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Molecular characterization of lumpy skin disease virus in specific Egyptian governorates using ORF 103 sequencing during early 2023

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ABSTRACT

In April and May 2023, five skin nodule samples were collected from cattle suspected to have lumpy skin disease (LSD) in three governorates in Egypt: Dakahlia, El-Menia, and El-Fayoum. Lumpy skin disease virus (LSDV) was detected quickly and specifically using Taq-Man real-time PCR. All samples proving positive for the virus. Following that, one positive sample from El-Fayoum governorate was isolated on Madin-Darby bovine kidney (MDBK) tissue culture for three passages, and characteristic LSDV cytopathic effects (CPE) were observed. To molecularly characterize and differentiate the strain, the isolated sample was subjected to sequencing and phylogenetic analysis, with a focus on the partial sequence of ORF 103. The results showed that the isolated virus belonged to the field strain group of LSDV, which was separate from vaccinal strains, demonstrating that ORF 103 sequencing data can be used to differentiate between field wild strains and vaccinal strains, as well as other Capripoxviruses (CaPVs).

1. INTRODUCTION

Lumpy skin disease (LSD) caused by the lumpy skin disease virus (LSDV) which is a member of *Poxviridae* family, this virus primarily affects cattle and water buffaloes (Wainwright *et al.*, 2013). The disease is classified as notifiable disease with great economic impact as it has high morbidity rates with damaging of hides, decreased milk production, abortion and infertility (Gelaye *et al.*, 2015; OIE, 2008). LSD was initially detected in Egypt in 1988, and another outbreak occurred in 2006 (El-Bagoury *et al.*, 2018). The prevalence of the disease persisted, with reports of LSD in Egypt throughout the years 2012 and 2013 (Wainwright *et al.*, 2013). According to Hodhod *et al.* (2020) cases of LSDV infection were reported during 2020. LSDV is an arthropod-borne disease spread by vectors such as Aedes aegypti mosquitoes, ticks, stable flies, and biting midges. It is worth noting, however, that transmission via direct contact and ingestion is uncommon (Al-Salihi, 2014). LSD manifests as a biphasic fever accompanied by the presence of numerous well-defined nodules on the skin, lacrimation, increased nasal and pharyngeal secretions, loss of appetite, and noticeable swelling of superficial lymph nodes. These clinical manifestations often lead to pneumonia as a prevailing outcome (Al-Salihi and Hassan, 2015). According to the Tulman *et al.* (2001) LSDV has double stranded DNA genome which demonstrates a remarkable degree of conservation, exhibiting over 95% homology. The genome of LSDV spans a length of 151 kbp and encompasses a central coding region responsible for encoding 156 presumed genes. The diagnosis of LSDV relies on either clinical manifestations or laboratory techniques (OIE, 2010). Laboratory diagnosis involves the identification of the virus through Transmission Electron Microscopy (TEM) and Histopathological examination (Tageldin *et al.*, 2014). The isolation of LSDV using MDBK tissue culture or other tissue cultures generated from bovine, ovine, and caprine sources is also required for disease diagnosis (Möller *et al.*, 2019; El-Desawy, 2008). The rounding of individual cells, clustering of perished cells, and breakdown of cell monolayers characterise the cytopathic effect (CPE) of LSDV on MDBK cells (Ayelet et al., 2014). LSDV can also be isolated successfully using pathogen-free embryonated chicken eggs (SPF-ECEs) (El-Bagoury *et al.*, 2018). The Enzyme Linked Immunosorbent Assay (ELISA) is recognized as a swift serological method for identifying LSDV, complemented by the serum neutralization test (SNT), which serves as the golden standard serological technique for detecting LSDV antigens or antibodies (Tuppurainen and Oura, 2012). Serological diagnosis of LSDV is also achievable through methods such as the Agar Gel Precipitation Test (AGPT) and the Fluorescent Antibody Technique (FAT) (Nashwa *et al.*, 2017). Using polymerase chain reaction (PCR) for LSDV molecular identification overcomes technical difficulties, time limits, and costs associated with viral isolation and serological testing. Real-time PCR has become widely used for the rapid diagnosis of numerous animal viral infections, providing rapid and surprisingly sensitive detection of LSDV across a wide range of samples. It yields quantifiable data and is a simpler, faster, and more sensitive alternative to standard PCR procedures (Nashwa *et al.*, 2017; Vidanović *et al.*, 2016). The occurrence of an LSDV outbreak presents a substantial economic jeopardy to the regional economy, underscoring the imperative of adopting stringent disease surveillance strategies. The central aim of this study is to

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assess the feasibility of promptly identifying LSDV through real-time PCR. Furthermore, the virus will be isolated using MDBK tissue culture. Subsequent sequencing will be conducted to the molecular and phylogenetic attributes of the isolate, aiming to discover any genetic variations and to determine the strain's affiliation with specific clades within the phylogenetic framework.

2. MATERIAL AND METHODS

2.1. Ethical Approval

The experimental procedures were executed within the Virology Research Laboratory, Virology Section, Faculty of Veterinary Medicine, Benha University, Egypt. These procedures received endorsement from the Animal Ethical Committees of Benha University, with the ethical approval code (BUFVTM 12-01-23) and within Animal Health Research Institute (AHRI), Giza, Egypt according to its regulations concerning virus identification, biosafety measures, and good microbiological practices

2.2. Virological samples

In the months of April and May in the year 2023, a total of five skin nodule samples were gathered from cattle exhibiting characteristic clinical indications of LSD, including fever, edema, and skin nodules. These samples were procured from three distinct governorates in Egypt, namely Dakahlia, El-Menia, and El-Fayoum. Following collection, proper labeling and transportation procedures were adhered to, and the samples were subsequently stored at a temperature of -80 °C, following the guidelines outlined by OIE, (2010). This storage protocol was implemented to ensure their preservation until they were ready to be utilized for real-time PCR, isolation on MDBK tissue culture, and sequencing procedures.

2.3. TaqMan real-time PCR

The gathered samples underwent a meticulous preparation process, and the extraction of viral DNA was executed using the G-SPIN™ Total DNA Extraction Kit (iNtRON Biotechnology, Korea), in accordance with the manufacturer's stipulated guidelines. Following successful extraction, the obtained DNA was preserved at a temperature of -20°C, to be employed in subsequent procedures. The subsequent mixing process involved the utilization of a commercial kit termed the LSDV dtec-qPCR test (GPS, Spain). The kit's components were rehydrated and employed as per the manufacturer's guidelines. In the procedure, a sterile 0.2 ml tube was utilized, and a final volume of 20 µl was prepared by skillfully combining the following elements: 4 µl of the stable qPCR mix (5x), 1 µl of the specific primer/probe, 10 µl of DNase/RNase-free water, and 5 µl of the designated template (comprising sample, positive control, or negative control). The mixture was subsequently introduced into the Applied Biosystems™ StepOne real-time PCR machine, adhering to the cycling conditions specified by the manufacturer. This encompassed an initial activation phase for one cycle at 95°C for 15 minutes, succeeded by 40 cycles of denaturation at 95°C for 15 seconds and hybridization/extension with data collection at 60°C for 1 minute. The cycle threshold (Ct) was chosen by the manufacturer and set at 35. The positive control was an integral part of the contents supplied within the commercial kit.

2.4. Isolation of LSDV on MDBK cell line

One sample was prepared and isolated on MDBK tissue culture for three passages according to OIE (2010). The growth media for MDBK cells consisted of Dulbecco' s Modified Eagle' s Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. The sample was prepared in a 10% suspension of maintenance media (DMEM) supplemented with antibiotics. In the flask containing MDBK cells, 300 µl of the prepared sample suspension added, while non-infected cells were used as a negative control. The flasks were incubated at 37ºC for one hour to allow virus adsorption and prevent dryness of the cell monolayer. After incubation, the monolayer was washed, and maintenance medium was added. The flasks were observed daily under a microscope to detect the development of CPE. When CPE was observed, infected cells were processed by freeze-thaw cycles and centrifugation to remove debris. Samples were then stored at -80ºC for subsequent passages. Virus isolates from the third passage were labeled then stored at -80ºC for further identification. The isolation was then confirmed by conventional PCR.

2.5. Conventional PCR for detection of ORF 103 of LSDV

For the identification of LSDV nucleic acids within MDBK cells, a conventional PCR approach was employed. The AmpliTaq Gold™ 360 Master Mix (Applied Biosystems™ Thermo Fisher Scientific, Waltham, MA, USA) was chosen as the amplification reagent and the PCR reaction was conducted in adherence to the manufacturer's prescribed procedures. The PCR primers, LSDVF (5'- ATG TCT GAT AAA AAA TTA TCT CG -3') and LSDVR (5'- ATC CAT ACC ATC GTC GAT AG -3'), were acquired from Bio Basic, Canada Inc., and these primers were designed to target a 570 bp fragment of the LSDV ORF 103 gene, as detailed by Zhu *et al.* (2013. The PCR reaction was comprised of a total volume of 25 µl, with 6 µl of extracted DNA, 12.5 µl of 2x AmpliTaq Gold™ 360 Master Mix, and 1 µl of 20 pmol forward and reverse primers, subsequently supplemented to the final volume using Nuclease-free water. The BIO-RAD® PCR system T100 thermocycler (BioRad, Hercules, California, USA) was used for DNA amplification, with a predetermined cycling procedure. This procedure included a cycle of 10 minute initial denaturation stage at 95°C, followed by 40 cycles of denaturation at 95°C for 60 seconds, annealing at 50°C for 60 seconds, extension at 72°C for 60 seconds, and a 10 minute final extension phase at 72°C. Both positive and negative controls were integrated into the procedure. Following PCR, the resultant products were subjected to 1.5% agarose gel electrophoresis in accordance with the methodology outlined by Sambrook *et al.* (1989). The ensuing gel images were recorded and subsequently examined employing the Molecular Imager Gel Doc™ XR+ Imaging system (BIO-RAD), alongside the utilization of Image lab™ software. To ensure accuracy, the observed DNA band, aligned with the projected size, was cross referenced with a 100 bp DNA molecular weight marker

2.6. Partial sequencing of ORF 103

To enable viral DNA sequencing, the PCR amplicons were subject to purification via the QIAquick® Gel Extraction Kit (Qiagen, Germany). Following purification, the partial sequence of the ORF103 gene was established through direct sequencing. This sequencing endeavor involved the utilization of the Applied Biosystems™ 3500 Series genetic analyzer system, which employed the Sanger sequencing technique along with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). To eliminate dye terminators, CENTRI-SEP Spin Columns

(PRINCETON SEPARATIONS, Inc., Adelphia, NJ, USA) were employed. The selection of sequencing primers and the cycling protocol remained consistent with those detailed previously

2.7. Alignment and phylogenetic analysis

To facilitate sequence alignment and phylogenetic investigation, the acquired partial sequence of the LSDV ORF103 gene was formally submitted to the GenBank database as LSD03/EGYPT/FAYOUM/2023/ORF103, and subsequently assigned the accession number (OR359631). To conduct database searches, the BLAST similarity search tool was employed, accessible through the GeneBank database maintained by the National Centre for Biotechnology Information, located in Rockville Pike, Bethesda, MD (available at http://www.ncbi.nlm.nih.gov). Sequence alignment and analysis were performed using BioEdit software alongside the ClustalW method (Hall, 1999), which facilitated the alignment of ORF103 sequences, including the one derived from this study. For the purposes of phylogenetic assessment, MEGA 7 software was utilized, employing the Neighbor-Joining method (Kumar *et al.*, 2016).

3. RESULTS

3.1. TaqMan real-time PCR

Every tested sample yielded a positive outcome for LSDV when assessed through real-time PCR, showcasing a range of distinct Ct values spanning from 19 to 23 (Fig. 1and Table. 1)

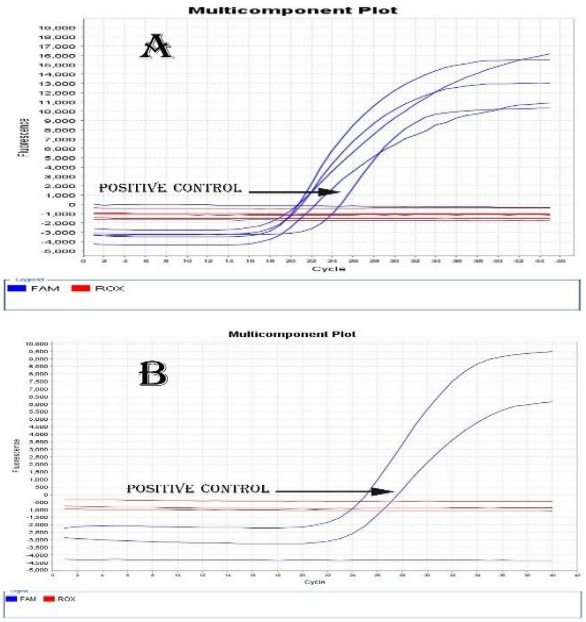

Fig. (1): Multicomponent plot of real-time PCR of skin nodule samples, (A) revealed four samples with Ct values ranging from 18 to 21, whereas (B) showed one sample with a Ct value of 23.

Table. (1): Samples details and their Ct values for real time PCR.

Sample no.	Governorate	Sample type	Ct value
1	Dakahlia	Skin nodule	18
2	El-Menia	Skin nodule	19
3	El-Menia	Skin nodule	20
4	El-Fayoum	Skin nodule	19
5	Dakahlia	Skin nodule	23

3.2. Isolation of LSDV on MDBK cell line

The sample that underwent inoculation led to the manifestation of CPE distinctive to LSDV, demonstrated through three successive blind passages in the MDBK cell culture. The discernible CPE traits encompass cell rounding, cellular aggregation, and the merging of cells to create clusters. Over time, the intensity of CPE escalated, ultimately resulting in the detachment of approximately 70-90% of the cell sheet. This observation was compared with the appearance of a standard, non-infected monolayer serving as the negative control (Fig. 2: A and B)

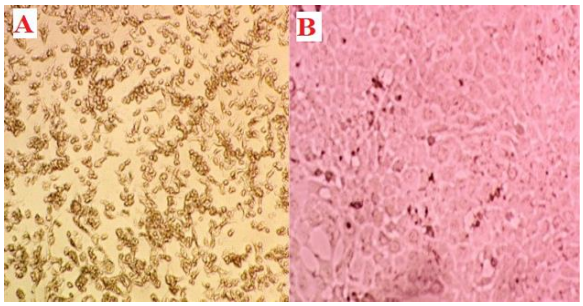

Fig. (2): (A) The characteristic CPE of an LSDV positive sample on MDBK cells, demonstrating cell rounding, aggregation, and the presence of cell clusters. (B) Non-inoculated MDBK cells as a negative control. The microscope had a 10X magnification power.

3.3. Sequencing and phylogenetic analysis

The isolated sample from the El-Fayoum governorate underwent partial sequencing of the ORF 103 gene, followed by its submission to the GenBank database. Through a comprehensive multiple alignment analysis of the LSD03/EGYPT/FAYOUM/2023/ORF103 strain, a genomic similarity spanning from 100% to 99% was observed with field strains of LSDV, as those represented by accession codes (MK342935.1, MN592992.1, and MN995838.1). Conversely, a 98.6% genetic congruence was noted with vaccinal strains of LSDV, as this identified by the accession code (KX764644.1). Interestingly, the strain demonstrated less than 98% genetic similarity with GPV strains. Phylogenetic analysis, as illustrated in Fig. (3), highlighted that detected LSDV strain, in conjunction with field strains, shares a common evolutionary branch, while vaccinal strains form a distinct branch. Additionally, the analysis unveiled a close genetic relationship between LSDV and GPV, even though they originate from different .

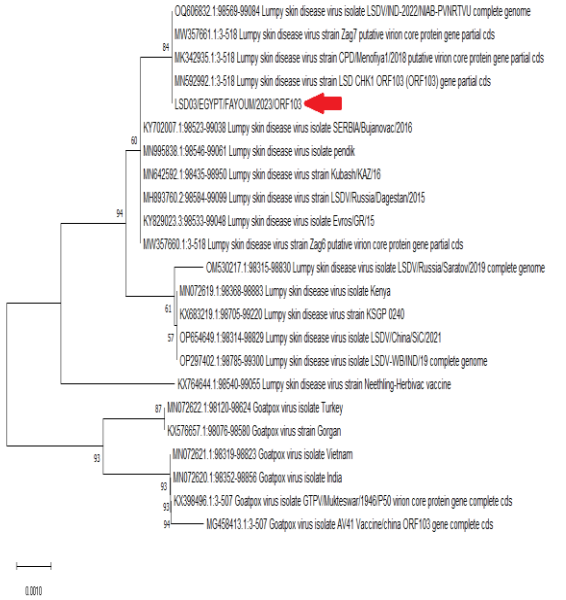

Fig. (3): presents a phylogenetic tree that has been constructed using the sequence of the LSDV ORF 103 gene. This graphical representation visually communicates the relationships among various strains, including the specific LSDV strain examined within this study (denoted by red arrow), along with other LSDV and GPV strains cataloged in the GeneBank database. The creation of the tree was executed through the utilization of MEGA 7 software, employing the Neighbor-Joining (NJ) statistical method.

4. DISCUSSION

LSD holds the classification of a notifiable disease as stipulated by the OIE. This classification underscores the disease's substantial impact on animal health and trade. LSDV's capacity to infect cattle regardless of age or gender results in substantial economic losses, both at the local and international levels (OIE, 2008), and from this point there is a significant need for rapid detection and tracking of the virus. In accordance with Vidanović *et al.* (2016), skin nodule biopsy samples are noted for exhibiting the lowest Ct values, rendering them highly suitable for testing purposes. Thus, in this investigation, we relied upon Taqman real-time PCR to rapidly and precisely detect LSDV within skin nodule samples. The outcomes consistently affirmed the presence of the virus across all tested samples. This alignment of results finds concurrence with the findings of El-Bagoury *et al.* (2023), who highlighted the efficacy of real-time PCR as a sensitive, prompt, and accurate diagnostic method for LSDV in various sample types, encompassing skin nodular samples. Subsequently, this study conducted trials to isolate LSDV on MDBK tissue culture. The virus is known to be capable of isolation on tissue cultures derived from bovine, ovine, or caprine sources (OIE, 2010). The results of this study revealed the presence of the characteristic CPE associated with LSDV, such as cellular rounding and cell aggregation leading to the formation of clusters. These outcomes align with the observations of Nashwa *et al*. (2017), who reported similar results subsequent to isolating LSDV from skin biopsy samples on MDBK tissue culture for three successive passages. Traditionally, CaPVs were deemed to be specific to particular host species. However, recent reports have highlighted instances of cross-infection, challenging the notion that Caps can be identified solely based on the host animal species. This is due to the existence of close antigenic relationships among these viruses. As a result, molecular techniques have become indispensable for precise strain differentiation. The subsequent phase of this study involved the sequencing and subsequent phylogenetic analysis of an isolate sourced from the El-Fayoum governorate. This process hinged on the partial sequence of ORF 103. This specific ORF encodes a virion core protein, as elucidated by Hala *et al.* (2021). The conducted phylogenetic analysis delineated distinct clades for LSDV field strains, LSDV vaccinal strains, and other CaPVs. Notably, the outcomes revealed that the studied strain belongs to the field strains of LSDV. Moreover, the analysis indicated that CaPVs members, including LSDV and GPV, have divergent clades, tracing back to distinct ancestral origins. These findings harmonize with the observations made by Hodhod *et al.* (2020), El-Habbaa *et al.* (2021), Hala *et al.* (2021), Yanni *et al.* (2021), who also relied on ORF103 for the molecular and phylogenetic characterization of LSDV. This outcome further underscores the conserved nature of the LSDV genome, as elucidated by Samia *et al.* (2022).

5. CONCLUSIONS

In this study, the application of TaqMan real-time PCR demonstrated its efficacy in swiftly detecting LSDV within skin nodule samples. The successful isolation of LSDV on MDBK tissue culture yielded notable results, confirmed by conventional PCR. Molecular methodologies, specifically ORF 103 sequencing and subsequent phylogenetic analysis, provided valuable insights into distinguishing LSDV strains and elucidating their relationship with other CaPVs. These findings significantly enhance our comprehension of LSDV epidemiology. Moreover, we emphasize the need for further exploration and comprehensive molecular characterization of LSDV to reinforce the effectiveness of effective vaccine production for disease management strategies.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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