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Isolation and molecular characterization of Lumpy skin disease virus from cattle and the detection of anti-viral antibodies in buffaloes

SARITA YADAV[1], ASHOK BOORA[1✉], PARVINA DEVI[1], NISHU[1], NISHA VERMA[1], INDERJEET SINGH[2] and ANIL KUMAR[3]

ICAR–Central Institute for Research on Buffaloes, Hisar, Haryana 125 001 India

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Lumpy skin disease (LSD) is a re-emerging transboundary World Organisation for Animal Health (OIE) listed viral disease of cattle and water buffalo (Davies 1991). Lumpy skin disease virus (LSDV), is an enveloped virus, with a double-stranded DNA genome of 151-kbp size, from family Poxviridae (genus *Capripoxvirus*) (Tulman *et al.* 2001).The *Capripoxvirus* genus also comprises sheeppox virus (SPPV) and goatpox virus (GTPV), which show a high degree of similarity to LSDV (Abutarbush and Tuppurainen 2018). As per the official data, 3.3 million cattle (1,84,447 deaths) had been affected by LSD till 2023 (Kumar *et al.* 2023). Lumpy skin disease virus is primarily transmitted through blood-feeding insects vectors, such as stable flies, mosquitoes, and ticks, but can also spread through direct contact with infected animals or contaminated objects and infected semen (Gelaye and Lamien 2019). Infected animals may exhibit mild or no clinical signs to severe form progressing to fever, lymphadenopathy, skin nodules, ulceration, decreased appetite and decreased milk production (Bedeković *et al.* 2018). The P32 is a capripoxvirus specific structural protein homologous to P35 envelope protein of vaccinia virus, encoded by H3L gene localized in the virus envelope (Tulman *et al.* 2001). This major envelope protein, a cell surface binding protein of capriviruses contains neutralizing epitopes, is strongly antigenic and binds virus particle to virus attachment sites on surface of host cells (Heine *et al.* 1999).

India is the largest milk producing nation in the world, accounting for almost 20% of the global milk production. Dairy farming is an integral part of rural livelihoods in India, particularly for small and marginal farmers. Molecular epidemiology of the LSD is essential for understanding the spread and evolution of LSD virus, development of new treatments and control strategies and preventing future outbreaks. The present study aimed at the detection of antibodies against LSDV in buffaloes, which were showing mild nodular lesions on skin, a characteristic of LSD. This was followed by isolation and molecular characterization of the LSDV in cattle from field outbreaks in villages of Rajasthan under ICAR-Farmer FIRST Project and suspected LSD field outbreak in buffalo from Bathinda, Punjab during the period 2021 and 2022.

Samples from skin lesions were collected from cattle (n=4) and buffaloes (n=6) during LSD suspected outbreaks in three villages (Chhani Bari, Jhansal/Moda Khera and Biran Utarada) of Bhadra tehsil, Hanumangarh district, Rajasthan during 2021 and Bathinda, Punjab during 2022. The cattle were showing typical clinical signs of LSD such as fever, oculo-nasal secretions, corneal opacity, characteristic skin nodules, limb odema, anorexia, drop in milk production and general depression (Fig. 1). In contact, apparently healthy buffaloes were not showing any specific clinical signs other than deep seated mild nodules of 25-50 mm size, suspected of LSD. Skin lesion samples from affected cattle were easily taken, but deep seated mild skin nodules from buffaloes were taken using scalpel blade. Briefly, total DNA was extracted from the samples (skin lesion homogenates 10% w/v, cell cultures) with Dneasy Blood and Tissue kit (Qiagen) as per the manufacturer's instructions. The eluted DNA was screened for LSDV nucleic acid using World Organisation for Animal Health (WOAH) recommended p32 envelope protein gene (LSDV074) based capripoxvirus-specific PCR, amplifying the desired 192 bp region (Ireland and Binepal 1998). Lumpy skin disease virus DNA was detected in all the skin samples from cattle (n=4) and none of the samples from buffaloes (n=6) turned positive in capripoxvirus-specific PCR targeting LSDV074 gene. Isolation of the virus from PCR positive skin triturates in 10% Dulbecco's phosphate buffered saline was done by infecting Madin-Darby bovine kidney (MDBK) cells in 25 cm^2 culture flask as per the standard protocol. Characteristic cytopathic effects (CPE) were observed (n=4, cattle) 3-4 days post-infection followed by 3× blind passage (Fig. 2).

Present address: [1]ICAR-Central Institute for Research on Buffaloes, Hisar, Haryana. [2]Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. [3]Indian Council of Agricultural Research, New Delhi. ✉Corresponding author email: Ashok.boora@icar.gov.in; ashokcirb@gmail.com

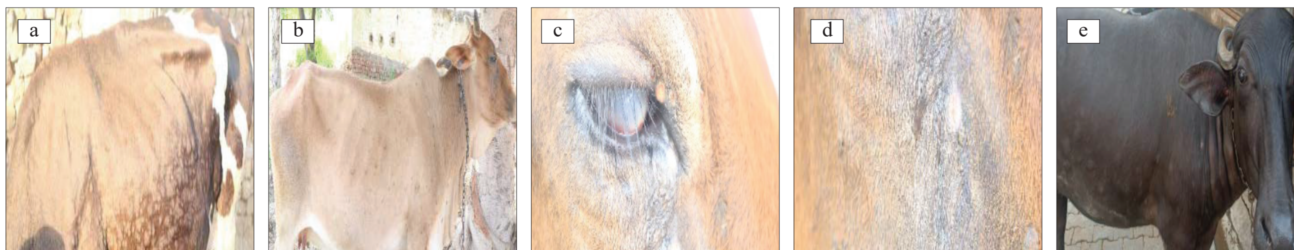
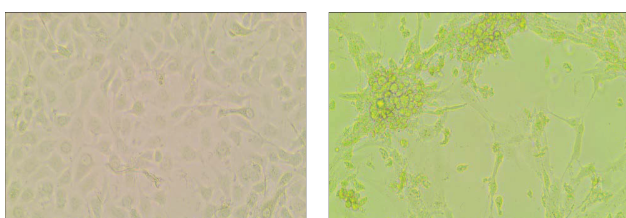


Fig. 1. Clinical signs in cattle and buffaloes suspected for LSD: (a,b) Typical skin nodules; (c) corneal opacity; (d) skin lesions ulceration and necrosis; (e) mild nodules on skin of buffaloes.

Further confirmation and identification of the virus isolates were done by amplifying LSDV specific partial fusion protein gene, LSDV 117 and full length RNA polymerase subunit, RPO30 gene (Sudhakar *et al.* 2020) (Fig. 3 A and B). The first set of primers, forward 5′-ACTAGTGGATCCATGGACAGAGCTTTATCA-3′ and reverse (5′-GCTGCAGGAATTCTCATAGTGTTG TACTTCG-3′) amplified 472 bp fragment of LSDV 117 (Ireland and Binepal 1998, Sudhakar *et al.* 2020) in all the four virus isolates. Further, a fragment of 1385 bp was amplified covering the complete open reading frame (ORF) 606 bp of RNA polymerase subunit, RPO30 gene (LSDV036) using second pair of primers: RPO30-1 (5′-CTCTGTTCCAAACTAAATCAT-3′) and RPO30-2 (5′-TTTTTGTATTACCAATTTCTG-3′) (Zhou *et al.* 2012). All the LSDV-negative samples from buffaloes were also found negative for BHV-1 and BHV-2 infection in PCR.



a. Mock-infected cells b. Virus-infected cells

Fig. 2. Isolation of LSDV in Madin-Darby Bovine Kidney (MDBK) cells from skin lesion of cattle.

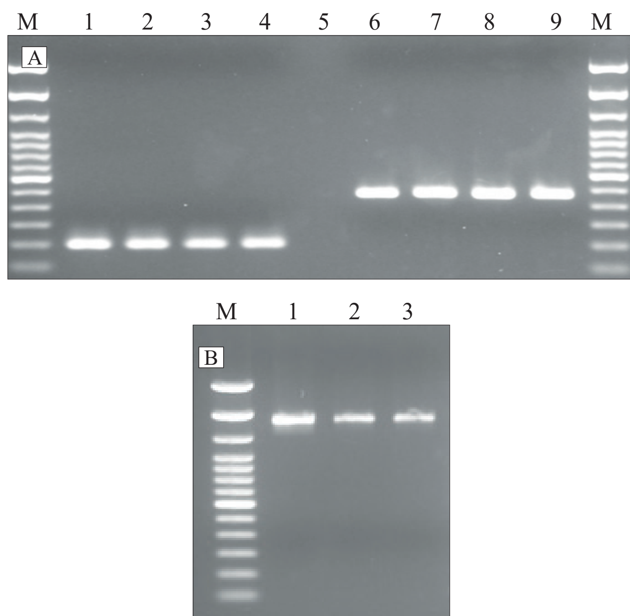


Fig. 3. (A) Agarose gel electrophoresis of PCR amplified gene product of LSDV074 gene (192 bp) in lane 1, 2, 3 and 4, M: 100 base marker, Lane 6, 7, 8, 9 for LSDV117 gene (472 bp) (B) LSDV RP030 gene/LSDV036 targeted by amplifying 1385 bp: Lane 1, 2 and 3, M: 100 base marker.

Virus neutralization assay was performed in buffalo sera samples in 96-well tissue culture plates using MDBK cell line as per standard protocol. Madin-Darby Bovine Kidney cells were seeded in 96-well plates to get 90% confluency. Serum samples from suspected buffaloes were diluted two-fold, in a volume of 50 µL in PBS. The diluted serum samples, negative serum, positive control, and negative control were pre-incubated with 50 µL of LSDV (10^4 PFU/ml) at 37°C for 1 h. The cells were then infected with the serum/virus mixture. Antibody titre was measured 48-96 h post-infection. The buffaloes with clinical manifestation of mild skin nodules showed an antibody titre of 1:16 to 1:64 (Table 1).

Though, buffalo is a susceptible host, only mild deep seated skin nodules suspected for LSD were reported from in-contact buffaloes under field condition in this study. None of the samples from buffalo were found positive for LSDV, BHV-1 and BHV-2 DNA by PCR. This was in agreement with the findings of (Elhaig *et al.* 2017). Though there were reports on LSDV in buffalo from Egypt (El-Nahas *et al.* 2011, Sharawi and Abd El-Rahim 2011). Davies (1991) reported that buffaloes did not show clinical

Table 1. Detection of LSDV DNA and anti-LSDV antibodies in clinical animals

Place	Species	LSDV DNA in skin scab*	Antibody titre
Chhani Bari	Cattle	Positive	NA
Jhansal/Moda Khera	Cattle	Positive	NA
Jhansal/Moda Khera	Cattle	Positive	NA
Biran Utarada	Cattle	Positive	NA
Chhani Bari	Buffalo	Negative	>1:64
Chhani Bari	Buffalo	Negative	1:32
Chhani Bari	Buffalo	Negative	1:32
Bathinda	Buffalo	Negative	1:16
Bathinda	Buffalo	Negative	1:16
Bathinda	Buffalo	Negative	>1:64

NA, serum not collected. *Capripoxvirus-specific PCR targeting p32 envelope protein gene (LSDV074).

Table 2. Pairwise comparison of nucleotide (nt) and amino acid (aa) sequence identities of RPO30 gene between the Indian isolate LSDV/India/RAJ/OP604055/2021 and other capripoxviruses

Description of sequence	Accession no.	LSDV/India/RAJ/OP604055/2023	
		Nucleotide sequence identity (%)	Amino acid sequence identity (%)
LSDV/SouthAfrica/Vaccine_LW/OM793609/1988	OM793609	98.8	99
LSDV/SouthAfrica/Neethling_vaccine_LW/AF409138	AF409138	98.8	99
LSDV/SouthAfrica/Herbivac/MK441838/2011	MK441838	98.8	99
LSDV/SouthAfrica/Lumpyvax_vaccine/KX764643/1999	KX764643	98.8	99
SPV/Nigeria/GU119924/1997	GU119924	93.6	93.5
SPV/Turkey/GU119916/1998	GU119916	93.6	93.5
GPV/Sudan/MN072624/2019	MN072624	98.5	97.5
GPV/Oman/MN072623/2019	MN072623	98.5	97.5
LSDV/Bulgaria/MT643825/2016	MT643825	99.8	99.5
LSDV/Russia/MH893760/2015	MH893760	99.8	99.5
LSDV/Serbia/KY702007/2016	KY702007	99.8	99.5
LSDV/China/MW355944/2020	MW355944	99.1	99.5
LSDV/HongKong/MW732649/2020	MW732649	99.1	99.5
LSDV/Vietnam/MZ577073/2020	MZ577073	99.1	99.5
LSDV/Thailand/ON152411/2021	ON152411	99.1	99.5
LSDV/India/Govindapalli/OM362831/2020	OM362831	100	100
LSDV/India/Kanakapura/OP903457	OP903457	100	100
LSDV/Kenya/MN072619/2019	MN072619	100	100
LSDV/India/WB/OP297402/2019	OP297402	100	100
LSDV/Mynamar/OM674463/2020	OM674463	100	100
LSDV/Nepal/OL689591/2020	OL689591	100	100
LSDV/India/ODI/MW452635/2019	MW452635	100	100
LSDV/Bangladesh/MT448692/2019	MT448692	100	100
LSDV/Russia/MT134042/2019	MT134042	100	100
LSDV/Israel/KX894508/2012	KX894508	99.8	99.5
LSDV/Kazakhstan/MN642592/2016	MN642592	99.8	99.5

form of LSD, although seroconverted.

Sequencing of full length RPO30 gene from one isolate, LSDV/India/Rajasthan/2021 was performed by Sanger's sequencing for further characterization and phylogenetic analysis. The nucleotide sequence was deposited in GenBank with accession number OP604055. Sequence alignment of full open reading frame (ORFs) of RPO30 gene sequence of Indian LSDV isolate of 2021, together with corresponding reference gene sequences of various capripoxviruses (LSDV field strains; goatpox and sheeppox strain retrieved from NCBI), was done using Clustal Omega for elucidating the phylogenetic analysis of these viruses. Best-fit nucleotide substitution model was calculated using JModel test (Posada 2008). PhyML program was used to construct Maximum Likelihood (ML) phylogenetic tree (Guindon and Gascuel 2003). Phylogenetic tree was edited in FigTree (Rambaut 2010) and Inkscape 0.48.4 (Inkscape1.2.2.) (Fig. 4). The results showed that RPO30 gene of LSDV/India/RAJ/OP604055/2021 sequenced as part of this study shared very close relationship with other LSDV isolated from different regions: nt/ aa sequence identities ranged from 98.8–100%/99-100% (Table 2). LSDV/India/Rajasthan/2021_OP604055 clustered with LSDV field strains from India, Kenya, Nepal, Bangladesh, Myanmar and Russia (LSDV/India/WB/OP297402/2019, LSDV/India/ODI/MW452635/2019, LSDV/India/Govindapalli/OM362831/2020, LSDV/Kenya/MN072619/2019, LSDV/Nepal/OL689591/2020, LSDV/Bangladesh/MT448692/2019, LSDV/Myanmar/OM674463/2020, LSDV/Russia/ MT134042/2019) sharing 100% nt and aa identities. Additionally, Indian LSDV strains clustered separately from the LSDV isolates from China, Vietnam, Hong Kong, Thailand and vaccine LSDV strains, sharing nt/aa sequence identities of 99.1/99.5 and 98.8/99%, respectively. This supports the conclusion that currently circulating field strains from India share a common ancestry with reference South African strain from Kenya (LSDV/Kenya/MN072619/2019), reflecting the circulation of same lineage in the Indian subcontinent. This is in accordance with the findings of Kumar *et al.* (2021) and Sudhakar *et al.* (2020).

Due to large genome size, only one gene is not sufficient to interpret the genetic relatedness of LSDV strains. The whole genome sequencing and analyses of the entire LSDV genome will greatly help in understanding the geographic origin, lineage and contribute further to molecular epidemiological study of Indian LSDV strains. To conclude, the present findings will contribute information to the

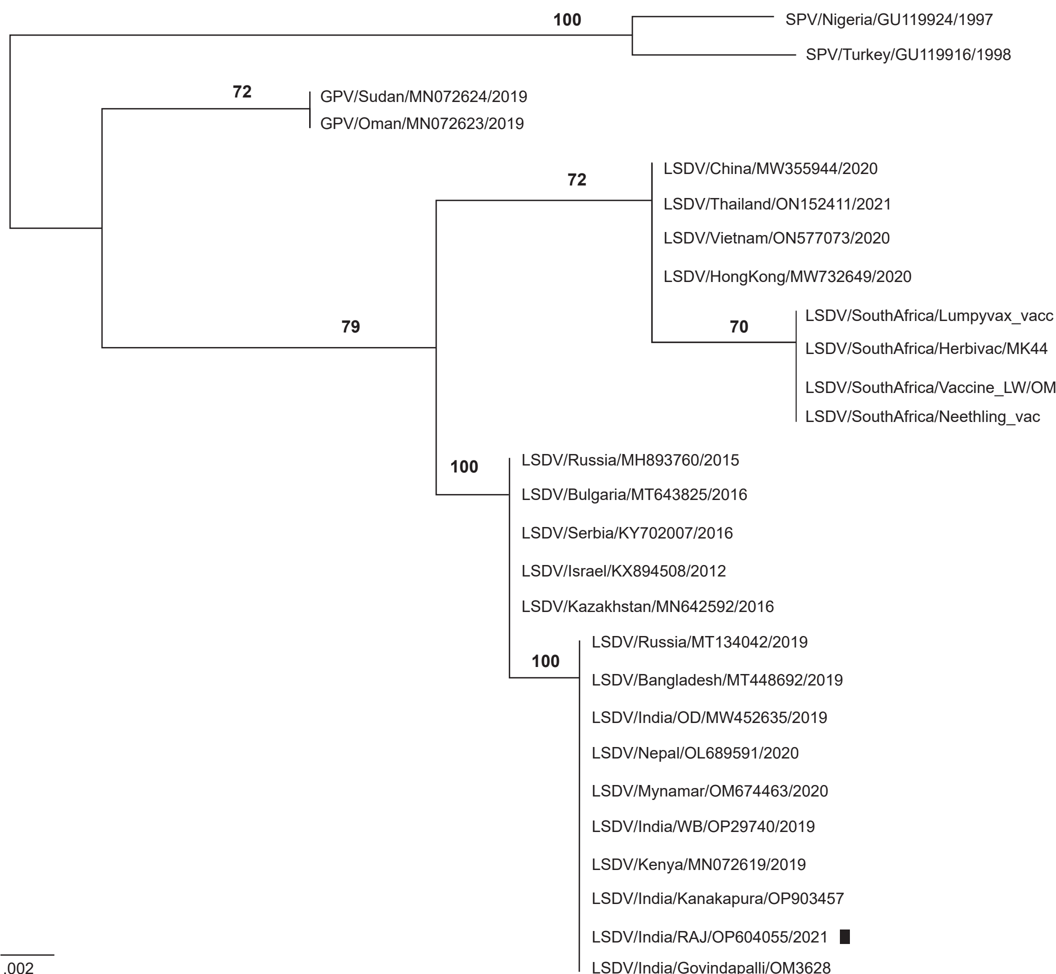


Fig. 4. Maximum likelihood (ML) unrooted phylogenetic tree of coding sequences of RPO30 gene comparing LSDV/India/RAJ/OP604055/2021 isolate (OP604055) with vaccine and field LSDV strain, goatpox and sheeppox strains from around the world. The Indian LSDV strain analysed in this study is shown in maroon colour and marked by a bold rectangle. Scale bar, 0.002 nucleotide substitutions per site.

currently available data on LSDV and warrants further molecular epidemiological study of the LSD disease in India, so that effective implementation measures can be adopted for the control and prevention of the disease.

SUMMARY

In the present study, four LSDV field strains were isolated from cattle in Bhadra tehsil, Hanumangarh district, Rajasthan under ICAR-Farmer FIRST Project and Bathinda, Punjab during the period 2021 and 2022. Apart from the characteristic clinical signs of LSD, the viral etiology was confirmed by PCR targeting LSDV074, LSDV 117 and LSDV036 genes, in addition to characteristic clinical signs LSD in cattle. Though, buffalo is a susceptible host, only mild deep seated skin nodules suspected for LSD were reported from in-contact buffaloes under field condition in this study. None of the samples from buffalo were found positive for LSDV, BHV-1 and BHV-2 DNA by PCR. Buffaloes exhibiting mild clinical signs in the current study showed an antibody titre of 1:16 to 1:64 by virus neutralization assay. No information is available regarding the seroprevalence of LSD in buffalo from India, therefore, it is imperative to further investigate the susceptibility of buffalo to LSDV infection. Based on the phylogenetic analysis of the ORF036 gene sequence, LSDV/India/RAJ/OP604055/2021 was in close relationship with LSDV from India, Kenya, Nepal, Bangladesh and Myanmar as they clustered together as a sub-cluster.

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