Research Article

# Molecular Characterization of Recent Field Isolate of Lumpy Skin Disease Virus Compared to the Vaccinal Strains

**Akram A. Salama[1], Moustafa A. Zaghloul[2*], Ahmed A. Zaghawa[1], Mohamed A. Nayel[1], Ahmed M. Elsafey[1], Mohamed F. Azooz[2], Hanem S. Harb[1] and Walid S. Mousa[1]**

*[1]Department of Animal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, University of Sadat City, El-Menofia, Egypt; [2]Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Agricultural Research Center (ARC), Cairo, Egypt.*

**Abstract** | The lumpy skin disease virus (LSDV), causes lumpy skin disease (LSD), is a transboundary viral disease of cattle that has a significant global economic impact. This study aims to identify the lumpy skin disease virus that circulated in El-Menofia Governorate between April 2020 and April 2022 by partially amplifying the EEV gene. Additionally, the amplified gene sequence was subjected to sequencing and phylogenetic analyses, and the acquired LSDV sequences were compared to those in GenBank. Using the standard polymerase chain reaction (PCR) and specific primers designed from the EEV gene, tissue samples from skin nodules (N = 40) and blood samples (N = 60) were collected from diseased animals. Sequencing and a phylogenetic tree construction were done on the amplified byproducts of samples from blood and nodules on cattle skin. By PCR technique, 55 samples out of 100 (55/100; 55%) were positive for LSDV. A representative sample was sequenced and had a very high identity percentage of > 99.7-100% with virulent LSDV isolated from Egypt (Sharkia Governorate) or different countries abroad (Kazakhstan, Turkey, Israel, and South Africa), while the identity with vaccinal strains of LSDV was 94.1%. The alignment analysis revealed a distinct 27-base deletion in the vaccine strains. Our results can be used to better understand the epidemiology of LSDV in Egypt and to build an effective disease control programme. Furthermore, our findings emphasize the importance of continuous surveillance and characterization of circulating LSDV strains, as well as the need to improve the DIVA strategy for distinguishing the vaccine strains from field viruses.

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***Correspondence** | Moustafa A. Zaghloul, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Agricultural Research Center (ARC), Cairo, Egypt; **Email:** moustafazaghlool@gmail.com
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## Introduction

The lumpy skin disease virus (LSDV) causes the potentially lethal viral disease, lumpy skin disease, which affects cattle and water buffalo. It is amongst the most economically significant viral diseases and is categorized as a worldwide concern (Tuppurainen *et al.*, 2017).

LSDV belongs to genus Capripoxvirus (CaPVs), Chordopoxvirinae subfamily of Poxviridae family. It has a single, linear dsDNA that encodes for approximately 200 proteins. Poxviridae is divided into two subfamilies; Entomopoxvirinae, which is responsible for insect poxviruses, while Chordopoxvirinae is responsible for vertebrate poxviruses. LSDV, sheep poxvirus (SPPV) and goat poxviruses (GTPV) are belonging to genus Capripoxvirus (Sudhakar *et al*., 2020).

Despite the annual mass immunization of cattle with sheep pox vaccine in Egypt, LSD remains prevalent and LSDV thrives almost every summer the livestock industry in Egypt leading to a significant economic impact on the animal and byproduct trade.

Lumpy skin disease (LSD), a serious threat to stockbreeding, can cause acute or subacute illness in cattle and buffalo (Givens, 2018). Cattle of all ages and breeds are affected, but young animals and those lactating at their peak are highly susceptible (Tuppurainen *et al*., 2011). The World Organization for Animal Health (OIE) has classified LSDV as a notifiable disease due to its potential for rapid spread and significant economic losses (Tuppurainen and Oura, 2012).

Recently, LSD spreads in disease-free countries that emphasize the necessity for prevention of the disease transmission, as well as control and eradication (Sprygin *et al*., 2019). LSDV is a DNA virus, has 30 structural and nonstructural homologs of pox viral proteins. It shares genetic and antigenic similarities with Sheep Pox Virus (SPPV) and Goat Pox Virus (GTPV) (Givens, 2018).

Clinical symptoms of LSDV include fever, in appetence, nasal discharge, salivation and lachrymation, swollen lymph nodes, a significant decrease in milk production, loss of body weight, and potentially mortality (Tasioudi *et al*., 2016). Furthermore, the disease is distinguished by hard, slightly elevated, confined skin nodules 2-7 cm in diameter that commonly form on the neck, legs, tail and back shortly after fever onset (Sevik and Dogan, 2017).

Myiasis increased by necrotic and ulcerative nodules (Beard, 2016). In some cases, oedema of the legs and lameness were also seen (Tuppurainen and Oura, 2012). LSDV can cause abortion, mastitis and orchitis (Radostitis *et al*., 2007; Awadin *et al*., 2011). Nodules, on the other hand, were not found in aborted fetuses (Sevik and Dogan, 2017).

Since its discovery in Zambia in 1929, LSDV has been recognized in a number of other African nations (Wainwright *et al*., 2013). The disease has been found in Saudi Arabia, Lebanon, Jordan, Iraq, Israel, Turkey, and Iran (Sameea *et al*., 2017). Since 2015 (OIE, 2015; Zeynalova *et al*., 2016), it has been extended to Russia, Azerbaijan, Armenia, Greece, Bulgaria, Albania, Kosovo, Serbia, and Montenegro. As a result, it is important to take into account the increased risk of disease spread to the rest of Europe and Asia. LSDV has global distribution.

LSDV was initially detected in Egypt in 1988, 1989 and then again in 2006, 2011 and 2014 (Elhaig *et al*., 2017). Furthermore, the disease resurfaced during the summers of 2016 and 2018, even in animals immunized with the Romanian SPPV vaccine (Allam *et al*., 2020). Every summer, LSDV is detected in Egypt as recorded by Keshta *et al*. (2020) and Rouby *et al*. (2021).

Lumpy skin disease has led to serious economic losses in affected countries. The disease causes a considerable reduction in milk yield (from 10% to 85%) due to high fever and secondary mastitis. Other consequences of the disease include damaged hides, decline of the growth rate in beef cattle, temporary or permanent infertility, abortion, treatment and vaccination costs and death of infected animals (Sevik and Dogan, 2017). There have been no reports on the incubation period of LSDV infection under field conditions (OIE, 2016). Although the morbidity rate varies between 5% and 45% (sometimes up to 100%), the mortality rate is usually under 10% (sometimes up to 40%) (Coetzer and Tustin, 2004). The severity of the clinical disease is often influenced by the animal's age, breed, immune status and production period (Tuppurainen *et al*., 2017).

Vaccination is considered to be an effective method of controlling LSDV (Constable *et al*., 2017). Despite the fact that vaccination programs currently use a variety of CaPVs vaccine strains, vaccination failures and recurrences of various outbreaks have been documented (Gari *et al*., 2011). Several live-attenuated CaPV vaccine strains, including the

Neethling strain of LSDV, Kenyan SPPV and GTPV, Yugoslavian strain of SPPV, Romanian strain of SPPV, and Gorgan strain of GTPV, are used for LSDV prevention and control (Gari *et al*., 2015).

The Egyptian authorities approved the immunization with an attenuated sheep pox vaccine to be a viable and effective control agonist LSD (Elhaig *et al*., 2017; Tuppurainen *et al*., 2017).

The aim of this study is to detect LSDV strains that have been circulating in Menofia Governorate, Egypt between 2020 and 2021 based on molecular identification of EEV gene followed by sequencing and phylogenetic analyses.

## Materials and Methods

*Ethical approval*

All animal handling procedures, as well as samples collection and disposal, were performed according to the pointers and recommendations of the European Communities Council Directive 1986 (86/609/EEC).

*Animal examination*

The study was conducted on beef male and female calves aged from one to two years old, which was located at Sadat City, El-Menofia governorate, Egypt. The study period extended about two years from April 2020 till April 2022 at which epidemics of LSDV in cattle in El-Menofia Province were evident. One hundred calves, lives in groups and reared of same hygiene and management conditions. During regular veterinary visits, clinical examinations and observations of diseased animals were carried out according to Sameea *et al*. (2017). Most of the infected individuals evaluated showed significant clinical symptoms such as fever, lameness, skin nodules dispersed on the vulva, teat, and forelimbs and some died.

*Samples collection*

Forty skin nodules were obtained aseptically from affected calves by washing and disinfecting the area and removing hairs with a sterile scalpel blade. The sample was collected in a sterile universal bottle containing antibiotics and antifungals and transferred to the laboratory in 50% glycerol saline. The tissue samples were then kept at -20°C for molecular characterization of LSDV using the procedures outlined in OIE (2016).

Sixty whole blood samples were collected with and without anticoagulant, then transported and stored at -20 °C in the laboratory. The blood samples were collected aseptically from feverish animals with temperatures ranging from 40 °C to 41 °C. The samples were then placed in a sterile test tube for serum separation, which was used for biochemical measurements. Samples were taken at (1st-2nd days) and (10-14th days) after the formation of cutaneous nodules.

Tissue samples were prepared following the recommended protocol OIE (2010). Skin scabs were collected from diseased animals with sterile scissors and placed into a sterile container with 50% glycerol buffer saline (pH 7.2). Briefly, scabs were placed in a sterile petri dish, cut into small pieces with sterile scissor and placed in a 1.8 ml cryotube with 1.5 ml physiological saline containing antibiotics and antifungal solution. Then, samples were homogenized by homogenizer, followed by three cycles of freezing (-20°C) and thawing (room temperature) for the homogenized suspension. Following centrifugation of the homogenized suspension at 3000 rpm/15 min, the supernatant was filtered by syringe filter (0.45mm) and the filtrate stored at -20 °C until using for molecular characterization.

*DNA extraction and PCR*

The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to extract the viral DNA from samples according to the manufacturer's instructions. LSDV was confirmed using a specific PCR assay targeting the Capripox EEV gene using the following pair of primers; forward primer 5′-ATGGGAATAGTATCTGTTGTATACG-3′ and reverse primer 5′- CGAACCCCTATTTACTTGAGAA -3′, to amplify a 958 bp amplicon for Chibssa *et al*. (2021).

In a 25-µl reaction, 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 of each primer at a concentration of 10 pmol, 5.5 µl of water and 5µl of DNA template were used. The reaction was carried out in a thermal cycler Applied Biosystem 2720. The PCR products were separated using gradients of 5V/cm electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer. The fragment sizes were determined using a gene ruler 100 bp ladder (Fermentas, Thermofisher, Germany). A gel

documentation system (Alpha Innotech, Biometra) photographed the gel and the data was processed using computer software.

*Sequencing of EEV gene and phylogenetic analysis*

The QIAquick PCR product purification kit (Qiagen) was used to purify the PCR products. For sequencing, a Perkin-Elmer Bigdye Terminator V3.1 cycle sequencing kit was used and a Centrisep spin column was subsequently used for purification. To confirm the sequence identity, DNA sequences were acquired using the Applied Biosystems 3130 genetic analyzer (HITACHI, Japan). A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al*., 1990) was first performed. Phylogenetic tree was performed in MEGA 11 using neighbor- joining (NJ) method (Tamura *et al*., 2021). Amino acids sequence alignment was constructed by BioEdit software version 7.1.3.0.

## Results and Discussion

Most of the infected cattle and buffaloes displayed typical clinical signs and symptoms such as fever (40 - 41.5°C), loss of appetite, nasal and ocular discharge and widespread skin nodules. Furthermore, the sick animals had swollen sub-scapular and pre-femoral lymph nodes, as well as a significant decrease in milk output. Necrotic plaques in the oral and nasal mucous membranes, ulcerated nodules and scabs were also observed in some affected calves. In the case of buffaloes, however, only tiny nodular lesions with no centralized ulcerations were found.

To validate the presence of LSDV viral DNA from suspected clinical cases, a conventional PCR was done using specialized primers that targeted the LSDV EEV gene at the 958 bp area, as shown in (Figure 1). The samples are collected from very closely related areas so we take one sample only. PCR revealed that (55 %) of the 100 samples taken from suspected clinical cases tested were positive. After amplification of the target gene, all DNAs taken from positive samples produced a distinct band in the gel. There was only one sequenced sample. This sequencing sample was translated to amino acids and registered in Gen Bank as OP484928.

Phylogenetic and nucleotide sequence distance analysis (Figure 2) were used to evaluate the genetic link between sequenced LSDV isolates from diseased cattle using EEV gene primers. These sequenced LSDV isolate was grouped with other LSDV isolates from Egypt and diverse regions in the same clade, as well as isolates from SPPV and GTPV from Gen Bank, to determine the identity and proximal region of disease transmission responsible for recurrent and ongoing outbreaks. The Egyptian field LSDV isolate was more closely related to LSDV sequences from Kenya, South Africa, Israel and Kazakhstan.

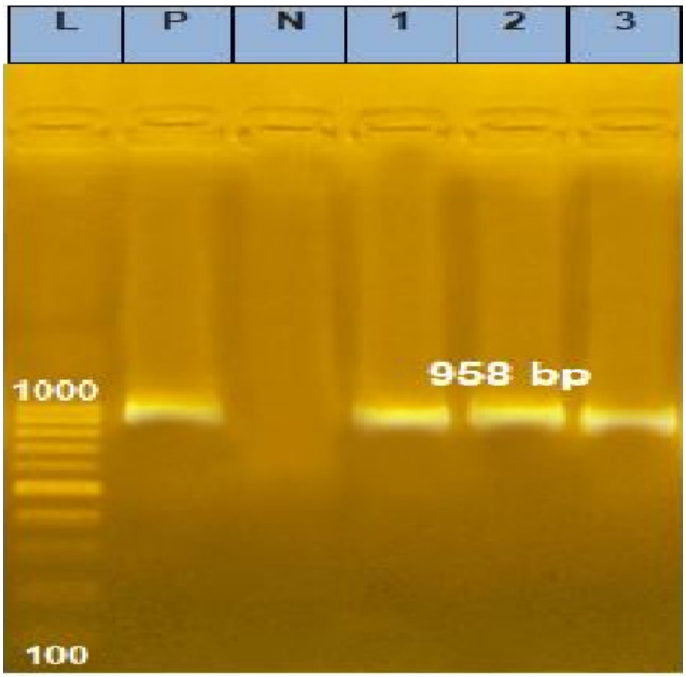


**Figure 1:** *Agarose gel electrophoresis picture of PCR product showing the amplification of fragment of EEV gene at 958 bp. P: Control positive; L: DNA ladder; N: Negative fragment of LSDV in samples; Lane 1: Positive fragment of LSDV from skin nodules; Lane 2: Positive fragment of LSDV in samples taken from vaccine as positive control; Lane 3: Positive fragment of LSDV in samples taken from vaccine as positive control.*

When analyzing nucleotide sequence of EEV gene of the LSDV Egyptian isolated strain in the current study showed 100% nucleotide identity with Egyptian LSDV (Al-Sharkia) OL690031.1 F11-LSD 2221 and OL690032.1 F12-LSD 2221 (outbreak 2021) and 99.8 % with South Africa LSDV MH670904, Egyptian MH639086.1 (outbreak 1988) and Israel LSDV KF985232.1 (outbreak 2006) and 99.7% with Israel LSDV KF985233.1, LSDV KF985233.1 and LSDV KF985233.1 (outbreak 2012).

Furthermore, when compared with LSDV vaccine strains, the LSDV strain showed nucleotide identities 99.7% with Kazakhstan MT130502.1 Lumpy skin

disease virus strain Neethling-RIBSP vaccine and 94.1% with South Africa lumpy skin disease virus strain Neethling-LSD vaccine-OBP KX764645.1. Lumpy skin disease virus strain SIS-Lumpyvax vaccine KX 764645.1.

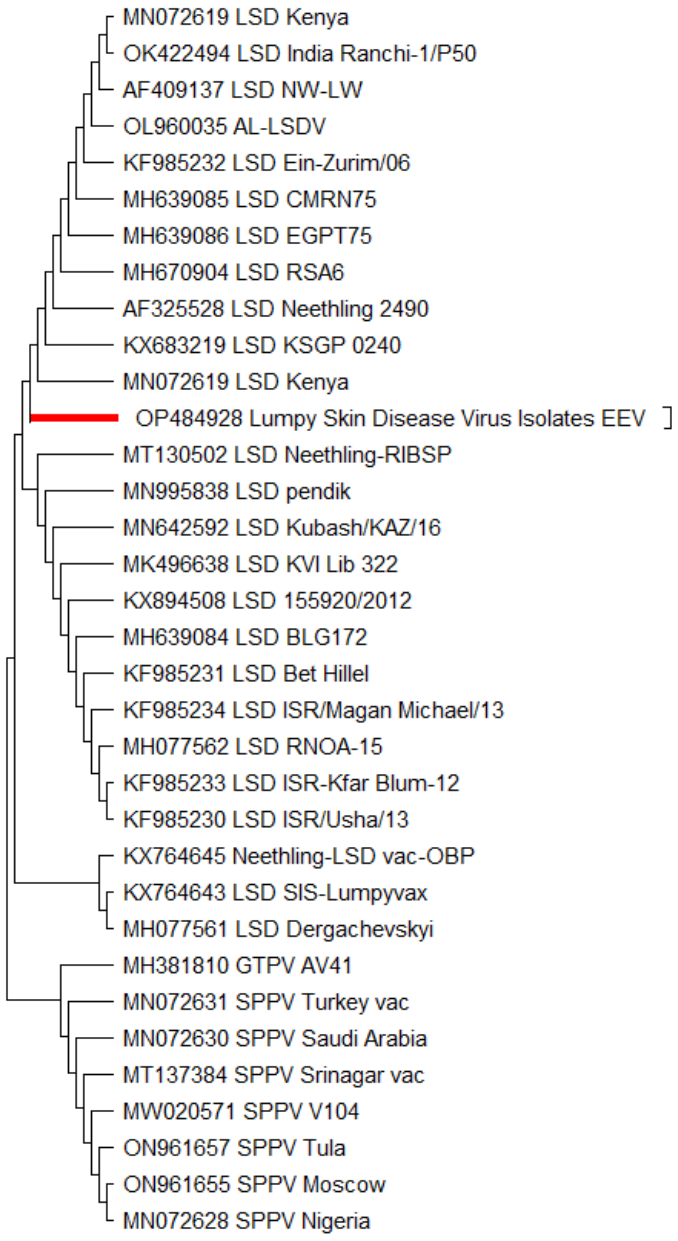


**Figure 2:** *Neighbor joining Phylogenetic tree of based LSDV isolate (OP484928) and vaccinal strains based on nucleotide sequences of EEV gene and drawn by MEGA 11.*

Pair wise nucleotide and deuced amino acids sequence alignments of the partial nucleotide sequences of the EEV gene showed major sequence differences between the vaccine strain and the LSDV field isolate (Figures 3 and 4). The pair wise nucleotide sequence alignment showed that the vaccine strains carries 27 bases (deletion) (Figure 4) less than the virulent virus in the extracellular enveloped virions (EEV) gene and have substitutions at bp: A 116255 G, C 116442 T, T 116514 C, A 116602 T, A 116601 T, T116627 C, G 116630 A. There is a deletion in LSD virulent virus nucleotide sequence at bp 600, 797, 857, 865, 870, 873 and 883.

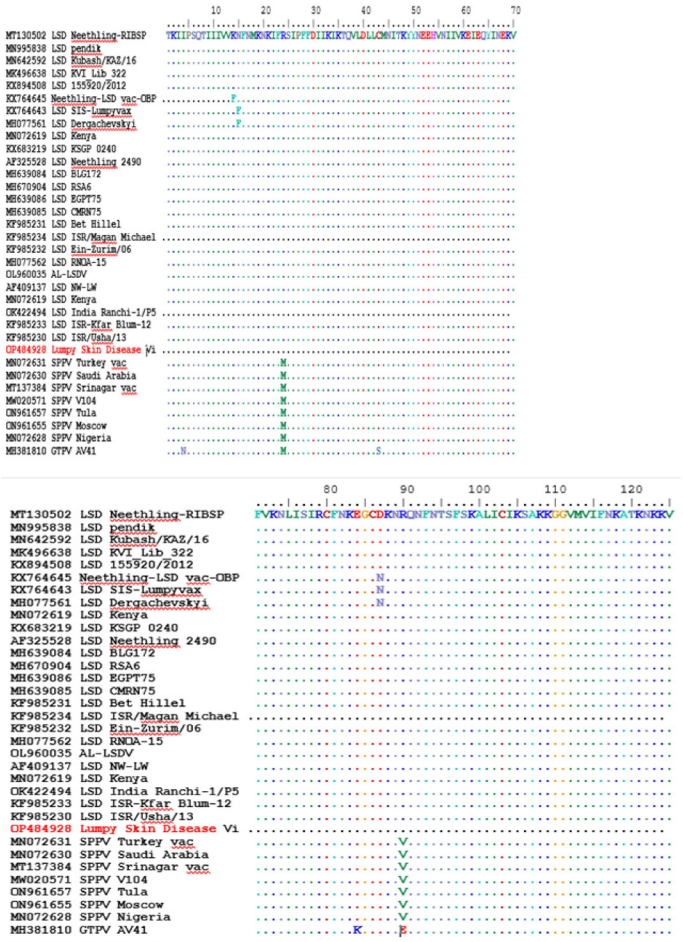

**Figure 3:** *Deduced amino acids multiple sequence alignments of the EEV gene. The Egyptian LSDV (Accession No. OP484928) isolate were aligned with representative LSDV sequences retrieved from GenBank, Lumpy skin disease virus strain Neethling-LSD vaccine-OBP (Accession. No. KX764645.1), Lumpy skin disease virus strains SIS-Lumpyvax vaccine (Accesion No. KX 764643.1). The dots indicate the identical nucleotides in the alignment and digit detect the changes between sequences.*

Deuced amino acids sequence alignments (Figure 3) showed that there are substitutions in LSD virulent virus amino acids sequence in comparison to vaccine strains at: M 24 R, V 90 R and N 89 D.

Lumpy Skin Disease (LSD) is an infectious disease of cattle caused by Lumpy Skin Disease Virus (LSDV) of the family Poxviridae. Currently the disease has been emerged as a devastating threat for the large domesticated ruminants in Asia, Europe, Africa and the Middle East including Egypt.

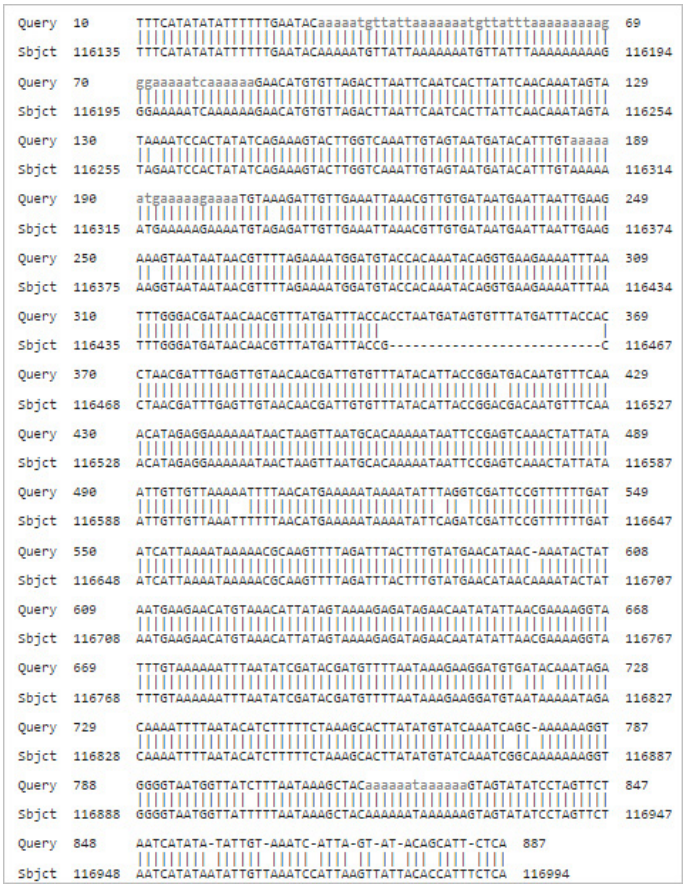


**Figure 4:** *Pair wise nucleotide sequence alignment. LSDV (Query) aligned against Lumpy skin disease virus strain Neethling-LSD vaccine-OBP (acc. KX764645.1) (Subject).*

Our results revealed that PCR assay detects LSDV in 55 samples out 100 skin nodules and blood samples from the corresponding infected cattle.

Sequencing of the PCR amplicons and phylogenetic analyses were helpful to trace the new LSD outbreak and can be useful in developing genetic tools for epidemiological investigations and development of effective LSDV vaccines (Varshovi *et al*., 2009). To prevent the introduction of LSDV from neighboring countries during importation, it is strongly advised to use the PCR assay during quarantine examination for live domestic and wild bovine species, as well as bovine semen from endemic countries (Tuppurainen *et al*., 2017).

The evolutionary analysis of the EEV gene sequences was able to classify the CaPVs into three separate groups: LSDV, SPPV and GTPV. Our results showed that LSDV isolates being collected from El-Menofia province, Egypt were closely related to LSDV isolates previously reported in Kazakhstan, Turkey, Israel, South Africa and Kenya. The BLAST similarity analysis was 99.7%-100% between the LSDV sequences and the sequences in GenBank

The ability to distinguish between field and vaccine LSDV strain was demonstrated in this investigation. Despite widespread vaccination, the disease appeared to be common in the Egyptian dairy farms. The Veterinary Services opted to utilize living attenuated vaccination based on the Neethling vaccine strain to stem the spread of LSD (Elhaig *et al*., 2017). One of the major issues with this method is the requirement to create a DIVA system that would allow differentiation between vaccinated animals from infected ones.

The initial step in this strategy was to detect enough variations between the various nucleotide sequences to distinguish the field strains from vaccine strains; the alignment revealed a unique 27-base deletion in the vaccine strains. The existence of the deletions in the vaccine strains was confirmed by sequence alignment. The discovery that the deletion happened exclusively in the vaccine strains and was not found in any virulent strain prompted the hypothesis that this sequences is necessary for virulence in field viruses and that when this 27-base segment is lacking, the virus is attenuated. The obtained findings agreed with Sophia *et al*. (2014).

LSDV is thought to be introduced during cattle importation from neighboring countries such as Kenya, South Africa and Israel, as a lack of strong quarantine measures results in the disease's quick spread.

## Conclusions and Recommendations

These findings highlight the significance of ongoing LSDV strain characterization and monitoring, as well as the need to continuously improve methods for distinguishing vaccine strains from field viruses.

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## Author's Contribution

All authors participated in the concept development, study execution, data analysis and interpretation processes. The first draught of the manuscript was written by all contributors. All authors had full access to all the study's data, accepted responsibility for the validity of the data and the accuracy of the analysis and gave their final approval before publication.

*Conflict of interest*

The authors have declared no conflict of interest.

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