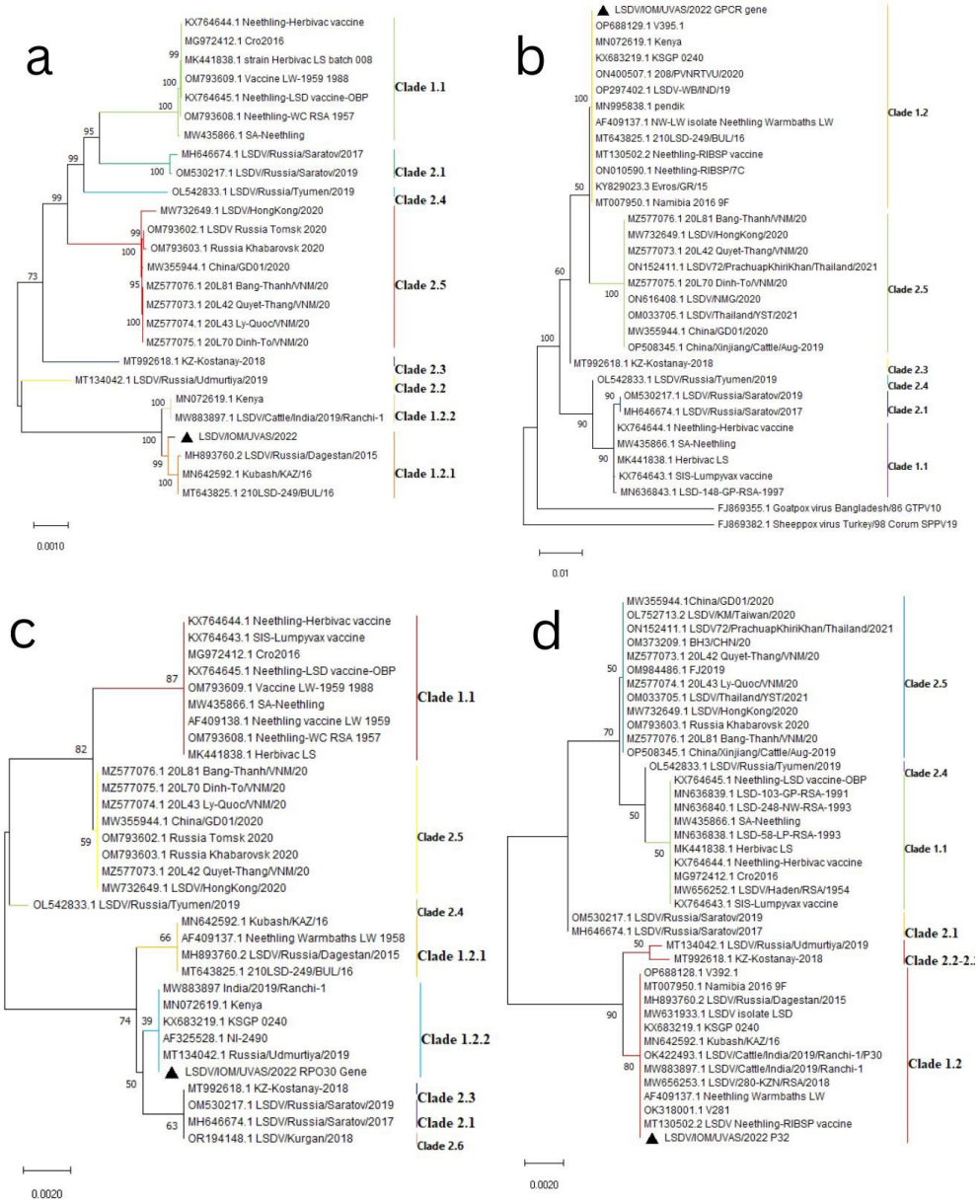

Fig. 2. Phylogenetic analysis of LSDV Isolates. This figure contains four panels (a, b, c, d) showing phylogenetic trees of LSDV isolates, (a) showing the phylogenetic tree of whole-genome of LSDV isolate, (b) Phylogenetic tree of *GPCR* gene showing clustering with clade 1.2, (c) showing the phylogenetic tree of *RP030* gene showing characteristic clustering with clade1.2.2, (d) Phylogenetic tree of *P32* gene clustering with 1.2 clade.

The phylogenetic analysis of the *P32* gene is presented in Figure 2d, indicating its placement within Clade 1.2. PASC analysis confirmed 100% identity of the *P32* gene sequence with isolates from Bangladesh, Namibia, Russia, Kenya, Kazakhstan, South Africa, Nigeria, and Morocco. Additionally, other strains exhibited above 99% similarity and originated from regions including Kazakhstan, Russia, Vietnam, Hong Kong, Thailand, China, Taiwan, South Africa, and Croatia details for sequence shown in Table V. *GPCR* (PP690778), *RP030* (PP690779), *P32* (PP690780) are the genes and their accession numbers submitted to NCBI GenBank.

Table IV: Data of strains used in phylogenetic analysis of *RP030* gene and their percentage homology with field strain.

Accession Number	Strain Name	Country	Year	Clade	Per. Homology
AF325528.1	NL-2490	Kenya	2006	Clade 1.2.2	100.00%
AF409137.1	Neethling Warmbaths LW 1958	South Africa	1958	Clade 1.2.1	99.83%
AF409138.1	Neethling vaccine LW 1959	South Africa	1959	Clade 1.1	98.84%
KX683219.1	KSGP 0240	Kenya	1974	Clade 1.2.2	100.00%
KX764643.1	SIS-Lumpyvax vaccine	South Africa	1999	Clade 1.1	98.84%
KX764644.1	Neethling-Herbiac vaccine	South Africa	2016	Clade 1.1	98.84%
KX764645.1	Neethling-LSD vaccine-OBP	South Africa	2016	Clade 1.1	98.84%
MG972412.1	Cro2016	Croatia	2016	Clade 1.1	98.84%
MH646674.1	LSDV/Russia/Saratov/2017	Russia	2017	Clade 2.1	99.83%
MH893760.2	LSDV/Russia/Dagestan/2015	Russia	2015	Clade 1.2.1	99.83%
MK441838.1	Herbivac LS	South Africa	2011	Clade 1.1	98.84%
MN072619.1	Kenya	Kenya	2019	Clade 1.2.2	100.00%
MN642592.1	Kubash/KAZ/16	Kazakhstan	2016	Clade 1.2.1	99.83%
MT134042.1	Russia/Udmurtiya/2019	Russia	2019	Clade 1.2.2	100.00%
MT643825.1	210LSD-249/BUL/16	Bulgaria	2016	Clade 1.2.1	99.83%
MT992618.1	KZ-Kostanay-2018	Kazakhstan	2018	Clade 2.3	99.83%
MW355944.1	China/GD01/2020	China	2020	Clade 2.5	99.17%
MW435866.1	SA-Neethling	South Africa	1959	Clade 1.1	98.84%
MW732649.1	LSDV/HongKong/2020	Hong Kong	2020	Clade 2.5	99.17%
MW883897.1	India/2019/Ranchi-1	India	2019	Clade 1.2.2	100.00%
MZ577073.1	20L42 Quyet-Thang/VNM/20	Vietnam	2020	Clade 2.5	99.17%
MZ577074.1	20L43 Ly-Quoc/VNM/20	Vietnam	2020	Clade 2.5	99.17%
MZ577075.1	20L70 Dinh-To/VNM/20	Vietnam	2020	Clade 2.5	99.17%
MZ577076.1	20L81 Bang-Thanh/VNM/20	Vietnam	2020	Clade 2.5	99.17%
OL542833.1	LSDV/Russia/Tyumen/2019	Russia	2019	Clade 2.4	99.50%
OM530217.1	LSDV/Russia/Saratov/2019	Russia	2019	Clade 2.1	99.83%
OM793602.1	Russia Tomsk 2020	Russia	2020	Clade 2.5	99.17%
OM793603.1	Russia Khabarovsk 2020	Russia	2020	Clade 2.5	99.17%
OM793608.1	Neethling-WC RSA 1957	South Africa	1957	Clade 1.1	98.84%
OM793609.1	Vaccine LW-1959 1988	South Africa	1988	Clade 1.1	98.84%
OR194148.1	LSDV/Kurgan/2018	Russia	2018	Clade 2.6	99.83%

Table V: Data of strains used in phylogenetic analysis of *P32* gene and their percentage homology with field strain.

Accession Number	Strain Name	Country	Year	Clade	Per. Homology
AF409137.1	Neethling Warmbaths LW	South Africa	1957	Clade 1.2	100.00%
KX683219.1	KSGP 0240	Kenya	1958	Clade 1.2	100.00%
KX764643.1	SIS-Lumpyvax vaccine	South Africa	1999	Clade 1.1	99.07%
KX764644.1	Neethling-Herbiac vaccine	South Africa	1999	Clade 1.1	99.07%
KX764645.1	Neethling-LSD vaccine-OBP	South Africa	1999	Clade 1.1	99.07%
MG972412.1	Cro2016	Croatia	2016	Clade 1.1	99.07%
MH646674.1	LSDV/Russia/Saratov/2017	Russia	2017	Clade 2.1	99.28%
MH893760.2	LSDV/Russia/Dagestan/2015	Russia	2015	Clade 1.2	100.00%
MK441838.1	Herbivac LS	South Africa	2011	Clade 1.1	99.07%
MN636838.1	LSD-58-LP-RSA-1993	South Africa	1993	Clade 1.1	99.07%
MN636839.1	LSD-103-GP-RSA-1991	South Africa	1991	Clade 1.1	99.07%
MN642592.1	Kubash/KAZ/16	Kazakhstan	2016	Clade 1.2	100.00%
MT007950.1	Namibia 2016 9F	Namibia	2016	Clade 1.2	100.00%
MT130502.2	LSDV Neethling-RIBSP vaccine	Kazakhstan	2018	Clade 1.2	100.00%
MT134042.1	LSDV/Russia/Udmurtiya/2019	Russia	2019	Clade 2.2-2.3	99.79%
MT992618.1	KZ-Kostanay-2018	Kazakhstan	2018	Clade 2.2-2.3	99.69%
MW355944.1	China/GD01/2020	China	2020	Clade 2.5	99.28%
MW435866.1	SA-Neethling	South Africa	1959	Clade 1.1	99.07%
MW631933.1	LSDV isolate LSD	Morocco	2017	Clade 1.2	100.00%
MW656252.1	LSDV/Haden/RSA/1954	South Africa	1954	Clade 1.1	99.07%
MW656253.1	LSDV/280-KZN/RSA/2018	South Africa	2018	Clade 1.2	100.00%
MW732649.1	LSDV/HongKong/2020	Hong Kong	2020	Clade 2.5	99.28%
MW883897.1	LSDV/Cattle/India/2019/Ranchi-1	India	2019	Clade 1.2	100.00%
MZ577073.1	1.20L42 Quyet-Thang/NVM/20	Vietnam	2020	Clade 2.5	99.28%
MZ577074.1	1.20L43 Ly-Quoc/NVM/20	Vietnam	2020	Clade 2.5	99.28%
MZ577076.1	1.20L81 Bang-Thanh/NVM/20	Vietnam	2020	Clade 2.5	99.28%
OK318001.1	V321	Nigeria	2018	Clade 1.2	100.00%
OK422493.1	LSDV/Cattle/India/2019/Ranchi-1/P30	India	2019	Clade 1.2	100.00%
OL542833.1	LSDV/Russia/Tyumen/2019	Russia	2019	Clade 2.4	99.17%
OL752713.2	LSDV/KM/Taiwan/2020	Taiwan	2020	Clade 2.5	99.28%
OM033705.1	LSDV/Thailand/YST/2021	Thailand	2021	Clade 2.5	99.28%
OM373209.1	BH3/CHN/20	China	2020	Clade 2.5	99.28%
OM530217.1	LSDV/Russia/Saratov/2019	Russia	2019	Clade 2.1	99.28%
OM793603.1	Russia Khabarovsk 2020	Russia	2020	Clade 2.5	99.28%
OM984486.1	FJ/2019	China	2019	Clade 2.5	99.28%
ON152411.1	LSDV/72/PrachuapKhiriKhan/Thailand/2021	Thailand	2021	Clade 2.5	99.28%
OP508345.1	China/Xinjiang/Cattle/Aug-2019	China	2019	Clade 2.5	99.28%
OP688128.1	V392.1	Bangladesh	2021	Clade 1.2	100.00%

DISCUSSION

LSD is recognized as a significant transboundary disease due to its potential spread across borders through animal trade, which poses implications for food security (Ahmad *et al*., 2023; Rossiter and Al Hammadi, 2009). Our study focused on the comprehensive analysis of the whole genome of LSDV and specific genes, including *RP030, P32* and *GPCR*, shedding emphasis on the molecular epidemiology and genetic diversity of LSDV circulating in Pakistan.

In Pakistan, limited research has been conducted on the molecular detection and phylogenetic analysis of LSD. Notably, Irshad *et al*. (2022) reported a low percentage (13.3%) of molecular positivity using PCR in a small sample size (n=15). However, recent studies, such as Ul-Rahman *et al*. (2022) utilized genes *RP030, P32*, and *GPCR* to characterize LSDV strains circulating in Pakistan, revealing clustering with strains from Asian, Middle Eastern, and African countries, including Thailand, China, India, and Saudi Arabia.

Our study significantly expands on these findings, demonstrating a higher positivity rate with 76.9% of samples testing positive for the *GPCR* gene (296/385 samples) by PCR. This type of reduction in positivity by PCR might because of breakage of cold chain and electricity load sheading which causes reduction in percentage positivity of samples by PCR. Furthermore, our LSDV isolate exhibited maximum sequence homology with strains from Kenya, India, Russia, Bangladesh, Myanmar, Nepal, and Morocco, underscoring the global dissemination and genetic diversity of LSDV strains. Until now there is no WGS published from Pakistan.

Phylogenetic analysis based on whole genome sequencing data revealed that our study isolates clustered within Clade 1.2, alongside strains reported from Bangladesh and India. This East African clade includes sequences from Kenya, highlighting potential transboundary transmission dynamics. Notably, subclade 1.2.2 encompasses strains specific to Indian and East African regions, while subclade 1.2.1 represents a Euroasian/African clade with strains from Kazakhstan and Europe. Clade 2.5 reported recombinant strains and strains from East and Southeast Asian countries (Thailand, China, Vietnam, Taiwan). Clade 1.1 and 2.5 are sister branches while 2.1 and 2.4 are sub-branched from Clade 1.1 and 2.5 (Breman *et al.*, 2023).

Pairwise alignment of our LSDV isolate's whole genome showed high similarity (99.94%) to the Neethling strain NI-2490 (NC003027) from Kenya and 99.93% to strains from Bangladesh and India (Putty *et al.*, 2023). Additionally, our isolate exhibited significant homology (>90.90%) with strains from India, Bangladesh, Russia, Serbia, Kenya, and Turkey, highlighting potential genetic relatedness and vaccine implications. This shows that the strain sequenced has greater homology to the vaccine neething strain of Kenya. Moreover Pakistan shares borders with India, China, Afghanistan, and Iran the uncontrolled movement of vectors, animals across the border and significantly influences the potential spread of viruses originating from neighboring countries (Hasib *et al.*, 2021; Sudhakar *et al*., 2022).

Furthermore, the *RP030* and *P32* gene analysis revealed clustering within Clade 1.2, with significant homology to strains from various countries, including Bangladesh, Namibia, Russia, Kenya, and India. PASC analysis highlighted the prevalence of identical or closely resembling LSDV strains in Pakistan, emphasizing the role of genetic similarity in the recurrence of LSDV outbreaks.

The pronounced genetic likeness observed in LSDV strains from diverse geographic areas, together with vaccine strains, supports the hypothesis that all capripox viruses have a shared genetic heritage and lineage (Tulman *et al.*, 2001). It is worth noting that the existing vaccines might offer sufficient protection against LSDV infection, as evidenced by previous studies (Klement *et al.*, 2020; Wolff *et al.*, 2021). This implies that vaccination initiatives could effectively manage LSDV outbreaks, especially in high-risk areas neighboring regions where outbreaks have been confirmed.

CONCLUSION

In conclusion, our study provides valuable insights into the molecular epidemiology and genetic diversity of LSDV in Pakistan. The widespread distribution and genetic relatedness of LSDV strains underscore the importance of international collaboration in disease control and surveillance efforts. Continued research and surveillance are essential to monitor the genetic drift in outbreak strains and develop effective vaccination strategies tailored to the specific strains prevalent in our region and neighboring countries. Moreover, our whole genome analysis of the local isolate provides updated information for studying LSDV and aids in the development of new vaccines. This research guides policymakers in taking appropriate steps to control future outbreaks by vaccinating the cattle population, controlling suspected routes of entry of the disease by blocking vectors, and properly implementing import regulations and quarantine protocols.

DECLARATIONS

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Ethical statement and IRB approval

The samples were collected during natural LSDV outbreaks across Pakistan as part of routine disease monitoring and control efforts. Institutional Review Board approval and ethical clearance were not required as the samples were collected under standard veterinary practices during outbreak investigations.

Statement of conflict of interest

The authors have declared no conflict of interest.

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