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Molecular detection, Isolation and characterization of lumpy skin disease virus in Andhra Pradesh, India

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Abstract
Lumpy Skin Disease (LSD) is an emerging transboundary disease in India caused by LSD virus that shares genetic relatedness with sheep pox and goat pox viruses in the genus *capripox* virus. In the recent past, India has experienced LSD outbreaks in several states. The current study deals with the recent LSD outbreaks (2022) from cattle in Andhra Pradesh (AP). A total of 89 outbreaks were recorded in AP from various districts. *Capripox* genus specific (P32 gene) PCR was used for identification of the disease. Out of a total number of 868 clinical samples tested, 178 samples were positive for LSDV genomic DNA with 20.51 percentage positivity. From the positive samples, 21 samples representing different geographical regions of the state were processed for isolation of virus in MDBK cell line and LSDV was isolated from 12 samples. Specific CPE was observed at 3rd blind passage in MDBK cell line. The virus was also adapted to vero cells wherein CPE was observed within the first adaptation passage. The isolates were confirmed as LSDV by ORF036 and ORF011 LSDV specific PCRs yielding 606 bp and 1134 bp PCR products respectively. The PCR product of the partial ORF036 gene from Vizianagaram district isolate was sequenced and phylogenetic analysis was performed. The isolate clustered closely with Indian isolates from Tamil Nadu, Karnataka, Haryana and Rajasthan states and also with field isolates from Bangladesh, Myanmar, and Nigeria. It clustered away from the LSDV Neethling vaccine strains, strains from Kenya (1958), heterologous vaccine strains and some Indian strains of 2019 outbreak.

Keywords: Lumpy skin disease virus (LSDV), lumpy skin disease (LSD), Andhra Pradesh, virus isolation, phylogenic analysis

Introduction
The lumpy skin disease virus (LSDV), which is a member of *capripox* genus in the Poxviridae family, causes lumpy skin disease (LSD) in cattle. Due to its significant economic effect and potential for rapid spread, the World Organization for Animal Health (OIE) has classified it as notifiable. (Tuppurainen *et al.*, 2017) [17] The disease was first observed in Zambia in 1929 (Kumar *et al.*, 2021) [8] and later in other African countries like Kenya in 1957. The first transboundary spread of the diseases from Africa to middle east Asia was reported in Israel in 1989. In July 2019, the disease first appeared in South Asian countries like Bangladesh, India and China. India reported its first lumpy skin disease outbreak in August 2019 in Odisha (Sudhakar *et al.*, 2020) [15]. The clinical manifestation of disease includes pyrexia which persists for 1-3 days (OIE. 2018) [12], increased nasal and pharyngeal secretions, lacrimation, anorexia, dysgalactia, pneumonia, enlargement of lymph nodes and eruption of nodules on the skin, which may cover entire body. Lesions are often found in the mouth and upper respiratory tract. The severity of the disease varies considerably between breeds and strains of cattle (Al-Salihi., 2014) [3]. Animals of all ages are susceptible but morbidity and mortality is more in young calves. Pregnant cattle may abort and intra uterine transmission is also reported (Rouby and Aboulsoudb, 2016) [13]. Bulls may become temporarily or permanently infertile and virus can be excreted in the semen for prolonged periods. (Sudhakar *et al.*, 2020) [15]. Many cattle suffer severe emaciation and loss of production for several months. The skin lesions cause permanent damage to the hides.
LSDV is a double stranded DNA virus with 151 kbp genome encoding 156 open reading frames (Lojkic *et al.*, 2018) [10] Sheep pox and Goat pox viruses are antigenically close to LSD virus. (Abutarbush *et al.*, 2018) [1]. As *capripox* viruses are cross reactive, heterologous goat or sheep pox vaccines can be employed to confer cross protection against LSD where homologous LSD vaccine is not available. (Tuppurainen *et al.*, 2014) [16].

Different PCR assays (both conventional and real time PCR assays) have been developed for detection of LSDV in clinical samples. Conventional gel-based PCR assay based on *capripox* genus specific P32 gene is simple and sensitive for detection in clinical samples (OIE 2018, Sudhakar *et al*., 2020, Kumar *et al.,* 2021) [12, 15, 8]. For further confirmation and molecular characterization of LSDV, species specific PCRs based on ORF036 encoding RNA polymerase subunit (RPO30) (Kumar *et al*., 2021 and Sudhakar *et al*., 2020) [8, 15], ORF011encoding G protein coupled receptors (GPCRs), ORF012 encoding ankyrin repeat (ANK), and ORF017 encoding LSDV fusion protein have been developed.

Virus isolation and neutralization tests are still the gold standard assays used for confirmation of disease. Isolation of the virus along with its propagation (Mashaly *et al.*, 2020) [11] helps in the creation of diagnostic assays and effective vaccines tailored to the specific area. As LSDV has narrow range of cell culture permissibility, it is traditionally grown on primary cells like lamb testicular cells primary Goat kidney cells (Kumar *et al.,* 2021) [8] and embryonated chicken eggs, However primary cells are more prone to contamination, time consuming and expensive to cultivate. Additionally cytopathic ovine pestiviruses present in primary cells of ovine or caprine origin may cause interference (OIE., 2018) [12]. To overcome these difficulties continuous cell lines like Maidin-Darby bovine kidney (MDBK) and vero (African green monkey kidney cells) are generally used. MDBK cells support good growth of LSDV and produce foci and characteristic cytopathic effect (Kumar *et al*., 2021, Fay *et al*., 2020 and Mashaly *et al*., 2020) [8, 6, 11]. Vero cells also produce characteristic CPE and good titres of virus after adaptation and are suitable for subsequent vaccine production (Kumar *et al.,* 2021 and Mashaly *et al.,* 2020) [8, 11].

Genetic characterization of circulating LSDV strains provide useful information on molecular epidemiology, tracing out the genetic diversity of evolving strains, advanced diagnostic approach, vaccine design and selection of appropriate vaccine candidate suitable to that particular area. RPO30 gene is considered to be the appropriate gene for phylogenetic differentiation of field strains of LDV from vaccine strains, hence it is commonly used for LSDV genetic tree calculation (Gelaye *et al.,* 2015: Agianniotaki *et al.,* 2017 and Sudhakar *et al.,* 2023) [7, 2, 14].

During the year 2022, LSD has affected many states in India. In comparison to the 2019 outbreak, the current outbreak in India caused wide variety of cattle fatality and morbidity rates. In Andhra Pradesh state, the disease was first confirmed during the year 2019 (Kavitha *et al.,* 2021) [9]. In 2022 starting from August onwards every district in the state was affected leading to a significant loss to the dairy farmers. The significant morbidity and rapid spread of disease in the 2022 outbreak in Andhra Pradesh when compared to the 2019 epidemic underscored the necessity for precise and rapid diagnosis and molecular identification of emerging strains. As a result, the current study was undertaken to investigate the molecular detection of LSD virus in clinical samples collected from suspicious animals of different geographical areas of the state. Isolation in MDBK cells and genome sequencing were used to confirm the virus identification. A phylogenetic investigation of one of the circulating LSDVs in Andhra Pradesh during the 2022 outbreak was carried out to look for any differences with the 2019 virus strains and vaccines currently being used against LSD.

Materials and Methods

Clinical evaluation and sample collection

Animals with characteristic nodular lesions suggestive of LSD were first observed in Srikakulam and Vizianagaram districts of Andhra Pradesh state during August 2022. Later similar cases were reported from all the districts of the state. Along with skin lesions, the animals were reported to be showing symptoms such as fever, nasal discharges, decreased milk yield, anorexia, edema in dependent parts especially in hind limbs and difficulty in walking. From August 2022 to February 2023, 868 samples (489 blood samples, 347 nasal swab samples, and 32 skin scab samples) were obtained from suspected animals of various districts in Andhra Pradesh. These samples were collected by qualified veterinarians working at field institutes according to the established guidelines with due consent from the farmers. The whole blood samples (5ml) were collected in EDTA/heparin tubes by jugular vein puncture. Nodular skin lesions (scabs) were aseptically collected by surgical excision under local anesthesia. Both nasal swabs and skin scabs were collected in sterile chilled PBS (OIE, 2018) [12]. All the collected samples were transported in cold chain to Veterinary Biological & Research Institute (VBRI), Vijayawada, Andhra Pradesh within 1-4 days of collection.

Sample Processing

The samples were processed and screened against LSDV at Veterinary Biological &Research Institute (VBRI), Vijayawada which is the Government of India authorized lab for LSD diagnosis in AP. Skin scab samples were triturated in a mortar and pestle with sterile sand after being cut into small pieces with a sterile scalpel and forceps. A 10%suspension was prepared with sterile PBS containing antibiotics (Penicillin 1000 IU/ml, streptomycin 1mg/ml and Amphotericin 2,5 µg/ml).The suspension was then freeze thawed thrice and centrifuged at 3000 rpm for 10 mins to remove any gross particles. The supernatant was collected and stored at -20 °C until use. The whole blood samples and nasal swab samples collected in EDTA tubes and sterile PBS respectively were stored at 4 °C till use.

Viral genomic DNA extraction

The viral genomic DNA was extracted from blood and nasal swab samples using DNeasy ® Blood and Tissue kit (Cat.No.69504, Qiagen, Germany) according to the manufacturer's instructions. Briefly, 200 µl of blood or nasal swab fluid was mixed with 20 µl of proteinase K and lysis buffer and incubated at 56 °C for 30 mins. After two washing steps, the DNA was eluted in 60 µl of elution buffer and stored at -20 °C till use. Nucleospin® Tissue kit (MACHEREY-NAGEL, Cat. No.740952.250) was used to extract genomic DNA from skin scabs and cell culture fluid according to the manufacturer's instructions. Briefly, 200 µl of scab tissue homogenate or cell culture fluid was mixed with 25 µl of protenase K and 185 µl of lysis buffer and incubated at 56 °C for 2-3 hours for skin scabs and 70 °C 15 mins for cell culture fluid. Then 200 µl of lysis buffer was further added and incubated at 70 °C for 10 mins. The second incubation step is not necessary for cell culture fluid. After two washing steps, DNA was eluted in 100 µl of elution buffer and stored at - 20 °C till further use.

Capripox genus specific and LSDV species specific Conventional PCR

All the samples collected from LSD suspected animals were initially tested for the presence of LSDV genome by *capripox* genus specific PCR as recommended by OIE (OIE, 2018) [12]. The virus isolates grown on cell lines were subjected to LSDV specific PCR with ORF036 (RPO30) and ORF011 (GPCR) partial genes. DNA amplification was carried out in a total volume of 25 µl with 12.5 µl of 2 X Emerald Amp MAX HS PCR master mix (Takara Bio Inc), 1 µl (10 pmol) each of forward and reverse primers, 5.5 µl of nuclease free water and 5 µl of template DNA for all the PCR reactions. The PCR assay for *capripox* genus specific PCR was carried out in a thermal cycler with initial denaturation at 94 °C for 5 mins, followed by 35 cycles of 94 °C for 1min, 50 °C for 30 sec, 72 °C for 1 min followed by final extension at 72 °C for 5 mins. Except for the Primer concentration of 20 pmol, the DNA amplification mix preparation for ORF030 and ORF011 is the same as for capripox PCR. The thermal conditions for the ORF036 (RPO30) PCR experiment are as follows: Initial denaturation at 95 °C for 2 mins followed by 35 cycles of 95 °C for 40sec, 52 °C for 1min and 72 °C for 40 sec, followed by final extension at 72 °C for 4 mins. The thermal profile for ORF011 (GPCR) is the same as that of ORF036, with a change in extension time to 80 sec. The details of primers used are tabulated below (Table 1).

Table 1: Primers used to amplify *Capripox* and LSDV specific gene segments

Type of primers	Gene/ORF	Primer sequence	Product size
Capripox	P32	F: 5'-TCCGAGCTCTTTCCTGATTTTTCTTACTAT -3'	192 bp
		R: 5'-TATGGTACCTAAATTATATACGTAAATAAC -3'	
LSDV	ORF036	F: 5'-ATGGATGATGATAATACTAATTCATATAG -3'	606 bp
		R::5'-TTATTTTTCTACAGCTCTAAACTTCG -3	
LSDV	ORF011	F: 5'-ATGAATTATACTCTTAGYACAGTTAG-3'	1134 bp
		R: 5'-TTATCCAATGCTAATACTACCAG-3'	

The PCR products were analyzed by gel electrophoresis in 1.5% agarose gel containing ethidium bromide (1 µg/ml) in 1X TAE buffer. The genomic DNA extracted from commercially available lyophilized Goat Pox vaccine (TSVBRI, Hyderabad) was initially used as positive control for standardization of *capripox* genus specific PCR.

Virus isolation on MDBK cell line

Virus isolation in cell culture was performed as per OIE protocol. LSDV isolation and propagation was carried out on Madin Darby Bovine Kidney (MDBK) cells and adapted to African green monkey kidney cells (vero). Both the cell lines were obtained from National Centre for Cell sciences (NCCS), Pune, India. The MDBK cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics (Penicillin 100 IU/ml, Streptomycin 100 µg/ml and amphotericin B 2.5 µg/ml) and 10% fetal calf serum (Himedia). Briefly, one ml of each sample (scab tissue clarified supernatant /nasal swab contents) was filtered through 0.45 µ syringe filter and inoculated into 25 cm^2 tissue culture flasks containing confluent monolayer. Cells were allowed to adsorb virus for 2 hours at 37 °C followed by addition of 7 ml of DMEM medium with 0.5% fetal calf serum. The flasks were checked daily for 7-10 days for cytopathic effect (CPE). If no CPE was observed by day 10, the culture was freeze thawed thrice, centrifuged and the clarified supernatant was inoculated into fresh MDBK cultures. The sequential blind passages were continued until CPE was observed. Once the characteristic CPE was observed, the isolates were subjected to LSDV ORF036 (RPO30) and ORF011 (GPCR) species specific PCR for further confirmation. The isolates were stored at – 80 °C until further use.

Adaptation of LSDV to vero cells

Vero cells were grown and maintained in Eagle Minimum Essential Medium (MEM) with Earle's Salts, NEAA, L-Glutamine supplemented with antibiotics and 10% fetal calf serum. To adapt LSDV to vero cells, 1ml of MDBK amplified LSDV was inoculated on 25 cm2 tissue culture flask with confluent vero cell monolayer and incubated at 37 °C for two hours followed by addition of MEM with 0.5% fetal calf serum. Flasks were observed for 5-10 days for CPE. If no CPE was observed until 10 days post infection, sequential passage was continued. The isolates were subjected to LSDV ORF036 (RPO30) PCR for confirmation.

Nucleotide sequencing and phylogenetic analysis

RPO30 gene was selected for phylogenetic analysis as it is the most commonly used gene for identity analysis. Using forward and reverse primers, the purified PCR product (606 bp) from ORF036 (RPO30) gene of the chosen LSDV isolate was sequenced. Nucleotide sequencing was done using Applied Biosystem Genetic Analyzer, CA, USA, with Sanger sequencing method. The obtained nucleotide sequence was subjected to NCBI GenBank nucleotide sequence blast (https://blast.ncbi.nlm.nih.gov) to compare the sequence identity with LSDV field isolates from different regions and time periods and homologous and heterologous vaccine strains.

The phylogenetic analysis was performed using Clustal W in MEGA 11 software (https://www.megasoftware.net) by employing neighbor joining method and phylogenetic reliability was evaluated using 1000 replicates bootstrap analysis.

Results and Discussion

Screening of clinical samples by conventional _capripox_ genus specific PCR

When clinical samples collected from LSD suspected cattle from 13 districts of Andhra Pradesh were tested by *capripox* genus specific PCR, amplification products of expected size (192 bp) for the partial fragment of P32 gene (Figure.1) were obtained from 11 districts. Out of 868 clinical samples (489 blood samples,347 nasal swab samples and 32 skin scab samples) tested by PCR, 168 samples including 46 blood samples, 110 nasal swab samples and 22 skin scab gave positive amplification with 20.51percentage positivity. Out of 168 positive samples, 21 Samples (13 nasal swabs and 8 skin scabs) were chosen from various geographical areas of the state for virus isolation study in animal cell lines.

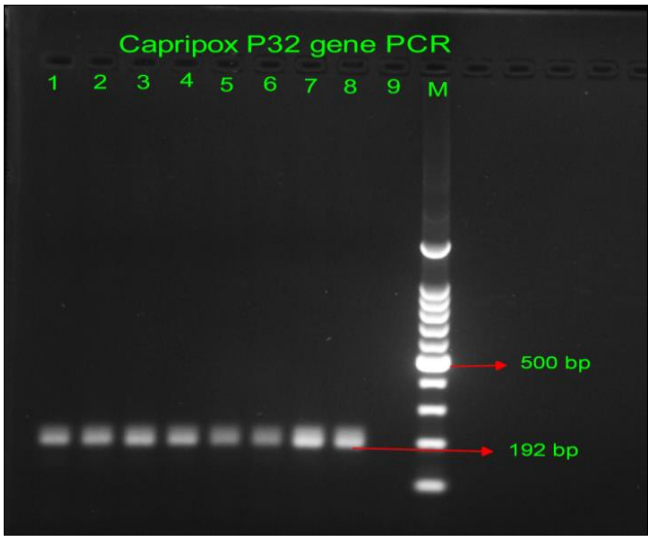
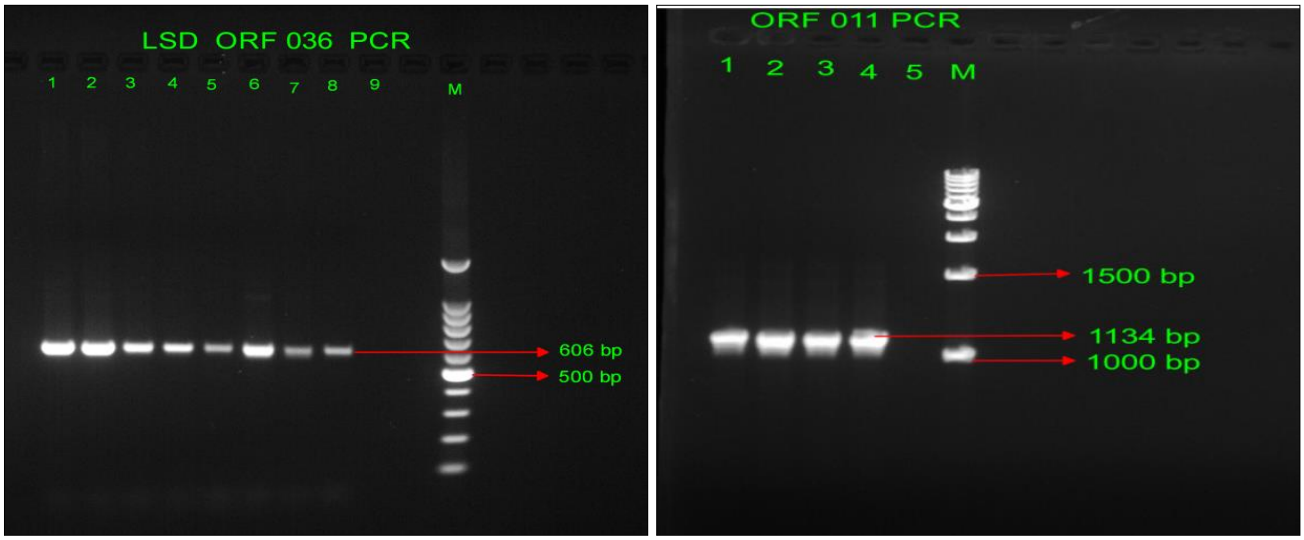


Fig 1: Capripox partial P32 gene based PCR for detection of LSDV in Clinical samples Lane 1 to 7: Positive clinical samples, Lane 8: Positive control, Lane 9: Negative control,. M: 100 bp DNA ladder

Virus isolation in MDBK cell line

The samples inoculated into MDBK cells did not produce any CPE until 2nd blind passage but were positive for presence of LSDV by PCR in the initial passages. Upon 3rd blind passage, MDBK cells started showing cytopathic changes like retraction and clustering of cells at72 hours post infection (hpi). Cell rounding, ballooning, degeneration of cells and vacuoles formation was evident at 5 dpi. (Fig. 4). Cells gradually detached from the surface and complete detachment was observed from 7th to 10th day post infection (dpi). The results are in agreement with findings made by Mashaly *et al*., 2020 [11] who reported primary isolation of LSDV in MDBK cells with characteristic CPE upon 3rd blind passage at72 hpi. Out of 21 samples processed for isolation, 12 samples (7 skin scabs and 5 nasal swabs) gave positive result. The specificity of CPE in MDBK cells was confirmed at different passage levels by both ORF036 and ORF011 PCRs. Specific 606 bp and 1134 bp products were amplified respectively for both genes from all the 12 isolates (Fig. 2). This study reports the first successful isolation of LSDV, directly in MDBK cell line from Andhra Pradesh state. The virus has adapted to MDBK cell line within 3 passages highlighting the fact that primary cell lines can be replaced with MDBK cell lines for virus isolation.



(A). ORF036 PCR (B). ORF011 PCR

Fig 2: ORF036 and ORF011 PCR for confirmation of LSDV CPE in MDBK cells

Lane 1 to 4: LSDV isolates at passage5 in MDBK cells, Lane 5 to 8: LSDV isolates at passage 3 in MDBK cells Lane 9: Negative control. M: 100 bp DNA ladder	Lane 1 to 4: LSDV isolates at passage5 in MDBK cells, Lane 5: Negative control M: 1kb DNA ladder

Adaptation of LSDV to Vero cells

In order to test the permissibility of virus to vero cells and to get good titre of virus, three isolates grown in MDBK cells up to 5th passage were inoculated onto vero cells for adaptation. Characteristic CPE was observed in all three samples at 1st passage level itself. Clustering and shrinkage of cells was observed at 48-72 hpi. At 5th dpi, cell rounding, ballooning, degeneration, and foci-type tiny plaques were detected across the monolayer. (Fig. 5). Detachment of cells from monolayer was observed at 7th-10th dpi. The CPE findings were in agreement with other previous reports (Ayelet *et al.*, 2014; OIE, 2018 and Mashaly *et al.*, 2020) [12, 11]. The specificity of CPE in Vero cells was confirmed with ORF 036 specific PCR wherein a specific 606 bp product was obtained from all the three isolates tested. (Fig. 3). In contrast to prior observations (Kumar *et al*., 2021) [8] who reported that clear CPE with plaques in vero cells was not evident upto15thblind passage. CPE was more visible in vero cells than in MDBK cells, and apparent CPE was found at the first adaption passage itself. This is in agreement with findings made by Mashaly *et al.*, 2020 [11] who reported that vero cells are best susceptible cells for propagation of LSDV giving higher virus titre at 72 hpi when compared to MDBK cells. From this study, it is more evident that LSDV can be propagated successfully in vero cells after initial 2-3 blind passages in MDBK cells.

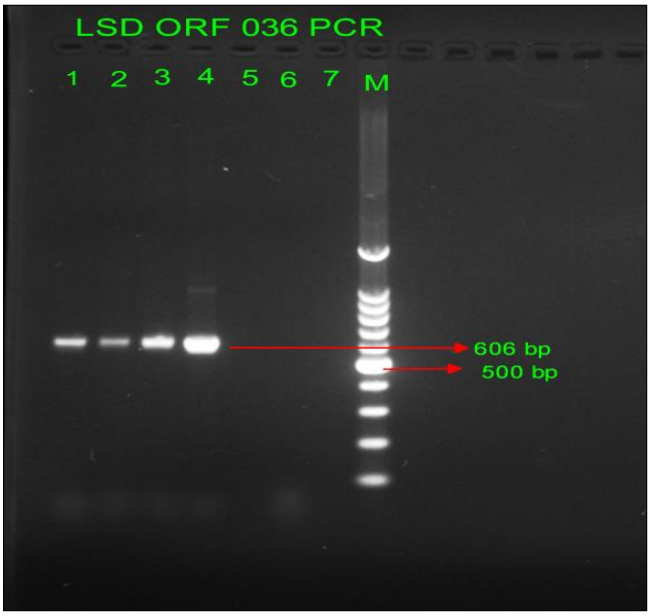


Fig 3: ORF036 PCR for confirmation of LSDV in Vero cells Lane 1 to 3- LSDV isolates at 1st adaptation passage in vero cells, Lane 4 positive control Lane 6 – Negatibe control, Lane 5&7- blank Lane M- 100bp DNA ladder

A. CPE in MDBK 5th dpi (4x)	B. CPE in MDBK 5th dpi (40x
C.CPE in MDBK 5th dpi (20x)	D. Uninfected MDBK cells 5th dpi (20x)

Fig 4: Cytopathic effect of LSDV isolates in MDBK cells
CPE – Cytopathic effect, dpi- days post infection, MDBK- Madin Darby Bovine Kidney cells,
(Images at different magnification powersusing Inverted microscope, Olympus)

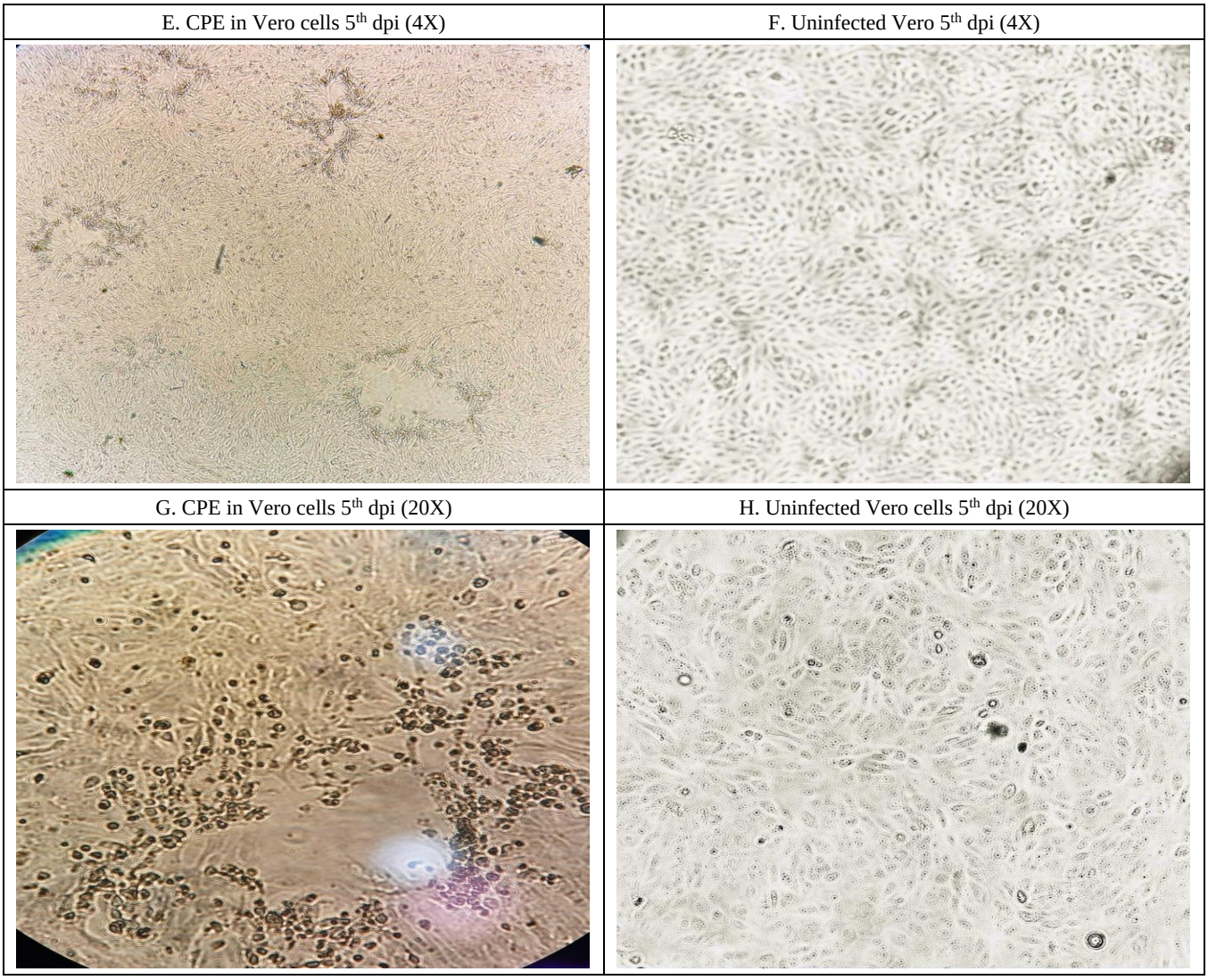


Fig 5: Cytopathic effect of LSDV isolates in vero cells at 1st adaptation passage
CPE – Cytopathic effect, dpi- days post Infection, vero- African green monkey kidney cells (Images at different magnification powrs using Inverted microscope Olympus)

Nucleotide sequencing and phylogenetic analysis

The PCR product of RP030 gene of one isolate (VZM 118) grown on MDBK cell line (Passage 5) from Vizianagaram district was sequenced. The Nucleotide sequences of the isolate showed highest similarity to the Tamil Nadu 2022 isolate with 96.50 percent identity. The isolate clustered closely with other field isolates of India (Karnataka, Haryana, Rajasthan and Odisha). The isolate also clustered with Bangladesh, Myanmar and Nigeria 2020 LSDV field isolates and clustered away from Neethling Vaccine strains (SG-1) and Kenya isolate of 1958. Heterologous vaccine strains like Sheep pox and Goat pox vaccines clustered separately from the isolate. It also clustered away with most Indian isolates from 2019 outbreaks especially the ones from Andhra Pradesh, highlighting the fact that increased morbidity and fatality during 2022 outbreaks might have been due to the variation observed between both the strains. However whole genome analysis of isolates from different regions and different time periods might be required to effectively determine if there is a significant genetic variation in the LSDV strains.

The present study reported the occurrence and spread of LSD in all parts of the state during August 2022 to February 2023. The state Animal Husbandry department has taken up preventive vaccination programme immediately with available heterologous goat pox vaccine as homologous LSD vaccine was not available in India. Once the disease was confirmed at laboratory level, all the white cattle in the villages located within5 kms radius of epicenter (Ring vaccination) were vaccinated. Further all the white cattle in border villages of the state were also vaccinated to limit the spread of the disease across state borders. The affected animals were isolated from the rest of the flock and treated with antibiotics and supportive therapy. Preventive vaccination with heterologous goat pox vaccine along with proper control measures limited the severity of the disease and number of cases reduced drastically by the end of January 2023.

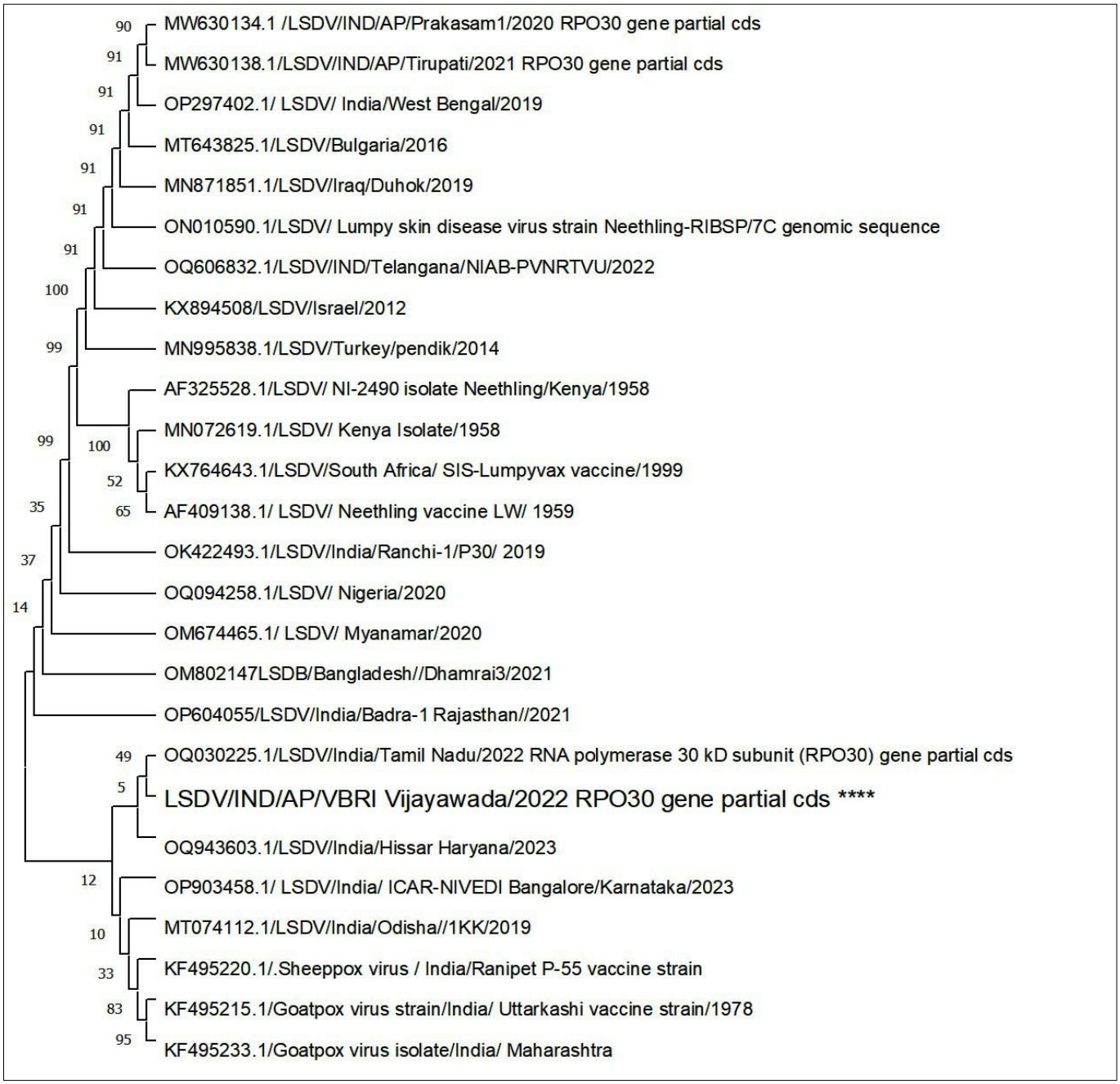


Fig 6: Phylogenetic tree of LSDV isolate of Vizianagaram district of Andhra Pradesh **** represents the sequence obtained from this study.

Conclusion

In conclusion, the study confirmed the wide spread occurrence of LSD in cattle in Andhra Pradesh, India during the year 2022. The identity of LSD virus was confirmed collectively based on clinical signs, amplification of capripox (P32) gene, virus isolation, LSDV specific (ORF036 and ORF011)) PCR and nucleotide sequencing. The study reports the isolation of LSDV in MDBK cells and also successful adaptation to vero cells. Phylogenetic analysis based on partial RPO30 gene (ORF036) sequences clustered the Vizianagaram isolate with other LSDV isolates of India. The isolate of the study has shown close proximity with 2022 isolate of Tamil Nadu which is a neighboring state of Andhra Pradesh. The isolate clustered away from LSDV Neethling vaccine strains and the Andhra Pradesh LSDV 2019 outbreak viral strains. However, genetic characterization of all LSDV isolates using whole genome analysis would provide a better understanding of the molecular epidemiology and genetic variation in emerging LSDV strains.

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