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Research Article

Maiden isolation and molecular confirmation of lumpy skin disease virus from cattle in West Bengal

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Abstract

To investigate the presence of lumpy skin disease virus (LSDV) in some suspected cattle of West Bengal (WB), the nodules and scabs were collected and virological/ molecular confirmation was performed. The skin biopsies (n=6) were collected from the representative animals (n=3) in virus transport medium and stored at 4ºC. Latter, LSDV-specific polymerase chain reaction (PCR), recommended by World Organization for Animal Health (WOAH, previously OIE), was performed. Chorioallantoic membrane (CAM) of sample inoculated embryonated chicken egg (ECE) showing pock lesions was triturated and inoculated into vero cell lines. Clear cytopathic effect (CPE) of virus was observed at 72 hours post inoculation. Capripox virus specific PCR confirmed the presence of LSD virus in the cell supernatant. We report LSDV isolation from field outbreaks using ECE and vero cell lines first time in WB. LSD cases of WB need urgent attention to formulate control strategies as the state shares international borders.

Keywords: DNA virus, Emerging disease, Lumpy skin disease virus, Skin nodules, Vector-borne disease

Highlights

- LSDV could be isolated from a field outbreak using ECE and vero cell lines
- The sampling site shared frequent legal /illegal cross-border movement of cattle
- Outbreak(s) of LSD need immediate attention to formulate control strategies

INTRODUCTION

Lumpy skin disease (LSD) is a vector-borne trans boundary poxvirus infection in cattle and buffaloes of all ages characterized by fever, nodules on the skin, mucous membranes and internal organs besides emaciation, enlarged lymph nodes, edema of the skin, and sometimes death (OIE, 2008). The disease is of economic importance (Feyisa, 2018; Gumbe, 2018) as it causes reduction in milk production, temporary or permanent sterility in bulls, temporary to permanent damage to the skin characterized by distinctive nodular lesions leading to detrimental effect on the commercial value of hides and death due to secondary bacterial infections (Amenu *et al*., 2018). LSD is caused by Lumpy skin disease virus (also known as neethling virus) which is a deoxyribonucleic acid (DNA) virus that belongs to the genus *Capripoxvirus* under *Poxviridae* family (Lubinga *et al*., 2014). Genetically, LSD virus is very similar to the other *Capripoxvirus* species such as Sheep pox and Goat pox virus. Transmission of LSD virus occurs predominantly by arthropods. Though no specific vector has been identified to date, mosquitoes (e.g. *Culex mirificens* and *Aedes natrionus*), biting flies (e.g. *Stomoxys calcitrans* and *Biomyia fasciata*) and male ticks (*Riphicephalus appendiculatus* and *Amblyomma hebraeum*) could play a role in the transmission of the virus. The incidence of LSD infection varied in different locations depending on the abundance and feeding behaviour of the arthropod vectors (Gubbins, 2019).

Lumpy skin disease is endemic in African and Middle Eastern countries and is also reported from south-east Europe, the Balkans and the Caucasus. Moreover, outbreaks of LSD have been reported in Russia, Turkey and Georgia (Tuppurainen and Oura, 2012). Laboratory confirmation of LSD is most rapid using a real-time or conventional polymerase chain reaction (PCR) method specific for capripox viruses in combination with a clinical history of skin nodules and enlarged superficial lymph nodes in cattle.

From a suspected field outbreak of LSD in cattle of South 24 Parganas district of West Bengal, an attempt was made to isolate LSDV using embryonated chicken egg (ECE) and vero cell lines and subsequently molecular confirmation of the virus was carried out.

MATERIALS AND METHODS

Sample collection: In September, 2019 during field

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visit to collect samples for our ongoing research projects, several LSD suspected infections were noted in cattle (Fig.1a, 1b, 1c) with skin nodules, fever and enlarged lymph nodes in Dakhshin Barasat [lat. 22.2251^{0}N; long. 88.4452^{0}E] of District- South 24 Parganas, West Bengal (India). Several verbal communications regarding the spread of LSD like infection in cattle in the same region were received earlier from the local veterinarians. The samples were collected from skin biopsies (nodules and scabs) and were processed in the laboratory of Veterinary Microbiology Department, Belgachia Campus, West Bengal University of Animal and Fishery Sciences (WBUAFS), Kolkata, India. Earlier, since higher percentage of scab samples were found positive for LSD (Sprygin *et al*., 2018; Sudhakar *et al*., 2020), nodules and scabs of affected skin were considered as clinical samples (Skin biopsies).

Skin biopsies (n=6) were taken from the affected animals (n=3) in virus transporting medium i.e. phosphate buffered saline (PBS, pH 7.2) and stored at 4^{o}C. The stored samples were divided into two parts. One part was used to extract DNA and capripox virus specific PCR was performed to confirm the presence of LSD virus. The other part of the samples was utilized for virus isolation and characterization.

DNA extraction: The viral DNA was extracted from the processed skin samples (n=6) using commercial DNA extraction kit (GCC Biotechnologies, India) and was confirmed as LSDV genome using World Organization for Animal Health (WOAH/OIE) recommended PCR (WOAH, 2024).

Polymerase chain reaction: In brief, PCR was performed using specific primers and cycle conditions described earlier (Ireland and Binepal, 1998). The two primers, forward (5′ -TCCGAGCTCTTTCCTGA TTTTTCTTACTAT-3′) and reverse (5′ -TATGGT ACCTAAATTATATA CGTAAATAAC-3′), were used to amplify a 192-bp fragment of the P32 envelope protein gene (LSDV074). The PCR reactions were prepared in a total volume of 25 µL consisting of 12.5 µL of 2X PCR master mix (GCC Biotech., India), 1µL (10 pmol) of each forward and reverse primers, 5.5 µL of nuclease-free water and 5 µL of template DNA. The PCR assays were carried out in a thermal cycler (Eppendorf, Sweden) with the following cycle conditions: 1 cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 50°C for 30 s, 72°C for 1 min. The final extension was carried out at 72°C for 5 min. Then each sample (10 µL) was mixed with gel loading dye and agarose gel (1.5%) electrophoresis was performed for 45 min at 85 volt using TBE (Tris-Borate-EDTA) buffer with a 100 bp DNA marker (Sudhakar *et al*., 2020).

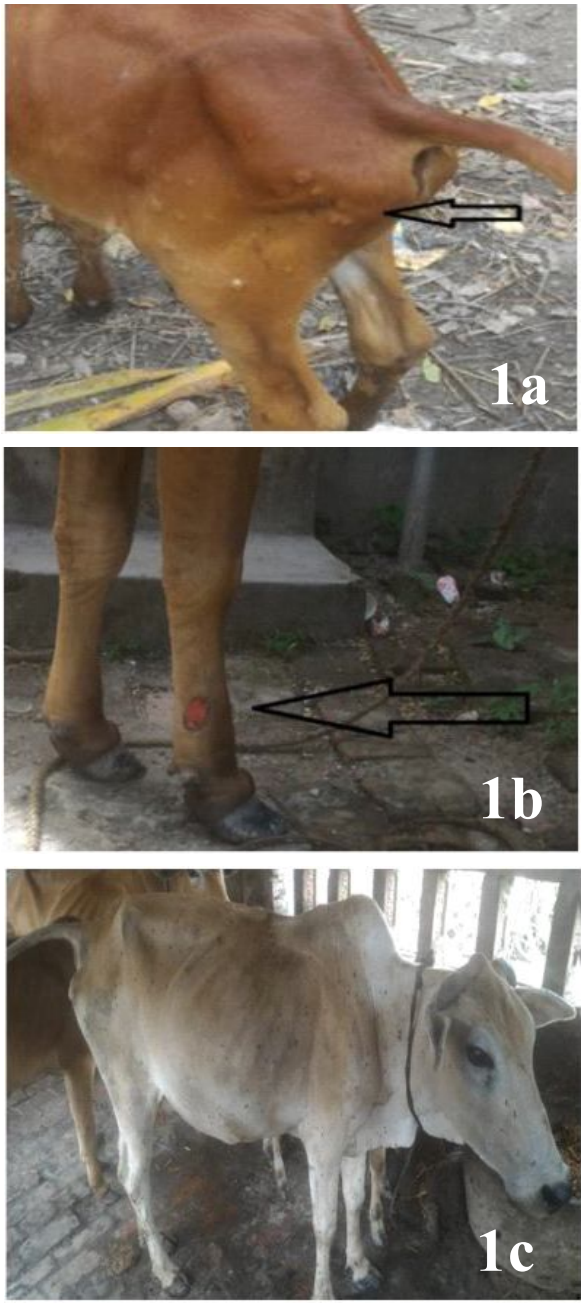

Fig. 1. Skin lesions of LSD affected cattle. (a) Localised skin nodules, (b) Ulceration and scab on skin, (c) Generalized skin lesion throughout the body

Embryonated chicken eggs: The other part of the samples were pooled to make one sample and was

triturated by pestle and mortar using sterile PBS (pH 7.2) with sterile sand, filter sterilized and inoculated into chorioallantoic membrane (CAM) of embryonated chicken eggs (ECE). At 72 hr post inoculation, the CAM was collected to observe any visible change.

Cell culture: CAM showing pock lesions (Fig. 3) was triturated by pestle and morter using PBS (pH 7.2), filter sterilized and the material was inoculated to 25 cm^2 flask (Nunc, Thermo scientific, USA) containing vero cell line (procured from National Centre for Cell Science, Department of Biotechnology, Government of India, Pune) maintained in DMEM (Dulbecco's Modified Eagle's medium, Sigma chemicals, USA), pH 7.2 containing antibiotics and 2% fetal calf serum (FCS, Sigma chemicals, USA). The virus isolation was done following the World Organization for Animal Health (WOAH)/OIE recommended techniques (WOAH, 2024) with some modifications such as use of vero cells in place of lamb testis cells and use of Dulbecco's modified Eagle medium (DMEM) instead of Glassgow's modified Eagle's medium (GMEM) as liquid medium. The flask was monitored every day for cellular pathology by inverted microscope (Zeiss, Germany) using 40X objective.

RESULTS

PCR confirmation: All the 6 samples obtained from skin biopsies showed desired positive bands in the agarose gel (Fig. 2) while analyzing in Gel documentation system (Enduro GDS, Labnet, USA).

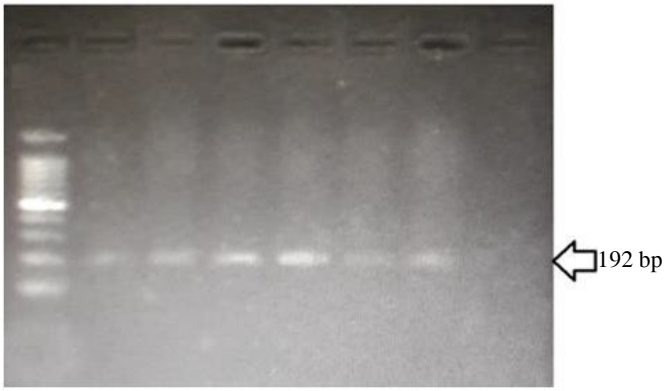

Fig. 2. Gel showing amplified 192-bp fragment of the P32 envelope protein gene product as assessed by polymerase chain reaction.
Lane-1: DNA Marker (100 bp ladder), Lane-2 through Lane 7- samples, lane 8- negative control

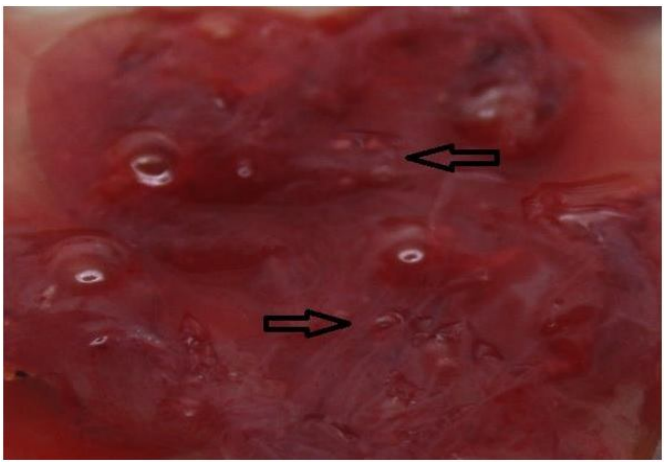

Fig. 3. Pock lesions on the Chorio-allantoic membrane (CAM) of inoculated Embryonated chicken egg (ECE)

Embryonated chicken eggs: Pock lesions were observed on the Chorioallantoic membrane (CAM) of inoculated Embryonated chicken egg (ECE) at 72 hr post inoculation (Fig. 3).

Cell culture: Clear cytopathic effect (CPE) of virus in vero cells was observed in form of rounding of cells and detachment of cell sheet at 72 hours post inoculation. Considerable CPE in form of mass mortality of cells and sloughing off the cell monolayer was observed at 96 hours post inoculation (Fig. 4). The cells were harvested by freezing and thawing and

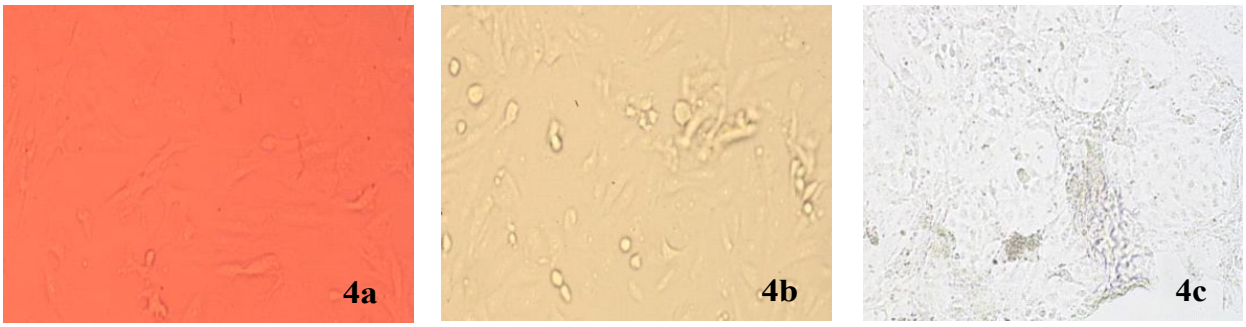

Fig. 4. Vero cell line- (a) control; (b) CPE at 72 hr post inoculation; (c) CPE at 96 hr post inoculation

were washed; DNA was extrated and capripox virus specific PCR was again performed to confirm the presence of LSD virus in the cell supernatant. The present findings indicate that LSD virus may be isolated in the laboratory from skin biopsies of affected cattle using ECE (CAM route) and vero cell lines for further studies.

DISCUSSION

Based on clinical signs, virus isolation and PCR, the LSD was diagnosed in cattle in West Bengal (India). It is now documented that LSD emerged in India in August 2019, first in Odisha state and spread to other areas (Sudhakar *et al*., 2020). Incidentally, the present work (isolation of virus from clinical samples) with the local samples of South Bengal was the first of its kind which has been documented in the present investigation. Actual work was done just prior to the period of COVID-19 pandemic. Due to pandemic situation, DNA samples could not be processed further for sequence studies.

From the present outbreaks, it seems that LSD has become an emerging infection in cattle in Southern part of West Bengal that needs immediate attention. Although the source of the infection could not be traced due to limited sampling, earlier study revealed that LSD strains involved in 2019 outbreaks in India and Bangladesh were very similar (Sudhakar *et al.*, 2022) and the present sampling site shared boundary with Bangladesh from where frequent legal /illegal cross-border movement of cattle was detected. This type of viral disease spreads fast in animal populations causing outbreaks in pockets leading to heavy economic loss to the farmers. Recently, central and western parts of India viz. Madhya Pradesh, Maharashtra, Rajasthan and Gujarat experienced outbreaks of LSD in cattle (Pandey *et al*., 2022; Yadav *et al*., 2024). It is felt that genomic analysis of the local isolates are needed to explore the origin and transmission routes of LSD e.g. trans-boundary route as also genetic mutation, if any, at local level. Moreover, immunogenicity should be tested for the local strains against African strains to find out their potentiality while developing new vaccines with local strains. Recently, the importance of genomic characterization of viral outbreaks is highlighted not only to monitor the frequency of mutations but also address its role in pathogenesis of LSDV as the outbreak continues (Yadav *et al*., 2024). With this aim, presently our laboratory is engaged to analyze the genetic variations among the local isolates. Our laboratory previously reported virus of other arbovirus disease of animals (viz. bluetongue) from South Bengal first time (Joardar *et al*., 2009). Awareness among the farmers for documentation of cases and proper early steps (e.g. vaccination with existing goat pox vaccine vis-à-vis LSD vaccine, vector control etc.) to prevent the spread of LSD infection in cattle and future disease outbreaks, if any, are warranted to avoid economic loss related to reduced production and productivity of cattle and buffaloes.

In short, LSDV could be isolated from a field outbreak using ECE and vero cell lines first time in West Bengal (India). Outbreak(s) of LSD need immediate attention to formulate control strategies as the sampling site shared boundary with Bangladesh from where frequent legal /illegal cross-border movement of cattle was detected.

List of abbreviations: CAM: Chorioallantoic membrane; CPE: Cytopathic effect; DMEM: Dulbecco's Modified Eagle's medium; ECE: Embryonated chick egg; FCS: Fetal calf serum; GMEM: Glassgow's modified Eagle's medium; LSD: Lumpy skin disease; WOAH: World Organization for Animal Health; PCR: Polymerase chain reaction; SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Conflict of interest: The author declares that there is no conflict of interests in the study.

Author's contributions: SNJ: Planned and supervised the work and wrote the manuscript; IS: Corrected/ modified the manuscript; MC, RB, AP, TM, SS: Involved in the sample collection and laboratory works. All approved the manuscript for publication.

Data availability statement: All the data and materials used to reach the conclusion of this study are available from the corresponding author on reasonable request.

Ethical statement: Samples were collected only after receiving verbal consent form the owners of the cattle.

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