Pakistan J. Zool., pp 1-8, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20220802170853

First Application of Extracellular Enveloped Viral Glycoprotein Gene Based DIVA - Approach with Molecular Characterization of Lumpy Skin Disease Virus in Al-Sharqia, Egypt

Hend E.M. Elsheikh[1]*, Mamdouh F. El-Mekkawi[1], A.A. Abou-Zaid[1] and Amal M. Abd El-Raof[2]

[1]Department of Animal Medicine, Division of Infectious Diseases, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt.
[2]Tissue Culture Unit, Virology Department, Animal Health Research Institute, Dokki, El-Giza, Egypt.

Article Information
Received 02 August 2022
Revised 06 August 2022
Accepted 10 August 2022
Available online 19 May 2023
(early access)

Authors' Contribution
MFE and AAA designed the study. HEME and AMA conducted the experiment. HEME prepared the first draft of the manuscript. MFE, AAA and AMA revised the manuscript.

Key words
Extracellular enveloped viral glycoprotein gene, DIVA approach, Molecular characterization, Lumpy skin disease, Cattle

ABSTRACT

Lumpy skin disease virus (LSDV) is a highly transmissible bovine disease caused by a virus belongs to Poxviridae family (genus *Capripox*). The disease was originally isolated from cattle in Egypt in 1988 and it later became widespread in the most of governorates. Because of the appearance of vaccine-associated disease recently, it is critical to differentiate infected from vaccinated animals (DIVA) strategies. Therefore, the aim of this study was to detect LSDV in suspected clinically diseased cows from 6 herds in Al-Sharqia governorate, Egypt, between May 2021-April 2022. Moreover, to detect whether this infection is due to a field strain or vaccine strain based on partial sequence of the EEV Glycoprotein gene that firstly used in Egypt, for LSDV detection from 2 infected cows and three types of live attenuated vaccines used in Egypt. In all, 42 of the 145 cows displayed characteristic LSD clinical signs in form of spontaneous eruption of many intradermal nodules varied in size and numbers. Conventional PCR was employed for LSDV confirmation as LSDV DNA was identified in 11 out of 12 (91.6%) samples [6/6 (100%) skin nodule biopsies and 5/6 (83.3%) nasal swabs] using EEV Glycoprotein gene. The nucleotide sequences of the EEV Glycoprotein gene of LSDV from 2 diseased cows aligned with those received from Gene Bank demonstrating that, the two detected LSDV were 100% similar and shared high sequence homology with the virulent strain from Egypt 1988; South Africa; Cameron; Kenya and Ein-Zurim/Israel with identities ranging from 99.7 % to 99.8%. Moreover, the nucleotide sequence alignment for LSDVs from 2 diseased cows and Al-Abbasya LSDV vaccine revealed the presence of 27 nucleotides that were absent in Romanian MEVAC-SPV and MEVAC-LSDV. So the conventional PCR targeting the partial EEV Glycoprotein gene is a quick and precise method for identifying LSDV. Also, the Partial sequence of EEV Glycoprotein gene has the ability to perform DIVA approach when use MEVAC LSD vaccine. In contrast it's not capable to do the same on Al-Abbasya LSDV vaccine due to unlikely presence of 27 nucleotides that specific for field strain in this type of vaccine.

INTRODUCTION

Lumpy skin disease (LSD) is highly transmissible bovine disease that can disseminate across borders. It is caused by LSDV in the genus *Capripoxvirus* (CaPV) under the Poxviridae family. The virus's Neethling strain is thought to be a prototype. Serologically, LSDV is related to goat poxvirus (GTPV) and sheep poxvirus (SPPV) (Fauquet *et al*., 2005).

Because of the large loss of productivity, irreversible damage to hides, infertility, and mortality in calves, LSD is one of Egypt's most economically significant viral illnesses of cattle (Tuppurainen *et al*., 2017). Fever, swollen lymph nodes, skin lumps, subcutaneous edema, ocular, nasal discharge, and excessive salivation are all signs of infection. The mucosal lining of the respiratory and digestive systems may develop ulcerative pox lesions (Elhaig *et al*., 2017). LSD was originally isolated and characterized from cattle in Egypt throughout two

* Corresponding author: hendelshikh@zu.edu.eg, hendelsheikh91@yahoo.com
0030-9923/2023/0001-0001 $ 9.00/0

epidemics in the provinces of Suez and Ismailia (House *et al*., 1990) and it later became widespread in the most of governorates (Hodhod *et al*., 2020). Understanding the genetic diversity of field isolates requires studying LSDV genetic characters during outbreaks. This genetic difference will help researchers pinpoint illness hotspots and the source of the LSDV virus that triggered infections (Ochwo *et al*., 2020). Vaccination with lumpy skin disease (LSD) is critical for animal health and farming economic viability. For the control of LSDV, either homologous vaccination containing live attenuation LSD virus (LSDV) or heterologous vaccines having live attenuated sheep pox or goat pox virus (SPPV/GTPV) can be applied. Despite SPPV/GTPV-based vaccines have a quite lower efficiency than live attenuated LSDV vaccines, they neither elicit vaccine-induced viremia, nor clinical reactions of the infection after immunization, that are induced by the multiplication activity of live attenuated LSDVs (Sprygin *et al*., 2020). Because of the appearance of vaccine-associated illness recently, it is critical to modify genomic DIVA approach (difference of infected from vaccinated animals) rely on LSDV circulating strain information. This necessitates regular testing and identification of LSDV field isolate (Badhy *et al*., 2021). So to distinguish between wild field kind LSDV generated from diseased cattle specimens and LSDV vaccination strains, the LSDV putative extracellular enveloped viral (EEV) Glycoprotein target genome was specifically chosen (Erster *et al*., 2019). A comparison of LSDV based on EEV Glycoprotein genetic sequences from wild field strains and vaccine strain indicated the presence of 27 nucleotides unique to the wild field strain type of LSDV that were lacking in vaccine strains (Badhy *et al*., 2021). This discovery emphasized the EEV gene as a key candidate target for the establishment of a 'DIVA' approach for detecting LSDV infections in herds inoculated with live attenuated vaccines. It was also demonstrated that the EEV Glycoprotein gene is a useful target for genetic differentiation amongst *Capripoxvirus* members. Therefore, the aim of this study was to detect and characterize LSDV in suspected clinically diseased animals from 6 different herds in Al-Sharqia governorate, Egypt, between May 2021 and April 2022 by using PCR targeting EEV Glycoprotein gene. Moreover, this study aimed to detect whether this disease is due to a field strain or a vaccine strain by applying DIVA technique based on partial sequence of the EEV Glycoprotein gene that is firstly used in Egypt for LSDV detection from 2 infected cows and three types of live attenuated vaccines (MEVAC-SPV, MEVAC- LSDV and Al-Abassya LSDV).

MATERIALS AND METHODS

Field examination

During the period between May 2021 and April 2022, we examined 145 cows belong to six herds from different regions in Al-Sharqia governorate, Egypt. In clinical examination, a total of 42 cows out of 145 revealed skin nodules, suggesting LSD infection according to Radostits *et al*. (2007). Their ages ranged from 1-6 years, and they were of both sexes. The majority of cattle's owners do not know if their animals have been vaccinated against LSDV or not, and if they have, they do not know what sort of vaccine was used.

Samples for conventional PCR

Twelve samples were taken from diseased cows: six biopsies of skin lesions and six nasal swabs.

Samples for partial sequencing using EEV Glycoprotein gene

Two field samples (skin nodules biopsies) were collected from 2 cows (first cow, during June 2021 after vaccination with unknown type of LSD vaccine submitted to Gene bank under name FI1-LSD-2021 with accession number OL960031 and second cow during November 2021 not known if vaccinated or not submitted to Gene bank under name FI2-LSD-2021 with accession number OL960032) and three types of vaccines.

Vaccines used in this study

Three types of live attenuated vaccines were used in this study. The first one was Romanian strain of sheep pox virus (SPPV) vaccine [manufactured by Middle East for Veterinary Vaccines MEVAC] and submitted to Gene bank under name ME-SPV with accession number OL960033. The second type is Neethling strain of Lumpy skin disease virus (LSDV) vaccine (manufactured by Middle East for Veterinary Vaccines MEVAC) and submitted to Gene bank under name ME-LSDV with accession number OL960034. The third type is Neethling strain of Lumpy skin disease virus (LSDV) vaccine [manufactured by veterinary Serum and Vaccine Research Institute of Al-Abassya] and submitted to Gene bank under name AL-LSDV with accession number OL960035.

Sample preparation

According to Abdallah *et al*. (2018), skin nodule biopsy specimens were cut into tiny pieces using a scalpel. Pieces were crushed in a mortar containing sterile sand, then passed to sterile tubes containing 10 mL of sterile phosphate-buffered saline (PBS) with PH 7.4 with antibiotic (Pencilline -Streptomycin- Amphotericine B Complex 10%). After

centrifuging the tubes at 5000 rpm for 10 min to remove the debris, the supernatant was poured to an Eppendorf tube for DNA extraction and kept at -80 °C until needed.

To prevent bacterial and fungal contamination, nasal swabs were taken on saline sol. with 10% pen-strop-amphotericin B and mixed well. The samples were centrifuged at 2000 rpm. The supernatant was pipetted into a sterile Eppendorf tube and kept at - 80°C until testing.

Viral DNA extraction

Viral particles were obtained from skin nodules and nasal swabs prepared according to the QIAamp DNA mini kit guidelines (source; Metabion, Germany).

Amplification and sequencing of the EEV glycoprotein gene

The EEV Glycoprotein gene F-5′-ATGGGAATAGTATCTGTTGTATACG-3′ and EEV Glycoprotein R-5′-CGAACCCCTATTTACTTGAGAA-3′ primers were developed to amplify sequences comprising the partial EEV glycoprotein (Menasherow *et al*., 2016).

Purification of the PCR products

Protocol for QIAquik PCR product purification using the QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA). On an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA), a purified PCR product was sequenced in both the forward and reverse directions. Using a Bigdye Terminator V3.1 cycle sequencing kit with a ready reaction (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No.4336817.

Phylogenetic analysis

A sequence comparison was done using the CLUSTAL W multiple sequence alignment system, version 1.83 of the MegAlign module of Lasergene DNAStar software Pairwise, which was developed by (Thompson *et al*., 1994) and Phylogenetic analyses were performed in MEGA6 using maximum likelihood, neighbor joining, and maximum parsimony (Tamura *et al*., 2013).

Sequences alignment

The alignments, annotations and assembly of the sequences were performed using CLC main workbench software version 7.5/ QIAGEN Company. https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-main-workbench.

RESULTS

Clinical findings

This study describes the clinical outcomes of LSDV infection in cattle from six herds in Al-Sharqia governorate, Egypt from May 2021 to April 2022. In all, 42 of the 145 animals displayed characteristic LSD clinical signs that varied in severity as illustrated in (Table I).

Diseased animal showed fever (40-41°C), anorexia, and a significant reduction in milk yield in lactating cows. The prescapular and prefemoral lymph nodes were enlarged. Spontaneous eruption of many nodules ranged in size (0.5 to 6 cm) in diameter and in numbers. The nodules are hard and rise somewhat above the surrounding skin (Fig. 1A, B) or sloughed split by a light circular pattern of hemorrhage creating distinct lesion of LSD Identified as “sit fast” in moderate and severely infected animals (Fig. 2). Nodules may coalesce, opened and widespread sloughed skin lesions may be developed in severely infected animals (Fig. 1C). Mucupurulent bloody nasal discharge was observed in severely infected animals with development of nodules on nasal mucosa (Fig. 1D). Some diseased animals developed edema in face and dewlap (Fig. 1E).

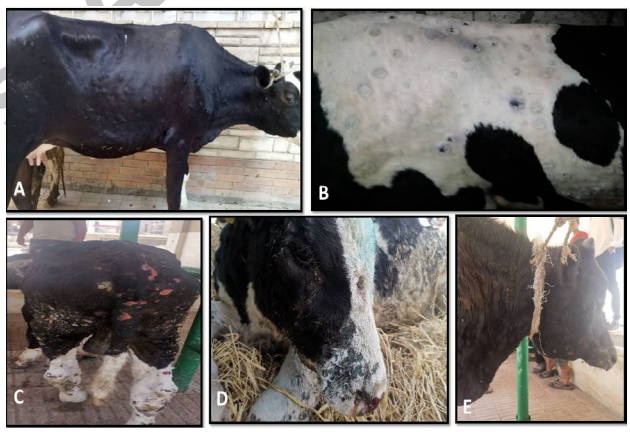

Fig. 1. Moderate and severe form of LSDV infection: (A, B) Multiple intradermal nodules distributed all over the animal bodies. (C) Widespread sloughed skin lesions in severely diseased animals. (D) Severe lacrimation, mucopurulent and bloody nasal discharge in diseased calves. (E) Edema in face and dewlap.

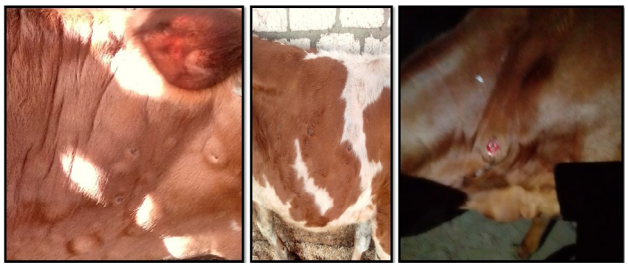

Fig. 2. Development of characteristic LSDV sit fast.

Table I. Degree of infection by LSDV in examined animals.

Severity of LSDV infection	Mild form of LSDV	Moderate form of LSDV	Severe form of LSDV
Temperature	Transient fever Slightly elevated 39°C for one day	Biphasic fever 39-40°C persist for 2-3 days then return to normal then elevated again after 4-5 days and may persist for up to 10 days	As moderate but temperature reach 41°C
Numbers, size and distribution of nodules	Less than 40 nodules usually closed not opened	Sudden appearance of large numbers of nodules usually more than 40 nodules may reach to 150 varied in size from .5 to 6 cm in diameter during the second rise in body temperature. Randomly distributed on neck, brisket, back, thorax limbs	As moderate but these nodules exceed 200 that distributed all over the body. These nodules coalesced, opened, large area of skin is sloughed leaving deep ulcers. Nodules present on mucous membrane of mouth, nose and respiratory system especially in young animals.
Sit fast	Not	Present	present
Edema in leg and brisket	Not	present	present
Course of disease	About 2-3 weeks	1-2 months	Prolonged recovery periods more than 2 months

Epidemiological findings

The morbidity rate was 42/145 (28.9%), mortality rate was 10/145 (6.9%) and case fatality rate was10/42 (23.8%).

Virus detection in clinical samples using conventional PCR

The LSDV was detected via conventional PCR depending on partial amplification of the EEV Glycoprotein gene (958-bp). The findings indicated that 11 of 12 (91.6%) of the samples were positive for LSDV [6/6 (100%) skin nodule biopsies and 5/6 (83.3%) nasal swab samples] (Fig. 3).

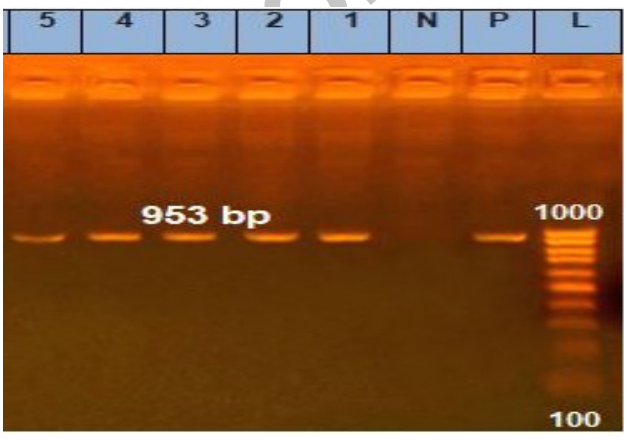

Fig. 3. Conventional PCR by EEV Glycoprotein gene: LSDV (amplicon of approximately 953 bp): 1000 bp DNA ladder (L), Positive control (P), Negative control (N). Lane (1, 2, 3, 4, 5): Revealed positive LSDV amplicons.

Application of DIVA approach

Conventional PCR was carried out on three different kinds of commercially available live attenuated vaccines that are routinely used in Egypt (Romanian strain of MEVAC-SPV, MEVAC -LSDV and Al-Abassya LSDV).

Sequencing and phylogenetic analysis

DNA sequencing of partial EEV Glycoprotein gene was performed for two representative samples, FI1-LSD-2021 and FI2-LSD-2021, as well as the three types of vaccines, (Romanian strain of ME-SPV, ME-LSDV, AL-LSDV). The nucleotide sequences of the EEV Glycoprotein gene from 2 diseased cows and three types of commercially available live attenuated vaccines aligned with those received from Gene Bank, demonstrating that the two LSDV isolates were 100% similar. The isolated LSDV shared high nucleotide sequence homology with a virulent strain from Egypt, 1988 MH639086 LSD EGPT75, as well as virulent strains from MH670904 LSD RSA6/South Africa; MH639085 LSD CMRN75/ Cameron; MN072619 LSD Kenya and KF985232 LSD Ein-Zurim/06/Israel and a vaccinal strain, KX683219 LSD vaccine, with identities ranging from 99.7 percent to 99.8 percent (Fig. 4). The partial EEV Glycoprotein gene nucleotide homology between the two field LSDVs in this study and the three vaccinal strains was 97.7% with (Romanian ME-SPV), 98.3% with ME-LSDV, and 99.8% with AL-LSDV (Fig. 4). Importantly, the nucleotide sequence alignment of the partial EEV Glycoprotein gene of the two LSDVs and Al-Abbasya (AL-LSDV vaccine) revealed the presence of 27 nucleotides that were absent in Romanian ME-SPV and ME-LSDV (Fig. 6).

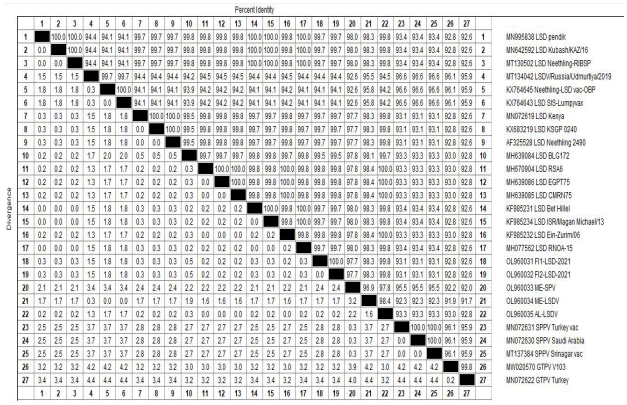

Fig. 4. The pairwise sequencing distance for the EEV Glycoprotein gene of local LSDV strains from two diseased cows in the governorate of AL-Sharqia and three types of live attenuated vaccines (MEVAC-SPV, MEVAC-LSDV and Al-Abassya LSDV) used in Egypt with the references LSDV, Sheeppox virus, and goatpox virus available in Genbank.

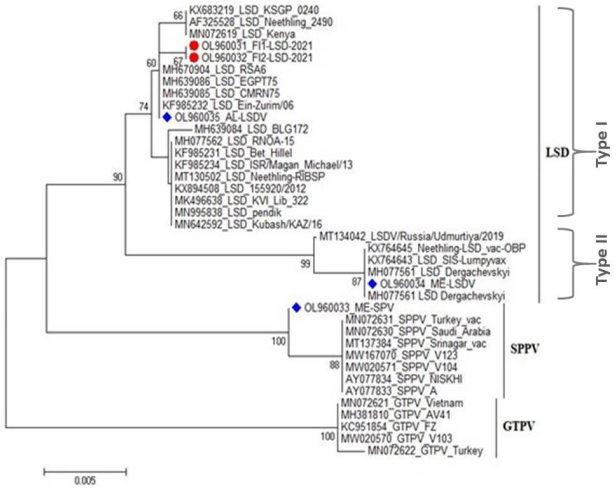

Fig. 5. phylogenetic tree based on LSDV strains taken from two sick cows and other LSDV strains from various countries, based on partial EEV glycoprotein gene sequences. The red circles indicate the two sequences discovered from this investigation. LSDV, SPPV, and GTPV sequences from Gene Bank were also examined. This study's three kinds of vaccines (MEVAC-SPV, MEVAC- LSDV and Al-Abassya LSDV) are all labeled in blue color.

Furthermore, phylogenetic tree based on partial EEV Glycoprotein nucleotide sequences gene revealed three distinct clusters exclusive to the LSDV, SPPV, and GTPV lineages (Fig. 5). Within the LSDV strains, two major sub clusters corresponding to field and vaccination strains were identified as Type I and Type II, respectively (Fig. 5). Moreover, when compared with documented sequences of LSDVs in Gene Bank (Fig. 6), demonstrated the presence of these 27 nucleotides in virulent strains from Egypt 1988 MH639086 LSD EGPT75 and from different regions such as MH670904 LSD RSA6/South Africa; MH639085 LSD CMRN75/ Cameron; KF985232 LSD Ein-Zurim/06/ Israel; MN072619 LSD Kenya, as well as the vaccination strain KX683219 LSD. In addition, the lack of 27 nucleotides differentiates them from the vaccinal strains KX764643 LSD-SIS-Lumpyvax and KX764645-Neethling-LSD-vac-OBP.

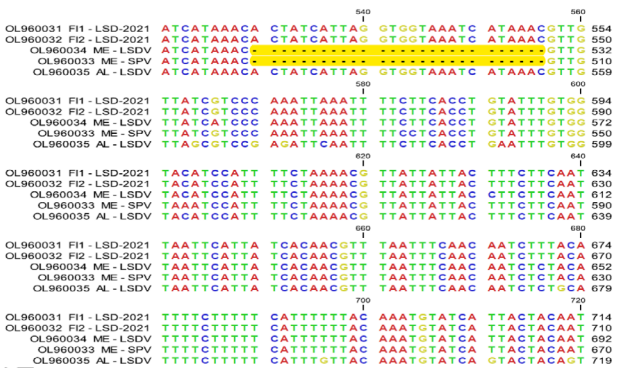

Fig. 6. Nucleotide sequence alignment of the partial EEV Glycoprotein gene of the 2 LSDV from 2 diseased cows and Al-Abbasya (AL-LSDV vaccine) revealed the presence of 27 nucleotides that were absent in Romanian ME-SPV and ME-LSDV (highlight yellow color).

DISCUSSION

Because of the expand of LSDV and the huge financial losses it has caused, the Office International des Epizooties (OIE) has classified LSDV to the list of notifiable cow diseases (Bowden *et al*., 2008). The LSD is categorized as an acute infectious disease with an incubation period of 7-14 days, beginning with fever and progressing to generalized cutaneous nodules (2–5 cm in diameter) and swelling of superficial lymph nodes (Elhaig *et al*., 2017).

The current study confirmed the presence of LSDV in diseased cows from 6 different herds in Al-Sharqia governorate, Egypt between May 2021 and April 2022. Out of 145 animals, 42 showed characteristic LSDV clinical manifestations. Most ill animals had fever (40-41°C), anxiety, anorexia, and a significant reduction in milk yield in lactating cows. The prescapular and prefemoral lymph nodes, in particular, were enlarged. Spontaneous eruption of many hard and raised intradermal nodules that ranged in size (0.5 to 6 cm) and number were recorded. All these symptoms coincide to those mentioned by (Abutarbush

et al., 2015; Elhaig *et al*., 2017). Some diseased animals developed edema in face and dewlap. This edema due to the ability of viral replication in cells such as endothelial cells in lymph vessels and vascular walls causes vasculitis and edema (Vorster and Mapham, 2008). In this study, the morbidity rate was 28.9 % while, the mortality rate was 6.9. Similar findings were reported by Coetzer (2004), who found that the morbidity rate of LSDV infection in cattle can range from 5 to 45 percent, with a mortality rate of less than 10% and Ayelet *et al*. (2014) noted that an investigation of LSD epidemics in Ethiopia indicated mortality rates of 5.9%. Higher mortality rate of 15.4 % was found in Oman by Tageldin *et al*. (2014). While Alemayehu *et al*. (2013) found a lower mortality of around 2% among cattle in Ethiopia.

Morbidity and mortality rates are influenced by cattle breed, host susceptibility, herd immune condition, and insects/mechanical vectors involved in viral transmission (Gumbe, 2018). For up to 70 years, the genome of the lumpy skin disease virus showed little genomic change (Roy *et al*., 2018). However, viruses may follow a different evolutionary pathway upon coevolution with different replication-competent field and vaccine isolates (Kononov *et al*., 2019). Since it is obvious that numerous diseases create identical symptoms, such as pseudo lumpy skin disease, dermodicosis, oncocercosis, and insect bite allergies (Alexander *et al*., 1957; Weiss, 1968). It is critical to obtain a definite diagnosis to make sure the effectively preventive and control actions for the herd through the use of PCR techniques. PCR is one of the fastest methods that can be used for viral identification in endemic regions with high sensitivity and specificity particularly in comparison to other ordinarily tools as virus isolation (Hodhod *et al*., 2020). In this investigation, conventional PCR was employed for LSDV confirmation, and LSD viral DNA was identified in 11 out of 12 (91.6%) samples [6/6 (100%) skin nodule biopsies and 5/6 (83.3%) nasal swabs] using EEV glycoprotein gene (958-bp). The findings are consistent with earlier studies that found that PCR may be utilized to identify LSDV in skin biopsy (Abdallah *et al*., 2018). Our finding supported by El-Nahas *et al*. (2011), which recorded that samples taken from skin lesions provide more positive PCR results than blood, nasal swabs, or those collected from septic viscera due to the higher concentration of virus inside the nodule**.** The nucleotide sequences of the Partial EEV glycoprotein gene from two detected LSDV of 2 diseased cows and three types of commercially available live attenuated vaccine aligned with those derived from Gene Bank, demonstrating that the two LSDV isolates were 100% similar. Moreover, the two LSDV isolates revealed strong nucleotide sequence homology with virulent strains from Egypt 1988 and other areas, including South Africa, Cameron, Kenya and Ein-Zurim/ Israel with identities ranging from 99.7 percent to 99.8 percent. This homology provides strong evidence of Kara *et al*. (2003), who stated that *Capripoxvirus* genomes are evolutionarily conserved, with more than 95 percent homology between LSD, sheep pox, and goat pox viruses. Also, Tulman *et al*. (2001) registered remarkably conserved *Capripoxvirus* isolates genomes with identities of at least 96%. This study proved the ability of partial sequencing of EEV glycoprotein to split the phylogenetic tree of CaPVs into three distinct clusters specific for LSDV, SPPV, and GTPV lineages, and the two LSDV were segregated into the LSDV not sheep pox, indicating that the sheep pox vaccination did not have a role in the establishment of these clinical symptoms. The above results validate those recorded by Saltykov *et al*. (2021) Who discovered that LSDV DNA was only detected in all specimens (blood and nasal swabs) of two diseased cows after vaccination with sheep pox vaccine, and that no SPPV vaccine-related genetic materials were found in the examined samples. As a result, these cattle did not have SPPV co-infection. The EEV glycoprotein gene sequence alignment exhibited a 27-nucleotide present in two detected LSDVs from 2 diseased cows which is typical of common field LSDV strain. The presence of 27 nucleotides in live attenuated vaccine of Al-Abbassia LSD is surprising result because these 27 nucleotides are related to field strain and so this vaccine segregated into the LSDV field strain rather than vaccine strain in phylogenetic tree and thus unable to differentiate field strain from this type of vaccine. Furthermore, the current investigation found that the Al-Abbassia LSD vaccination and vaccine strain KX683219 LSD were identical 100% identity. Previous research suggested that using the KX683219 LSD vaccine for immunization might result in the formation of extensive skin lesions (Abutarbush *et al*., 2016). Several research studies showed LSDV outbreaks in vaccinated flocks were caused by vaccination fails or the possible residual virulence of vaccinations like KX683219 LSD vaccine (Gelaye *et al*., 2015; Abutarbush *et al*., 2016). Previously, viruses identical to the KX683219 LSD vaccine, the LSDV NI2490 (1958), and the LSDV Kenya (1950) caused LSD epidemics in Kenya (Tulman *et al*., 2001).

CONCLUSION

LSD virus represents a big problem in Egypt although vaccination program. The conventional PCR technique targeting the partial EEV Glycoprotein gene is a quick and precise method for identifying lumpy skin disease virus. The sequencing of two LSDV from 2 diseased cows based on the partial EEV glycoprotein gene excluded out the idea

that the sheep pox vaccination was the cause of the infection in this investigation. Partial sequence of EEV glycoprotein gene has the ability to perform DIVA approach when use MEVAC LSD vaccine and Romanian strain of MEVAC SPV vaccine. In contrast it's not capable to do the same on AL-Abassia LSD vaccine due to unlikely presence of 27 nucleotides that specific for field strain in this type of vaccine. This study also demonstrated a clear inefficiency, lack of awareness small hold owners about vaccination of LSDV. As most owners do not know if their animals have been vaccinated or not, and if they have, they do not know what sort of vaccine was used, this information must be documented, and vaccination side effects must be monitored for three weeks following vaccination.

Statement of conflict of interest

The authors have declared no conflict of interest.

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