

Isolation and Molecular Characterization of Lumpy Skin Disease Virus from Kalubia Governorate

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Abstract

Lumpy skin disease virus (LSDV) was isolated from skin biopsies collected from clinically infected cattle at Kalubia governorate. The virus was isolated on chorio-allantoic membrane (CAM) of embryonated chicken eggs (ECs) and identified by agar gel precipitation test (AGPT) and indirect fluorescent antibody technique (IFAT) using specific antisera against LSDV. Further electron microscopic (EM) examination and polymerase chain reaction (PCR) for putative fusion protein gene have been adopted. Nucleotide sequencing of fusion protein gene indicated that the isolate was genetically related to the reference LSDV Neethling vaccinal strain.

Introduction

Lumpy skin disease virus (LSDV) is a member of genus capripoxvirus, together with sheep pox and goat pox viruses, within the chordo-poxvirinae subfamily of Poxviridae. The genomic sequence of LSDV, about 151-kbp in length, consists of a central coding region bounded by identical 2.4 kbp-inverted terminal repeats and contains 156 putative genes. Genomic comparisons revealed that LSDV is closely related to other members of the Chordopoxvirinae, it however contains a unique complement of genes responsible for viral host range and virulence (22). LSDV is the etiologic agent of an economically important disease of cattle in the Middle east and Africa (9). In affected cattle, LSDV exists in skin nodules, crusts of skin lesions, blood, saliva, nasal discharge, semen and milk (2).

In Egypt, LSDV was first isolated and identified from cattle during two outbreaks in Suez and Ismalia governorates on 1989 (6 and 12). Diagnosis of LSD is often based on characteristic clinical signs. However, mild and subclinical forms require rapid and reliable laboratory testing to confirm diagnosis. Laboratory diagnosis of LSD comprised either identification of the virus using: electron microscopy, egg inoculation, isolation in cell cultures, fluorescent antibody test; or detection of its specific antibody using serological tests. Several polymerase chain reaction (PCR) assays have been developed recently for more accurate and rapid detection of LSDV in suitable specimens (11 and 20).

the possible introduction of new strains of LSDV by the uninterrupted movement of animals across borders is a major constant threat so The key objective of this endeavor is to detect LSDV from clinically suspected skin nodules based on serological and molecular basis. That would secure proper vaccine formulation to control spread of LSDV in Egypt.

Materials and methods

2.1.Virus strain: Neethling type strain of LSDV (Reference strain) of a titer 10^6 TCID₅₀ was obtained from Virology Department, Animal Health Research Institute, Dokki, Giza, Egypt .This strain was used as a positive control for agpt ,ifat , and PCR.

2.2.Virological specimens: Twelve biopsies of intracutaneous skin nodules were collected from suspected naturally infected cattle with LSDV from different areas in kalubia governorate.

2.3. Embryonated chicken eggs (ECES): 10th day old Specific pathogen free (SPF) embryonated chicken eggs were purchased from Koum Oshim, farm, Fayoum, Egypt and used for virus isolation.

2.4. Antisera: Reference lumpy skin disease virus antisera were obtained from Virology Department, Animal Health Research Institute, Dokki, Giza and used for serological identification.

2.5. Antibovine IgG conjugated with fluorescein Isothiocyanate: It was developed in rabbits and supplied by Sigma and used in IFAT.

2.6. Virus isolation: Lumpy skin disease virus was isolated from collected samples on CAM of ECE according to (12).

2.7. Agar gel precipitation test (AGPT): It was applied according to (5).

2.8. Indirect Fluorescent Antibody Technique (IFAT): It was carried out for detection of LSD virus in CAM according to (19).

2.9. Electron microscope examination: It was applied for detection of virus in suspected tissue samples (nodules) using positive staining technique according to (14).

2.10. DNA extraction: Genomic DNA of neethling type vaccine virus and for DNA of the isolate from skin biopsy was extracted according to (17).

2.11. Polymerase chain reaction (PCR): It was performed according to (13). The PCR primers were developed from the gene for viral fusion protein with the following sequences; forward primer: 5'- TGTTGTACTTCGTCCTGTTTGAA -3' and reverse primer: 5'- CGACGATGATGAAACCAATG -3', the amplicon size of the PCR product is 412 bp. A final volume of 50 µl reaction mixture containing 5 µl of 10× PCR buffer (Promega), 1.5 µl of MgCl₂ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA template, 0.5 µl (1 U) of DNA polymerase (Promega), and 39 µl of nuclease-free water. The volume of DNA template (contains 200 ng) required may vary and the volume of nuclease-free water was

adjusted to the final volume of 50 μ l. Amplification was carried out on a Techne-TC-512 thermal cycler (Barloworld scientific Ltd, UK) for 35 cycles of denaturation at 95 °C for 10 s, annealing at 55°C for 10 s, and extension at 72 °C for 10 s. The 35 cycles were preceded with 1 cycle of 42 °C for 2 min and 94 °C for 10 min and followed by a final extension at 78 °C for 15 min. Positive (reference Neethling strain of LSDV) and negative (water) controls were included. Ten microliters of each amplified product were analyzed by agarose gel electrophoresis using a 100-bp DNA ladder (Promega) as a molecular marker on 1.5% agarose (FMC-Bioproducts, USA), containing 1 μ g/ml ethidium bromide

2.12. Direct sequencing of PCR amplicons: Two PCR amplicons derived from genomic DNAs of skin biopsies (one represented governorate and the other represented reference strain) were purified using Microcon columns (Amicon, USA) and directly sequenced in both directions with the same primers used to generate the PCR amplicons. Sequencing was done in an ABI PRISM system at the VACSERA using the dideoxy chain-termination method (18), based on the incorporation of fluorescent-labeled dideoxynucleotide terminators.

2.13. Computer-assisted sequence and phylogenetic analyses: The resulted nucleotide Sequence data of the selected PCR amplicons were compiled and compared to each other. By blast search for sequence homology via the GenBank database. The multiple nucleotide sequences were aligned by the Clustal W (1.82) program (21) of European Bioinformatics Institute (EBI, EMBL). Clustal W is a fully automatic program for global multiple alignment of DNA sequences. Phylogenetic correlation and tree construction were done using the PHYLIP and Tree view 32 (1.6.6) programs (8). All programs used in this study were accessed through their interactive web services.

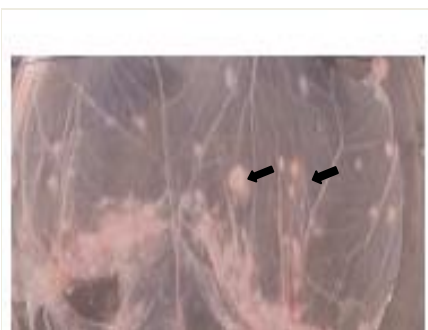
Results

Isolation of lumpy skin disease virus on CAM of ECE: LSDV was isolated on CAM of ECE and produced characteristic pock lesion. (Fig. 1).

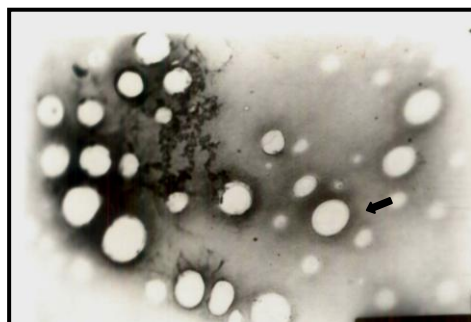
Serological identification of isolated LSDV: The LSDV antigens in infected CAM were detected by AGPT using reference LSDV. The isolated virus was identified also by IFAT. Table (1)

Electron microscopic examination: LSD virus appeared as ovoid in shape, with rounded ends. (Fig.2)

Molecular identification: The specific primers set amplified a DNA fragment of 412 bp equivalent to the expected amplification product (amplicon) size from LSDV. The LSD V reference strain and the local isolate had the same size of fusion protein gene fragment 412 bp, without significant differences between the strains (Fig.3). Subsequently, it was certain that these specimens contained DNA of LSDV. The nucleotide sequence data were comparatively aligned to each other and revealed close sequence identity (fig 4). Sequences in the alignment of the local LSDV isolates were subjected to blast search versus the GenBank database. The closest sequences to the local LSDV isolate were those of isolates/strains of LSDV, SPV and GPV. The phylogenetic tree produced confirmed the results obtained from both nucleotide sequence alignments and blast search as illustrated in (fig. 5). indicated that the isolate were identical to LSDV and closely related to SPV and GPV.



(Fig.1) characteristic pock lesion on infected CAM of ECE.



(Fig.2) LSDV isolate has ovoid shape With rounded ends

Table (1) Serological identification of LSDV isolates in ECE:

Number of skin nodules	Number of positive samples on ECE	Number of positive samples by serological identification	
		AGPT*	FAT**
12	7	4	5

*clear precipitation lines **yellowish green fluorescent

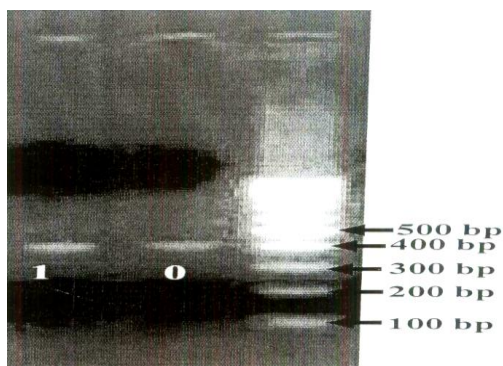


Fig.3. specific PCR product at the correct expected size of the fusion protein gene (412 bp), in the same pattern with no differences between reference strain and local isolate.

Lane M: High molecular weight nucleic acid marker

Lane 1: LSD virus isolates.

Lane 0: Reference LSD virus strain (Neethling type)

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Neethling strain AGTTGAATCACAGAGAGAAAAGTAGTGGTGACACGGGCATTATGAAGATA 50
Local isolate      . . . . . T . . . . . 50
Neethling strain AACTTTTGGACTTAAGCGAGGAGAAACCCAATATGATAAAAAATAAAAAAT 100
Local isolate      . . . . . 100
Neethling strain GATATTAAGAAAATAATTAATGAAAGATATAGCAATTATATTCTATCGA 150
Local isolate      . . . . . 150
Neethling strain CGACGATGAAATTTCTGACATTTTAAAAGATTTCATTTCATTAGTAACGAAG 200
Local isolate      . . . . . 200
Neethling strain AAATGCAAATAAAAGATTTTGTTTTAAGACTGTTGGTATTAGAAAAAACT 250
Local isolate      . . . . . 250
Neethling strain GTTTCAAACCGTCAGTAAAAGAATGCAATTCACTAAAAAATATTATTAAA 300
Local isolate      . . . . . C.G. A . . . G .GGCC .CC . . . . . T . G . . . . 300
Neethling strain AGATTAGAAAATCATATAGAACTATTAGAAAAAATATGATTGTTTTAAC 350
Local isolate      . . . . . G . . . . . 350
Neethling strain AAAAAAGGTAGATTTTCAAACAGGACGAAGTACAACAAGTTGGGAGGGGG 400
Local isolate      . . . . . 400
Neethling strain GTACGGNN 408
Local isolate      . . . . . 408

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Fig.4. Multiple alignments of nucleotide sequences for fusion protein gene of reference strain and local isolate LSDV.

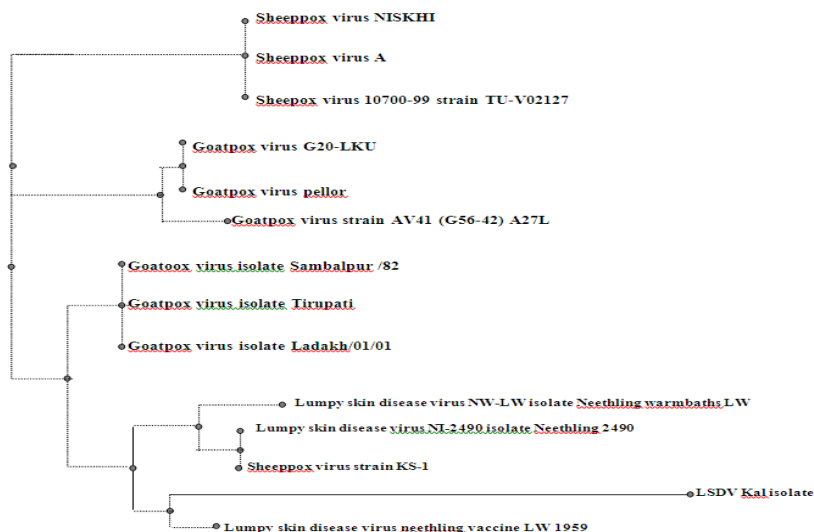


Fig.5. Phylogenetic tree based on the nucleotide sequence between aligned fusion protein gene sequences from LSDV Kal isolate and other published sequences.

Discussion

Lumpy skin disease (LSD) is a serious disease of cattle characterized by nodular cutaneous eruptions, lymphadenitis, and edema in one or more limbs (4). In the present study LSDV was isolated from samples collected from naturally infected cattle by inoculation on CAM of SPF-ECE. Characteristic pock lesions were observed after 1st passage and become clear after 3rd passage, this finding agrees with (10 and 12) who successfully cultivated LSDV on CAM of ECE and detected the characteristic pock lesions. Isolated LSDV was identified by serological tests, clear precipitation lines were appeared in AGPT by using reference LSDV antisera as recorded by Davies, 1991 and characteristic specific intracytoplasmic greenish yellow fluorescent granules were appeared in IFAT as demonstrated by (23). Electron microscope examination showed the characteristic ovoid shaped virion with "ball of wool" appearance, the same results were recorded by (1). Serological methods are useful for confirming retrospectively LSD but are too time consuming to be used as primary diagnostic methods (6 and 11). Serological assessment of antibodies to a capripoxvirus may sometimes be difficult due to the cross-reactivity encountered with other poxviruses as well as to the low antibody titers elicited in some animals following mild infection or vaccination (15).

Therefore, PCR was the test of choice for rapid detection and identification of the LSD outbreak causative agent. The PCR assay used in this work showed high specificity as a unique band of the expected size (~ 412 bp) was obtained for DNA samples derived from skin biopsies; and Neethling reference strain of LSDV. Multiple sequence alignments showed high homology percentage ($\geq 95\%$) of the nucleotide sequences between local isolates of LSDV and Neethling type strain. Nevertheless, blast searches over the Gene bank database

together with the phylogenetic analyses and sequence alignments revealed that local isolates of LSDV are highly related to not only other LSDV strains but also other Capripoxviruses (sheep and goat pox). These results coincide with the theory of that all capripoxviruses are genetically related and originated from one ancestor lineage (22). Capripoxviruses are not readily neutralized and neutralization studies cannot differentiate LSDV from sheeppox virus (SPV) and goatpox virus (GPV) (7). Cattle in contact with sheep or goats infected with SPV and GPV usually do not develop antibodies to capripoxviruses. However, cattle that have been vaccinated with SPV will develop neutralizing antibodies to LSDV (3). These results support the justified use of sheep pox virus vaccine for control of LSD (16).

In conclusion, the virus isolate from Kalubia is surely a LSDV which is closely related to other capripoxviruses of LSDV, SPV and GPV as revealed by the high nucleotide sequence identity and close branch distances in the phylogenetic tree. Selection and processing of clinical specimens, methods of DNA isolation, and PCR assay applied in this endeavor, presented a reliable laboratory diagnostic tool for LSDV.

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