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Molecular Characterization of lumpy Skin Disease Virus in Cattle (2017) Hala, A. Salem, Ebtsam, A. Abouelyazeed, Ali, H.M., Khattab, O. M and Saad, A. Moussa

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ABSTRACT

Lumpy skin disease (LSD) causes serious economic losses due to severe reduction in milk production, feed intake, and weight conversion, abortion, infertility, damage to cattle hide, suppressing immunity and deaths. LSD is considered a notifiable disease; Lumpy skin disease virus (LSDV) can be isolated from skin nodule collected from clinically infected cattle on chorioallantoic membrane (CAM) of embryonated chicken eggs (ECES) and identified by polymerase chain reaction (PCR). The present study describes the clinical and molecular diagnosis of lumpy skin disease (LSD) in Egypt during 2017. Twenty one cases of cattle suspected of being infected with (LSDV) depending on the clinical signs which appear clearly. All suspected animals were clinically examined and a tentative diagnosis of LSD was done. Specimens (skin nodules) were collected from infected animals for isolation on Specific pathogen free (SPF) embryonated chicken eggs (ECE) chorioalantoic membrane (CAMs) then examined by PCR assay to confirm existence of the disease causing agent in the suspected cases. The result of clinical examination revealed typical clinical manifestation of LSD with specific 570bp band that represent ORF103 gene of capripox virus was detected from selected sample by PCR. Phylogeny was proceeded using sequence of the partial ORF103 gene and by homology comparison with reference Lumpy skin viruses, SPVs and GPVs obtained from Gene Bank. The results showed 100% identity to LSDV/Menofiya1/18, LSDV/Menofiya 2/18, 99% LSDV/Kubash/KAZ/16, LSDV. Evros, 97% homology with goat pox virus GTPV. Sambalpur. KX398512.1 and 97% homology with sheep pox virus SPV/EG/AHRI/Wady El-gidid/MK 256477.1, SPV- El Minufiya - MF443334.1). In conclusion, PCR technique was rapid, sensitive and specific for detection of capripox virus especially LSDV in cattle.

INTRODUCTION

Lumpy skin disease (LSD) is viral disease of cattle that is caused by lumpy skin disease virus, LSD is caused by an enveloped double-

stranded DNA virus called LSD virus (LSDV), which together with sheep poxvirus (SPV) and goat poxvirus (GPV) constitutes the genus Capripoxvirus of the Chordopoxvirinae sub-

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family of the Poxviridae family (**Buller et al. 2005**). It causes abortion, infertility, and damage to cattle hide. LSD is considered a notifiable disease, and in affected countries, it results in serious restrictions to international trade (**Davies 1991 a, b, c**); **Tuppurainen and Oura 2012**). Soon after the fever, few to several variable-sized cutaneous nodules appear on different regions of the body. The entire body of the animal can be covered with nodules, and lesions may be seen in the mouth and nose as well as the mucous membranes of the eye in affected animals (**Haig 1957**; **Coetzer 2004**; **Babiuk et al. 2008**). Bulls may become permanently or temporarily infertile and the virus can be excreted in the semen for prolonged periods (**Irons et al. 2005**, **Şevik and Doğan 2017**).

Lumpy skin disease is considered as an economically important disease of cattle; serious economic losses can follow outbreaks that have a high morbidity and can produce a chronic debility in infected cattle (**OIE 2018**).

The economic losses due to this disease is due to reduced milk production, inappetite and weight loss, poor growth, abortion, infertility, skin damage and pneumonia especially in animals with mouth and respiratory tract lesions (**OIE, 2018**). Morbidity, mortality, and case fatality rates are influenced by many factors including the immune status of the affected cattle and the abundance of the vectors (**Thomas and Mare 1945**; **Tuppurainen and Oura 2012**). Experimental seminal transmission of LSDV in cattle has been reported recently (**Annandale et al. 2010 and 2014**). Although not commonly seen, the disease can be transmitted by direct contact (cutaneous lesions, saliva, respiratory secretions, milk, and semen) and using of contaminated needles (**Davies 1991a**; **Hunter and Wallace 2001**). Most cases are believed to result from transmission by an arthropod vector (**Chihota et al. 2001**). Lumpy skin disease (LSD) affects primarily cattle and occasionally buffalo (**Sharawi and El-Rahim 2011**; **El-Tholoth and El-Kenawy 2016**). The first diagnosis of LSD in Egyptian cattle was in the summer of 1989, followed by outbreaks in 2006, 2011, 2014, and 2017 (**Tuppurainen and Oura 2012**; **Abdallah et al. 2018**). In 2017, outbreaks of

LSDV in Egypt re-introduced of LSDV through imported cattle from Ethiopia or other endemic countries and unrestricted animals' movement across country borders is a major and constant threat for LSD (**Şevik and Doğan 2017**, **Hussein et al. 2017**). LSD is currently endemic in almost all African countries except a few northern countries (Libya, Tunisia, Algeria and Morocco) and in the Middle East (**Tuppurainen and Oura, 2012**; **Stram et al. 2008**; **Yeruham et al. 1995**). The disease has recently emerged in Israel, Jordan, Lebanon, Turkey, Iraq, Iran and Azerbaijan (**Tuppurainen et al. 2014**). With the exception of a few northern and southern African countries, all three capripox diseases co-exist in Africa. As a use of a homologous vaccine is recommended for all CaPVs, the molecular characterization of the causative agent will lead to better control of the spread of disease and will allow the use of the most appropriate vaccine

The objective of this study was to observe the clinical signs of the cattle infected with LSD, detect ORF103 gene of capripox virus from selected sample by PCR and Phylogeny was done using sequence of the partial ORF103 gene and by homology comparison with other capripox virus.

MATERIAL AND METHODS

Samples:

During 2017, a total of 42 samples (skin nodules) from cattle suspected to have LSD were submitted to the animal health research Institute (7 Menofya, 19 ElQalyubia, 5 Kafr Elshikh, 5 El-Behera, 3 Asuit and 3 Domiat). Skin nodules were aseptically collected from infected cattle with typical clinical signs of lumpy skin disease. Each sample was prepared for virological examination in 50 % glycerin buffer saline, 10% suspension in phosphate buffer saline (PBS) containing 100 IU/ml penicillin, 100 ug/ml streptomycin and 50 units mycostatin, then stored at -80°C till used. the mixture was incubated at 25 C 1 hr then frozen and thawed 3 times at -20°C and centrifuged at 3000rpm /10 min., the supernatant fluid was used as inoculums for virus isolation in embryonated chicken egg (ECE).

Virus Isolation:

SPF twelve days old ECE were inoculated with the prepared samples via CAM route which was used for virus isolation. Supernatant fluids of nodules injected on CAM. Samples were isolated in 12 days old in specific pathogen free (SPF) embryonated chicken egg (Koum Oshiem SPF, Fayoum, Egypt) and harvesting of CAMs.

DNA extraction:

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in kit. Extraction step for viral DNA directly from processed CAMs was done using a commercial kit (QIAamp viral DNA Mini Kit).

Polymerase chain reaction PCR:

Agarose gel electrophoreses Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation and allowed to cool at 70°C, then 0.5µg/ml ethidium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization. The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Twenty µl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. For gel analysis, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The run was stopped after about 30 min. and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software (Sambrook et al. 1989).

Oligonucleotide Primers and thermocycling.

Primers used were supplied from Metabion (Germany) are listed in table (1). Primers were utilized in a 25 µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermocycler.

Table (1) Oligonucleotide Primers and thermocycling.

Target gene	Primers	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
ORF 103	ATGTCTG ATAAAAA ATTATCT CG ATCCATA CCATCGT CGATAG	570	94°C 5 min.	94°C 30 sec.	52°C 40 sec.	72°C 45 sec.	72°C 10 min.	Zhu et al., 2013

Sequencing:**Purification and sequence of PCR product**

PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Big dye Terminator V3.1 cycle sequencing kit (PerkinElmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al. 1990) was initially performed to establish sequence identity to GenBank ac-

cessions. The phylogenetic tree was created by the MegA-5 (Tamura et al. 2007).

RESULTS**Isolation and identification of LSDV**

LSDV was isolated from skin nodule (n=21) collected from infected cows (3 Menofya, 11 ElQalyubia, 3 Kafr El-shikh, 2 El-Behera, 1 Asuit and 1 Domiat). based on the appearance of characteristic pock lesions on the CAM of ECE (Fig.1).



Fig. (1) Pock Lesions CPE of Lumpy Skin Disease Virus Isolated SPF ECE CAM.

Molecular analysis

Molecular Identification was done, The LSDV isolates were identified using conventional gel-based PCR and the specific primers set amplify a DNA fragment of 570 bp equivalent to the expected amplification product (amplicon)

size from LSDV ORF103 gene. The LSDV reference control vaccine strain and the local isolate from skin nodules, infected CAM had the same product size

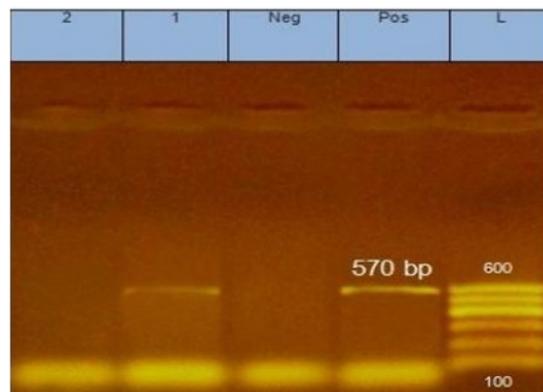


Fig. (2) 570 bp length DNA band of Lumpy Skin Disease Virus conventional PCR by ORF 103 Gene.

Analysis of sequenced isolate

Nucleotide sequences were analyzed with MEGA-5 program and Phylogenetic tree based on 570 nucleotide sequences ORF 103 gene constructed by the neighbor-joining method of (DNA Star software).

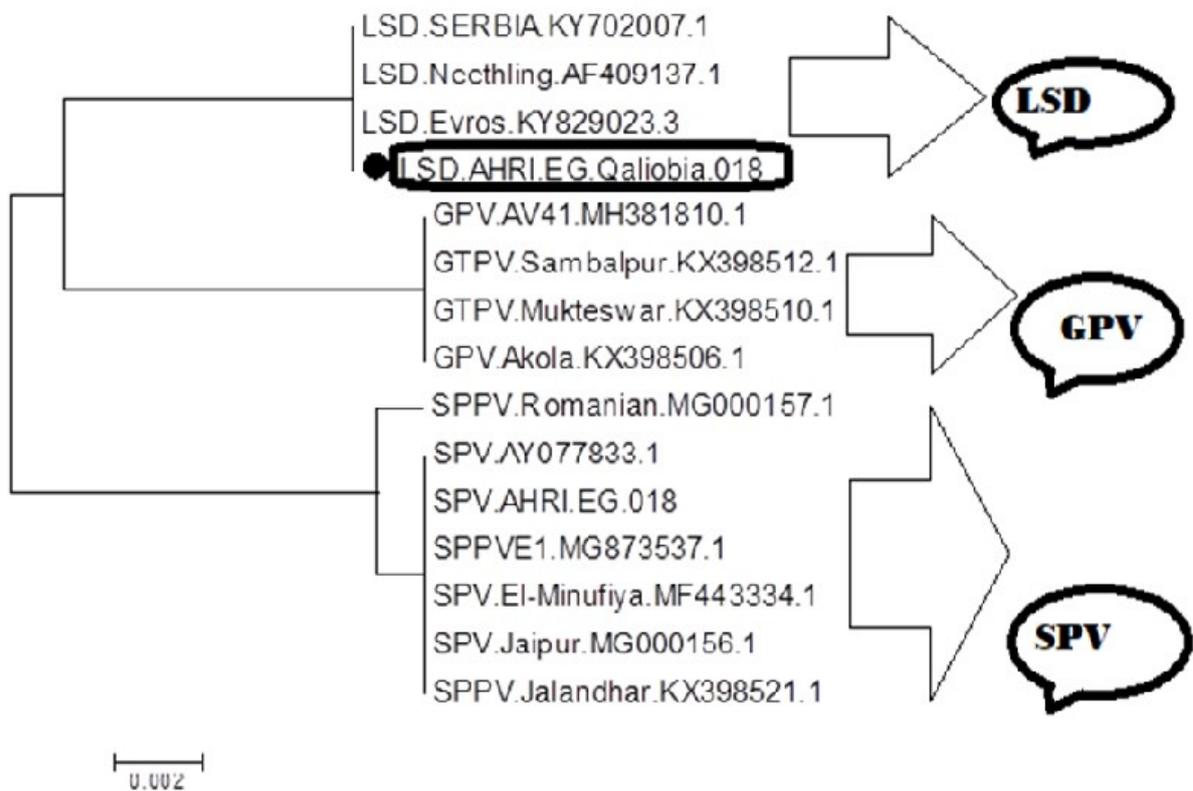


Fig. (3) Phylogenetic tree of (LSDV.EG.AHRI.018) based on 570 nucleotide sequences of ORF 103 genes drawn by (MEGA 5) & Sequences of other strains were obtained from GenBank.

DISCUSSION

Due to the rapid spread of LSDV and the severe economic losses caused, the Office International des Epizooties (OIE) includes LSDV in the listed notifiable disease of cattle (**Bowden et al. 2008**). LSDV was isolated from samples collected from naturally infected cattle by inoculation on CAM of SPF-ECE eggs. Characteristic pock lesions were observed after first passage and become clear after fifth passage (Fig.1). These findings come in complete agreement with those of **Kitching and Hammond (1992)** that successfully cultivated LSDV on CAM of ECE and detected the characteristic pock lesions. In affected cattle, LSDV exists in skin nodules, crusts of skin lesions, blood, saliva, nasal discharge, semen and milk (**Weiss 1968**). There is only one serotype of LSD virus which is very closely related serologically to the virus of sheep and goat pox (SGP), in which it cannot be distinguished easily by routine virus neutralization tests (**Burdin 1959**). 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 (**Lamien et al. 2011**). 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 (~ 570 bp) was obtained for ORF103 gene of LSDV DNA samples derived from infected CAM, and Neethling reference strain of LSDV. The genomic sequence of LSDV, about 151-kbp in length, consists of a central coding region bounded by identical 2.4 kbp-inverted terminal repeat and contains 156 putative genes. Genomic comparison 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 (**Sharawi and Abd El-Rahim 2011**). PCR is also preferred over other reliable methods of virus isolation and electron microscopy because these are not readily available and time consuming (**El-Kholy et al. 2008; Awad**

et al. 2010). Genome detection using Capripox virus-specific primers for the attachment protein and fusion protein a gene has been reported, and several conventional and real-time PCR methods have been established to be used on blood, tissue and semen specimens (**OIE 2018**). Lumpy skin disease (LSD) is one of the most important endemic infectious viral diseases of Africa including Egypt (**Elhaig et al. 2017**) and can be transmitted to the susceptible host by haematophagous insects, such as mosquitoes and stable flies (**Lubinga et al. 2014**). Both sex and all ages of cattle are the natural host; both zebu and exotic breeds are susceptible and are severely affected (**Al-Salihi 2014**). LSD can be speculated easily in the affected animal from the clinical picture of the disease. In the current study the typical clinical signs of LSD were observed ranging from acute disease to severe clinical manifestations including fever, enlarged superficial lymph nodes and edema in the dewlap and legs passing on appearance of skin nodules which are very characteristic for the disease which is attributed to the virus tropism to skin tissues and internal organs (**Tuppurainen et al. 2005; Gari et al. 2010; Constable et al. 2017**). Molecular detection of LSD viral attachment protein gene was carried out on nodular tissue samples using PCR which is quick, sensitive technique (**El Kholy et al. 2008; Awad et al. 2010; Raof et al. 2010; El-Kenawy and El-Tholoth 2011**). The results of PCR in the present study certified infection of the cattle by LSDV through observation of PCR positive band at the expected size of 570 bp fragment. These findings were consistent with the results of **El-Nahas et al. (2011); Sharawi and Abd El-Rahim (2011); EL-Khabaz (2014)**. With further identification by molecular characterization of virus isolate using PCR with primers specific to ORF103 gene (570bp) such that the PCR product could be used to detect LSDV in skin biopsy, and harvested CAMs of ECE (Fig.2) due to successive targeted the LSDV envelope protein-like gene to amplify the specific products from the extracted DNA products and the bands were clear and sharp by increasing the DNA concentration from target and were sensitive to detect LSDV strain in its original skin samples and their resource and this result agrees with previous results (**Sohair and Gaafar**

2016). This indicates that there was less nucleotide exchange through the years between the outbreaks. This finding correlates with the known fact that Capripox viruses are highly conserved within and among their species (**Babiuk et al. 2008; Tulman et al. 2002**). Finally, phylogenetic reconstructions were performed to determine the genetic relationship among Qaliobia isolates as well as other CaPV isolates (Fig. 3)

The studied LSDV isolate ORF 103 gene sequence had been deposited in gene bank under accession number MN792930 and it was shown 100% nucleotide genetic similarity with LSDV CPD/Menofiya 1/2018 MK 342935.1, LSDV CPD/Menofiya 2/2018 putative virion core protein gene MK 342936.1, 99% Identity with LSDV/Kubash/ KAZ/16/MN642592.1, LSD. Evros. KY829023.3, SERBIA/ Bujanovac /2016/KY702007.1, LSDV NW-LW Neethling Warmbaths LW/AF409137.1 and LSDV/Kenya/MN072619.1, 97% homology with goat pox virus GTPV. Sambalpur. KX398512.1, GTPV. Mukteswar. KX398510.1, GPV. Akola. KX398506.1 and GPV. AV41. MH381810.1, 97% homology with sheep pox virus SPV/EG/AHRI/Wady El-gidid core protein gene MK256477.1, SPV - El- Minufiya - MF443334.1, SPPVE1 - MG873537.1, SPV- Jaipur - MG000156.1, SPPV - Jalandhar - KX398521.1, SPV - AY077833.1 and SPPV - Romanian - MG000157.1.

In conclusion: PCR assay should be applied beside virus isolation and existence of characteristic pock lesions of SPF ECE CAMs for LSDV infection and we try to molecularly characterize LSDVs circulating in Egypt (2017).

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