RESEARCH ARTICLE

Molecular Detection and Genetic Analysis of Lumpy Skin Disease Virus (LSDV) in India

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Abstract

Lumpy skin disease (LSD) is a contagious trans-boundary viral disease of cattle, caused by lumpy skin disease virus (LSDV) under the genus Capripoxvirus of Poxviridae family. The genus Capripoxvirus also comprises goatpox virus (GTPV) and sheeppox virus (SPPV), which affect sheep and goat respectively and are antigenically similar to LSDV. LSD has recently been spread in Asia following outbreaks in the Middle East and Europe. The present study is aimed with the objective of molecular characterization and genetic analysis of LSD virus obtained from the infected cattle in Puducherry, India. PCR confirms the presence of the LSDV genome in the clinical sample. Phylogenetic analysis and complete assessment of multiple sequence alignments between LSDV, GTPV and SPPV P32 gene sequences revealed least variation between sheeppox virus and LSD virus (1.5% variation) and highest variation between sheeppox and goatpox virus (2.8% variation). Based on the present study, it is also confirmed and concluded that P32 gene can be used for differentiating LSD, sheeppox and goatpox viruses. The phylogenetic analysis revealed that the LSDV under this study was clustered in a separate clade with the other LSDV sequences of Turkey, Kazakhstan, Kenya, Russia, Serbia, Nigeria and other Indian LSDV sequences available in the GenBank. The query LSDV sequence was closely related with other LSD viruses circulating in India suggesting a single LSDV strain is circulating in the country. This result underlines the importance of continuous monitoring and characterization of circulating strains.

Key words: Lumpy skin disease virus (LSDV), Capripoxvirus, P32 gene, Polymerase chain reaction, Sequence analysis, Phylogenetic analysis.

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Introduction

Lumpy skin disease (LSD) is a trans-boundary viral disease affecting cattle, caused by lumpy skin disease virus (LSDV). LSD virus is an enveloped, ovoid, linear, double-stranded DNA virus classified under the genus Capripoxvirus of *Poxviridae* family (Yilmaz *et al*., 2017). The genus Capripoxvirus also comprises goatpox virus (GTPV) and sheeppox virus (SPPV), which affect sheep and goats respectively and also are antigenically similar to LSDV (Lojkic *et al.,* 2018). Capripoxviruses are cross-reactive within the genus; therefore SPPV- or GTPV-based vaccines have been used to provide cross protection against LSDV (Abutarbush *&* Tuppurainen, 2018). LSD is a notifiable disease by the World Organization for Animal Health (OIE) because of its potential rapid spread and substantial economic losses to the livestock industry. LSD was first reported in Zambia in 1929 (MacDonald, 1931) and spread rapidly in the cattle population across African countries (Tuppurainen and Oura, 2012). Until 1984, LSD was maintained within the countries of sub-Sahara Africa. The first confirmed transcontinental spread of LSD from the Africa was reported in Israel in 1989 (Yeruham *et al.,* 1995). In India, the disease was first reported in November 2019 (Sudhakar *et al*., 2020).

The occurrence of LSD causes significant economic loss with the decreased milk production, permanent damage of hides, and loss of draft, thus constitute a serious hazard to the food security of the people in the affected areas (CABI, 2019). LSDV has a narrow host range and does not infect non-ruminant hosts (Shen *et al*., 2011). Clinically, LSD has been reported only in cattle. Even in close contact with infected cattle, sheep and goats never developed LSD (Davies, 1991). The high concentrations of virus in the skin may also contribute to the spread of LSDV via insect vectors (Bowden *et al*., 2008). The clinical picture starts with fever (40–41.5˚C), which persists for 1–3 days. Nodular dermatitis, a common feature appears in the skin of LSD infected cattle within 1–2 days, which gradually become harder and necrotic thereby inducing severe discomfort, pain and lameness

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(Edelsten, 2014). This is accompanied by increased nasal and pharyngeal secretions, lachrymation, enlargement of lymph nodes, anorexia, dysgalactia, general depression and a disinclination to move (Tasioudi *et al*., 2016). Although the mortality rate is usually low (1–5%), the morbidity rate can be as high as 100% (Casal *et al.,* 2018). The morbidity rate varies according to the immune status of animals and frequency of mechanical vectors.

Clinical history, clinical signs, and symptoms of infected animals can be used to make a tentative LSD diagnosis. The serological diagnostic test cannot distinguish SPPV, GTPV and LSDV. Hence identification of these pathogens needs molecular based test such as polymerase chain reaction (Hosamani *et al*., 2004). The confirmation of LSD depends upon the conventional or real-time PCR specific for Capripoxvirus (Alemayehu *et al*., 2013). The skin lesions give more positive result in PCR than the blood or septic viscera due to the greater load of viral particles sheltered in the nodule (OIE, 2021). There are 49 genes that are conserved in all pox viruses including capripox viruses (Gubser *et al*., 2003). SPPV, GTPV and LSDV genomes are approximately 151 kbp and are strikingly similar to each other, exhibiting 96% nucleotide identity over their entire length. P32 is the major immunodominant gene having a size of 969 bp in LSDV and GTPV and 972 bp in SPPV (Chand *et al*., 1994). Also P32 gene sequencing data is widely used to differentiate SPPV, GTPV and LSDV and phylogenetic analysis of capripox viruses (Zhou *et al*., 2012; Bhaswanth *et al*., 2020).

The emergence of increased incidence of LSDV outbreaks in recent years makes it mandatory to understand and analyze the prevalent circulating LSDV strains. This requires constant monitoring and characterization of LSDV field strains. Therefore, the present study was aimed with the objective of characterizing the LSDV field strains. Comparative analysis with the goatpox and sheeppox virus strain and phylogenetic relationship based on P32 gene sequences were also carried out.

Materials and Methods

Clinical sample

The scab materials from the nodular lesions over the skin were collected from a suspected outbreak of LSDV in Puducherry, India with the clinical signs of fever, mucopurulent nasal discharges and widespread nodular skin lesions over the body. The scab materials were pooled and collected in phosphate buffer saline (PBS) with antibiotics and transported on ice to the laboratory. They were grounded up in approximately 4 mL PBS containing Penicillin-Streptomycin antibiotics using pestle and mortar. The suspension was clarified by centrifugation at 10,000 g for 15 min and subjected to DNA extraction using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH), according to the manufacturer's instructions.

Amplification of the P32 Gene and Analysis of the Amplicon

The extracted DNA from the samples was amplified for P32 gene by polymerase chain reaction of LSDV using the primer pair A95: CACGGATCCATGGCAGATATCCCATTA and B7: AACAAGCTTACTCTCATTGGTGTTCGG, which amplified a fragment of 1006 bp for LSDV and GTPV and 1009 bp for SPPV covering the entire stretch of P32 gene (Hosamani *et al.,*2004). The PCR reaction mix contained 100 ng template DNA, 5µL 10X PCR buffer, 2 mM MgCl2, 2 µL of 20 mM dNTPs, 10 µM of forward and reverse primers, 2U of Taq DNA Polymerase (New England Bio Labs) and the volume was made up to 50 µL with Nuclease Free water (NFW). The thermocycling conditions were as follows: 5min at 94^{o}C (initial denaturation), 35 cycles of 1 min at 94^{o}C (denaturation), 1 min at 56^{o}C (annealing temperature), 1 min at 72^{o}C (extension), followed by final extension (72^{o}C for 10 min) and hold at 4^{o}C. The amplified products were confirmed by resolving at 1.5% agarose gel electrophoresis and visualized under UV transilluminator (Syngene, U.K). Then the confirmed PCR products in the gel were excised for Gel extraction for further sequencing and sequence analysis.

Sequencing and Sequence Analysis

The amplified PCR products from the clinical samples were gel extracted and sent for singlepass sequencing. The primer pair A95/B7 was used for sequencing that covers the P32 gene of the goatpox virus previously isolated and stored in the Department of VMC, RIVER, Puducherry. The amplified products were sent for custom sequencing for both direction (5'-3' and 3'-5') using the automated sequencer, Applied Biosystem 3100. The specificity of the sequences with respect to the P32 gene of goatpox virus was determined using BLAST [Basic Local Alignment Search Tool] (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Further, the query nucleotide sequences were aligned with corresponding P32 gene of LSDV, GTPV and SPPV sequences available in GenBank using multiple alignment program, Clustal Omega (http://www.ebi.ac.uk/clustalomega/). For sequence alignment analysis, the sequences representing the full P32 gene were compared with those available in the GenBank databases of National Centre for Biotechnology Information.

Phylogenetic Analysis

The phylogenetic relationship based on the nucleotide sequences of the P32 gene of LSDV, GTPV and SPPV sequences was analyzed. From the aligned query nucleotide sequences, the phylogenetic tree was constructed with various LSDV, GTPV and SPPV P32 gene sequences obtained from different parts of the world (GenBank) with MEGA11 program using the Maximum Likelihood method based on the Tamura-Nei model (Tamura *et al.,* 2021).

Results and Discussion

Amplification of P32 Gene

The PCR amplification of P32 gene was carried out as per standard protocol. The scab material screened for LSDV was found positive by PCR. Skin lesions yield more positive results in PCR than other samples due to the greater load of virus sheltered in the nodule (OIE, 2021). The LSDV suspected scab materials, GTPV field strain (MN688757) and SPPV vaccine (positive control) yielded an approximately 1000 bp products as depicted in Fig 1.

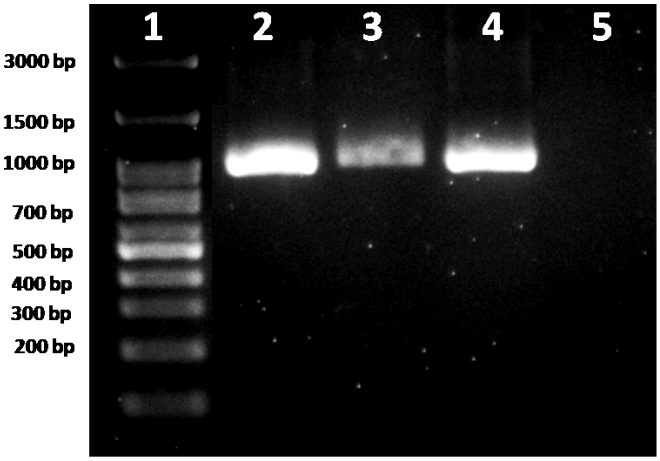

Fig 1: Agarose gel electrophoresis: The PCR amplification showing approximately 1000 bp amplicon targeting P32 gene using A95 and B7 primer pair. **Lane 1** - DNA Marker, **Lane 2** – Sheeppox commercial vaccine used as positive control with size of 1009 bp, **Lane 3**- GTPV field strain (MN688757) with size of 1006 bp, **Lane 4** - LSDV field samples with size of 1006 bp and **Lane 5** – Negative control showing no band.

Sequencing and Sequence Analysis

Upon Basic Local Alignment Search Tool (BLAST) analysis and the sequence alignment, the specificity of the sequence of P32 gene of the clinical sample was found to be maximally identical (98-99%) with P32 gene of Lumpy skin disease virus strains available in the GenBank. The sequence data of the clinical sample was checked for quality by chromatograph using Snapgene software version 4.2. The sequences were edited and the contigues were generated using the ClustalW program of Mega 7.0. The sizes of the contigues obtained were 969 bp for the LSD and Goat pox field strain and 972 bp for sheeppox vaccine. The aligned sequence of the P32 gene of LSD virus under this study were deposited in the Genbank, National Centre for Biotechnology Information (NCBI) Database and was assigned GenBank accession numbers MW815879.

The aligned amino acid variations of the query and reference sequences available in the Genbank are depicted in the Table 1. Eight non-synonymous mutation were observed at amino acid positions Gly26→Asp, Lys46→Asn, Leu49→Phe, Val93→Ala, Tyr136→His, Met290→Ile, Asn305→Asp and Val323→Ile accounting for 2.5% variation (i.e. 97.5% homology) at P32 gene between the LSDV sequences (MW815879), the GTPV sequences (MN688757, MN688758, MN688759) characterized in our previous study and few other GTPV sequences of India and the neighboring countries. Similarly, five non-synonymous mutations were observed at amino acid positions Leu49→Phe, Leu62→Phe, Ser132→Leu, Ile134→Thr and Asn305→Asp accounting for 1.5% variation (i.e. 98.5% homology) between the LSDV sequences (MW815879) and the SPPV sequences of Indian and other countries (MG000157, MW167070, FJ882029, MH198040, MN072629). Aspartic acid at the 54th amino acid position was absent in the LSDV sequences as expected. Two non-synonymous mutations were observed at amino acid positions Val98→Ala and Ile274→Val accounting for 0.6% variation (i.e. 99.4% homology) at P32 gene between LSD vaccine strain (KX764643) and the LSDV field strain under study. In contrast, nine non-synonymous mutation were observed at amino acid positions Asp26→Gly, Asn46→Lys, Leu62→Phe, Ala93→Val, Ser132→Leu, Ile134→Thr, His136→Tyr, Ile290→Met and Ile323→Val accounting for 2.8% variation (i.e. 97.2% homology) at P32 gene between SPPV vaccine strain and the GTPV field strains identified in our previous study. In addition, Aspartic acid at the 54th amino acid position were also absent in the GTPV sequences. The aligned amino acid variations of the query and the reference sequences available in the Genbank are depicted in the Table 1. Between LSDV, GTPV and SPPV P32 gene sequences; least variation was observed between the sheeppox virus and the LSD virus (1.5% variation) and highest variation was observed between the sheeppox and the goatpox virus (2.8% variation).

Phylogenetic Analysis

Furthermore, the sequencing data were used for phylogenetic analysis by comparing them with various goatpox and sheeppox virus sequences including vaccine strains retrieved from GenBank to elucidate the genetic relatedness of this virus (Fig 2). The phylogenetic analysis revealed that our LSDV query sequence was clustered within subgroup I together in a seperate clade with the P32 gene LSDV sequences of Turkey, Kazakhstan, Kenya, Russia, Serbia, Nigeria and other Indian LSDV sequences available in the GenBank. The LSDV sequences from China, Hong Kong, Vietnam, Taiwan, Saudi Arabia and some other Russian sequences are segregated from the Indian isolates, clustering within subgroup II. The previous studies have demonstrated that the LSDV virus strains could be divided into subgroups (Badhy *et al.,* 2021). This infection is mainly transmitted mechanically by arthropods and also spread across countries and continents by the live animal movements (Sprygin *et al.,* 2019). This potentiates the further spread of LSD across geography which necessitates the need increased surveillance and monitoring. The query sequence was closely related with other LSD viruses circulating in India suggests that a single LSDV strain is

Table 1: The amino acid sequence variation at P32 gene of the LSD query sequence, Goat-pox field strains and other reference sequences available in the Genbank.

	Place of Collection	GenBank Accession ID	Aminoacid position at P32 gene															
			26	46	49	54	62	93	98	115	132	134	136	156	274	290	305	323
LSDV	LSD Vaccine Strain Lumpyvax	KX764643	Asp	Asn	**Phe**	-	Phe	Ala	**Val**	Ser	Leu	Thr	His	Phe	**Ile**	Ile	**Asp**	Ile
	Puducherry India	**MW815879**	Asp	Asn	**Phe**	-	Phe	Ala	Ala	Ser	Leu	Thr	His	Phe	Val	Ile	**Asp**	Ile
	WB India	OK422493	Asp	Asn	**Phe**	-	Phe	Ala	Ala	Ser	Leu	Thr	His	Phe	Val	Ile	**Asp**	Ile
	Ranchi India	MW452625	Asp	Asn	**Phe**	-	Phe	Ala	Ala	Ser	Leu	Thr	His	Phe	Val	Ile	**Asp**	Ile
	ODI India	MW452622	Asp	Asn	**Phe**	-	Phe	Ala	Ala	Ser	Leu	Thr	His	Phe	Val	Ile	**Asp**	Ile
	Kenya	MN072619	Asp	Asn	**Phe**	-	Phe	Ala	Ala	Ser	Leu	Thr	His	Phe	Val	Ile	**Asp**	Ile
	Nigeria	OK318001	Asp	Asn	**Phe**	-	Phe	Ala	Ala	Ser	Leu	Thr	His	Phe	Val	Ile	**Asp**	Ile
	Russia	OM793602	Asp	Asn	**Phe**	-	Phe	Ala	**Val**	Ser	Leu	Thr	His	Phe	Val	Ile	**Asp**	Ile
	Taiwan	OL752713	Asp	Asn	**Phe**	-	Phe	Ala	**Val**	Ser	Leu	Thr	His	Phe	Val	Ile	**Asp**	Ile
GTPV	GTPV Vaccine Strain	KX576657	**Gly**	**Asn**	Leu	-	Phe	**Val**	Ala	Ser	Leu	Thr	**Tyr**	Phe	Val	**Met**	Asn	**Val**
	Puducherry India	**MN688757**	**Gly**	Lys	Leu	-	Phe	**Val**	Ala	Ser	Leu	Thr	**Tyr**	Phe	Val	**Met**	Asn	**Val**
	Puducherry India	**MN688758**	**Gly**	Lys	Leu	-	Phe	**Val**	Ala	Ser	Leu	Thr	**Tyr**	Phe	Val	**Met**	Asn	**Val**
	Puducherry India	**MN688759**	**Gly**	Lys	Leu	-	Phe	**Val**	Ala	Ser	Leu	Thr	**Tyr**	Phe	Val	**Met**	Asn	**Val**
	Tamilnadu India	KY508697	**Gly**	Lys	Leu	-	Phe	**Val**	Ala	Ser	Leu	Thr	**Tyr**	Phe	Val	**Met**	Asn	**Val**
	Mukteswar India	AY159333	**Gly**	Lys	Leu	-	Phe	**Val**	Ala	**Pro**	Leu	Thr	**Tyr**	**Ser**	Val	**Met**	Asn	**Val**
	Maharastra India	FJ748488	**Gly**	Lys	Leu	-	Phe	**Val**	Ala	Ser	Leu	Thr	**Tyr**	Phe	Val	**Met**	Asn	**Val**
	China	EF522176	**Gly**	Lys	Leu	-	Phe	**Val**	Ala	Ser	Leu	Thr	**Tyr**	Phe	Val	**Met**	Asn	**Val**
	Iran	MK948083	**Gly**	**Asn**	Leu	-	Phe	**Val**	Ala	Ser	Leu	Thr	**Tyr**	Phe	Val	**Met**	Asn	**Val**
SPPV	SPPV RF Vaccine Strain	MG000157	Asp	Asn	Leu	**Asp**	**Leu**	Ala	Ala	Ser	**Ser**	**Ile**	His	Phe	Val	Ile	Asn	Ile
	Jammu & Kashmir India	MW167070	Asp	Asn	Leu	**Asp**	**Leu**	Ala	Ala	Ser	**Ser**	**Ile**	His	Phe	Val	Ile	Asn	Ile
	Pune India	FJ882029	Asp	Asn	Leu	**Asp**	**Leu**	Ala	Ala	Ser	**Ser**	**Ile**	His	Phe	Val	Ile	Asn	Ile
	Karnataka India	MH198040	Asp	Asn	Leu	**Asp**	**Leu**	Ala	Ala	Ser	**Ser**	**Ile**	His	Phe	Val	Ile	Asn	Ile
	Turkey	MN072629	Asp	Asn	Leu	**Asp**	**Leu**	Ala	Ala	Ser	**Ser**	**Ile**	His	Phe	Val	Ile	Asn	Ile

circulating in the country at present. Continuous monitoring of LSDV outbreak and complete genome characterization (Full genome sequencing) need to be studied for devising effective control measures.

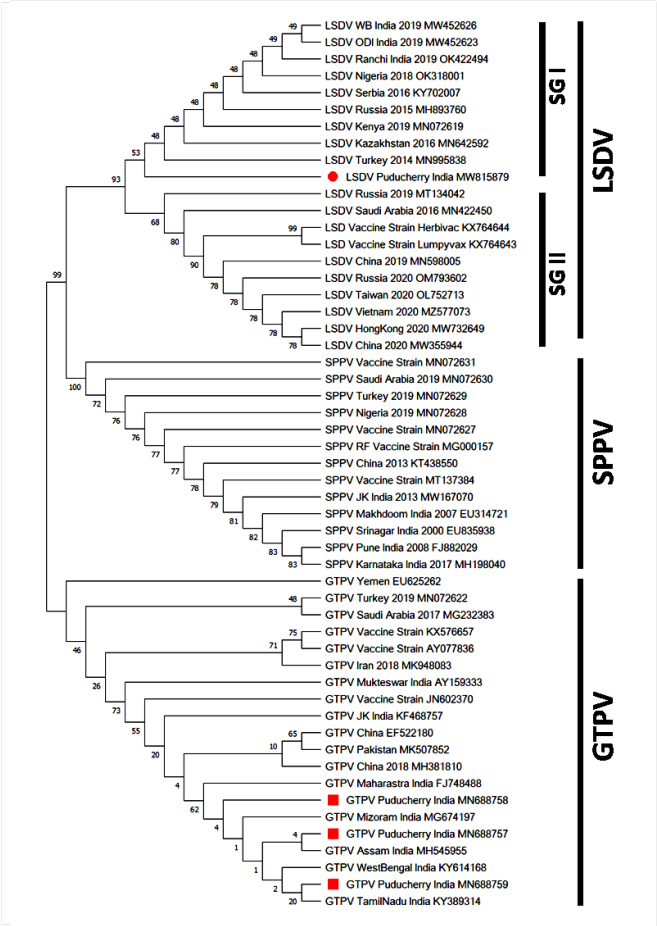

Fig. 2: Phylogenetic analysis: The evolutionary history was inferred by using the Maximum Likelihood tree based on the Tamura-Nei model. The LSD query sequence (MW815879) shown with solid circle, Goatpox field strains (MN688757, MN688758 & MN688759) with solid square and other reference sequences available in the Genbank. Bootstrap values are shown next to the branches in the phylogenetic tree.

Conclusion

Based on the present study, it also confirmed and concluded that P32 gene can be used for differentiating LSD, sheeppox and goatpox viruses. Based on phylogenetic analysis and detailed inspection of multiple sequence alignments between P32 immunodominant gene sequences; least variation was observed between sheeppox virus and LSD virus. The phylogenetic analysis revealed that the LSDV under this study was clustered in a separate clade with the other LSDV sequences of Turkey, Kazakhstan, Kenya, Russia, Serbia, Nigeria and other Indian LSDV sequences available in the GenBank. It was also shown that the query sequence was also closely related with other LSD viruses circulating in India suggesting a single LSDV strain is circulating in the country. This result underlines the importance of continuous monitoring and characterization of circulating strains.

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