**Original Paper****Genetic diversity of LSDV isolates from Menofeia Governorate, Egypt, summer 2019 based on the G-Protein coupled chemokine receptor (GPCR) gene.**Eman E. A. Rashed¹, Omayma A. Shemies¹, Saad S. A. Sharawi²¹Department of Virology, Animal Health Research Institute, Dokki, Egypt.²Department of Virology, Faculty of Veterinary Medicine, Benha University, Egypt**ARTICLE INFO****Keywords**

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ABSTRACT

An outbreak of lumpy skin disease (LSD) was recorded among cattle in summer 2019 at Menofeia Governorate, Egypt. Lumpy Skin Disease Virus (LSDV) isolation from skin nodules of suspected cattle (n=35) on embryonated chicken eggs chorioallantois membranes (ECs CAMs) revealed pock lesion on CAMs after three passages. Twenty-six isolates were confirmed positive for LSDV by PCR using specific primers designated for the G protein-coupled chemokine receptor (GPCR) gene with the predicted amplicon size of 554 bp. Sequencing and analysis of the obtained amplified segment of GPCR for the three isolates were deposited in GenBank by accession numbers OP881988, OP881989 and OP881990. Phylogenetic analysis and several alignments of the resulting sequences of GPCR gene of recent LSDV circulating in Menofeia Governorate fell within the cluster of fields LSDV virulent strains with high nucleotide identity and did not reveal significant genetic variations when compare with local reference strains as well as vaccinal strains. These viral isolates have a high identity, in the range of 97.5%–100% with LSDV either isolated from Egypt as LSD Egy/BSU-1 (KJ561442) or different foreign countries such as Turkey (KR024774), and identity percentage was 95.9% with the LSDV vaccinal strains as LSD SIS-Lumpyvax (KX764643) and in the range of 92.6 to 93.2 with sheep poxvirus (SPPV) and goat poxvirus (GTPV) strains.

1. INTRODUCTION

Lumpy Skin Disease Virus (LSDV) has DNA genome of 151 kbp that codes for 156 putative genes (OIE, 2020). Lumpy Skin Disease Virus (LSDV), Sheep pox virus (SPPV), and Goat pox virus (GTPV) have high antigenic resemblance and genetic identity (> 96%) (Tulman et al., 2002). Lumpy skin disease (LSD) is clinically distinguished by fever, cutaneous nodules on the body, and enlarged superficial lymph nodes (Ochwo et al., 2020). Morbidity rates can range from 1% to nearly 100%, with mortality rates between 1% and 3%. (Tuppurainen et al., 2017; OIE, 2018). In Egypt, first outbreak of LSD was recorded in 1988 (OIE, 2020) later, many epidemics were noted in various years (2006, 2015, 2016) (Sharawi and Abd El-Rahim, 2011; Tamam et al., 2018; Rouby et al., 2019). In various countries, homologous and heterologous live attenuated Sheep pox virus (SPPV) or Goat pox virus (GTPV) vaccines were used with varying degrees of achievement to control LSD (Klement et al., 2020). In spite of the Egyptian authorities' annual mass vaccination, LSDV still mingles nearly every summer in Egypt (Hodhod, 2020). LSD is typically diagnosed using different techniques. Serological diagnosis is time-consuming but not distinguish between various capripoxviruses members (El-Bagoury et al., 2009). Capripoxviruses are GPCR homologues that are responsible for cell proliferation as well as suppressing the host's antiviral effect (Kostenis, 2004). Members of the genus Capripoxvirus can be identified using phylogenetic analysis of the GPCR gene,

clearly separated into distinct and clear sets (Le Goff et al., 2009).

The present work was carried out for the isolation of LSDV by passage on CAM of SPF-ECE then identification of GPCR gene using PCR. Studying homology and diversity of LSDV isolates in comparison to reference and vaccinal LSDV strains by sequencing and phylogenetic analysis based on GPCR gene.

2. MATERIAL AND METHODS

All procedures were approved by Animal Ethical committees, Benha University with ethical approval number (BUFVTM 23-10-22).

2.1. Area of the study and sampling:

During the summer 2019, Thirty-five skin nodules samples were aseptically collected from cattle at different localities in Menofeia Province (Shebein El-Kom, Quesna, Berket El-Sabea, Menouf and Alshuhada) showed severe symptoms where nodules scattered all over the body parts. These samples were labeled, transported, and stored at -80 °C according to OIE (2018) until isolation.

2.2. Specific pathogen free embryonated chicken egg (SPF-ECE):

In the present study, eleven -day-old SPF-ECE were supplied by Animal Health Research Institute (AHRI) Dokki- Giza and used in the trial for LSDV isolation from the prepared suspected LSDV skin nodules.

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2.3. Isolation of LSDV virus:

Trials for isolation of LSDV were performed for three blind passages on the CAM of SPF-ECE (House et al., 1990).

2.4. DNA extraction:

DNA was extracted from local LSDV isolates using QIAamp DNA Mini Kit according to the manufacturer's instructions. Briefly, 200 µl of the local LSDV isolates with 30 min of lysis step at 56 °C, and the DNA was eluted in 50 µl of elution buffer included in the kit. The DNA concentration was checked by UV spectrophotometer (Eppendorf BioSpectrometer kinetic), and the DNA was stored at -20 °C for PCR assay (Sambrook et al., 2000).

2.5. Oligonucleotide primers:

The PCR primers were produced by Bio Basic, Canada Inc. of Markham, Canada to amplify the GPCR gene that is specific for the LSDV and had the following sequence: LSDVF (5'-AGT ACA GTT AGT AGC GCA ACC-3') and LSDVR (5'-GGG TGA ACT ACA GCT AGG TAT C-3') to amplify a 554 bp fragment of the GPCR gene (Le Goff et al., 2009).

2.6. Polymerase chain reaction:

The PCR reaction was carried out according to the procedures of Le Goff et al. (2009), in a total volume 25 µl was conducted 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, then complete up to a final volume with Nuclease-free water. DNA amplification was performed in BIO-RAD® PCR system T100 thermocycler (BioRad, Hercules, California, USA) preheated and adjusted at cycling protocol: initial denaturation at 95 °C for 10 min., then 40 cycles of denaturation at 95 °C for 60 sec., annealing at 50 °C for 60 sec., and extension at 72 °C for 60 sec., with final extension at 72 °C 10 min.

2.7. Detection of the PCR amplified products:

The amplified PCR products (10-15 µl) were analyzed by 1.5% agarose gel electrophoresis, according to Viljoen et al. (2005). The DNA band of predicted size visualized and detected using Molecular Imager Gel Doc™ XR+ Imaging system (BIO-RAD) using Image lab™ software for analysis of gel images in comparison with 100 bp DNA molecular weight marker.

2.8. Sequencing and phylogenetic analysis:

Three of the positive samples were chosen as representative samples from each locality that gave a strong band on the gel electrophoresis after PCR to ensure good results and fidelity of sequencing of the specific gene after being purified from the gel. The three positive samples by conventional PCR were purified by QIA quick gel extraction kit (Qiagen, Germany), Sequencing was completed by 3500 XL Genetic Analyzer in the Genome Research Unit, Animal Health Research Institute (AHRI), Egypt.

2.9. Publishing of the LSDV isolate sequences at gene bank:

Sequence data of GPCR for the three isolates were sending to GenBank database and it was published below accession numbers OP881988, OP881989 and OP881990.

2.10. Multiple alignment and phylogenetic analysis:

Nucleotide sequences of GPCR genes of LSDVs were searched in the GenBank database using BLAST (National

Center for Biotechnology Information, Rockville Pike, Bethesda, MD) resemblance search option. LSDV GPCR gene coding domain sequences chosen from GenBank data, involving sequences recorded in this study for phylogenetic analysis. Multiple nucleotide sequence alignments were achieved by Bioedit with ClustalW method and Phylogenetic analyses were achieved by maximum likelihood (ML), neighbor joining and maximum parsimony in MEGA6 (Kumar et al., 2018).

3. RESULTS

3.1. Isolation of LSDV from skin nodules on ECE:

Out of thirty-five samples, twenty-six were induced characteristic pock lesions (numerous, small, scattered white foci) on CAMs after three successive passages. The pock lesions became more prominent after six days of inoculation at the 3rd passage. (Table 1, Fig. 1).

Table 1 Results of isolation of suspected LSDV skin nodules on CAM of SPF ECE.

Localities	No of skin nodules samples	No. of positive samples	Positive percent
Shebein El-Kom	9	6	66.7%
Queesna	8	7	87.5%
Berket El-Sabea	6	4	66.7%
Menouf	5	3	60%
Al Shuhada	7	4	57%
Total	35	26	74.3%

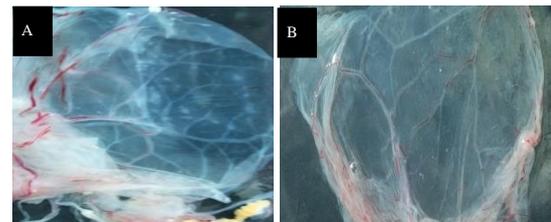


Fig. 1 A: Characteristic pock lesions of suspected skin nodules LSDV samples on CAM of SPF ECE after the third passages. B: Control normal non- infected CAM of SPF ECE.

3.2. Detection of LSDV specific DNA by PCR:

PCR assay was carried out using LSDV-specific primers for the amplification of a DNA fragment of 554 bp of the GPCR gene in Suspected LSDV isolates. PCR amplified products were analyzed by agarose gel electrophoresis, which revealed that 26 viral isolates were positive with a clear and obvious band and a predicted size of 554 bp for the GPCR gene fragment. None of the negative controls created any amplicons (Fig. 2).

3.3. Multiple sequence alignment and phylogenetic analysis:

Sequences were allied with GPCR gene sequences of CaPVs existing in the GenBank by ClustalW2. The generated phylogenetic tree of GPCR gene sequences revealed 4 genetically clear cluster lineages (LSDV field isolates, SPPV, GTPV, and LSDV vaccine strains) (Fig. 3). In the present study, the epidemic strains (characterized with red rings) are observed in the LSDV field isolates group.

A comparison of the nucleotide sequences of the GPCR gene of our local LSDV isolates (Fig.4 & Fig.5 and Fig.6) showed close nucleotide sequence homology (100% identity) and a very high percentage of identity range from 97.5-100% with all virulent LSDV isolated from various epidemics in Egyptian governorates (MN207143-KP071937- LC573970- MF156211), and in Turkey(KR024774), Sudan (FJ869369), KSA (MN422452)

and inda (OP056771) (Fig.4) .This percentage of identity dropped to be 95.9% with LSDV vaccine strains as LSD SIS- Lumpyvax(KX764643), LSD Herbivac LS(MK441838) and Neethling-LSD vac – OBP(KX764645), and in the range of 92.6% to 93.2 % with SPPV as SPPV Rumanian Fanar (KF495237) and GTPV strains as GTPV Pellor (AY077835) (Fig.4) . The sequences of the GPCR gene of the three local isolates were registered in GenBank as: LSDRashed/ Menofeia/ Egy2019-1, LSDRashed/Menofeia/Egy2019-2 and LSDRashed/Menofeia/Egy2019-3, with accession numbers OP881988, OP881989 and OP881990, correspondingly.

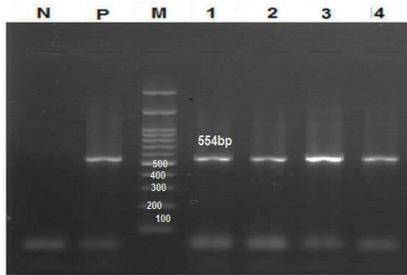


Fig. 2 Agarose gel electrophoresis of amplified PCR products of local isolates based on G-protein- coupled chemokine receptor gene. Lane M: High molecular weight nucleic acid marker (100 bp). Lane N: Negative control. Lanes 1-4: positive local isolates samples (554 bp). Lane P: positive control.

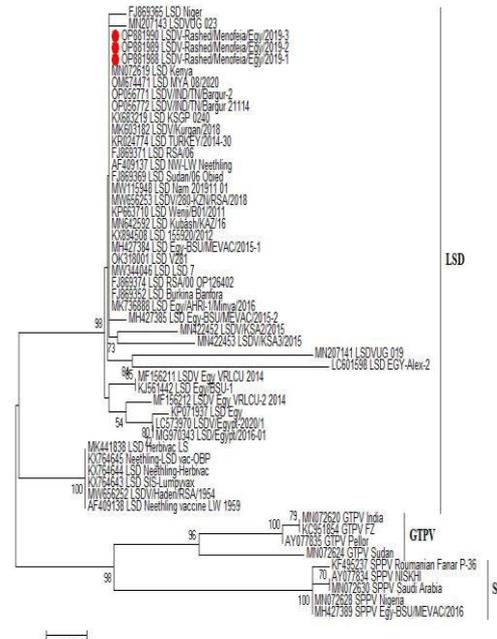


Fig. 3 Phylogenetic tree based on GPCR Nucleotides sequences of local LSDV isolates, red rounds, with other CaPVs whose GPCR genes were from Gen Bank Database by using maximum likelihood method.

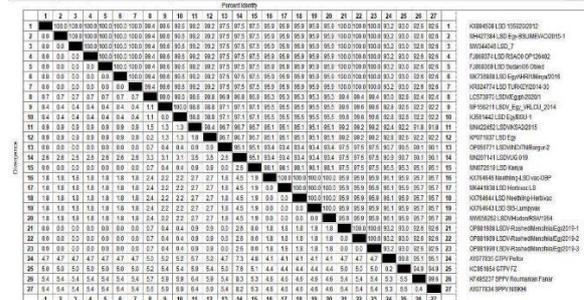


Fig. 4 Pair wise sequence distance for GPCR gene of local LSDV isolates with reference LSDV, sheep pox virus and goat pox virus published in GenBank.

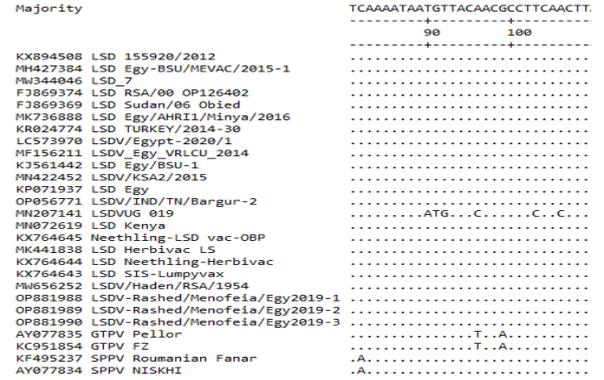
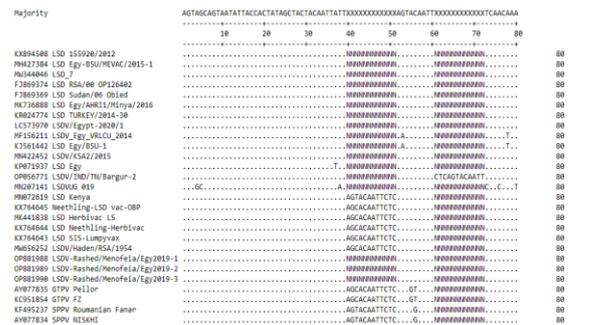


Fig. 5 Multiple alignments of first 100 nucleotides of LSDV detected from our isolates with sequences of CaPVs recovered from GenBank

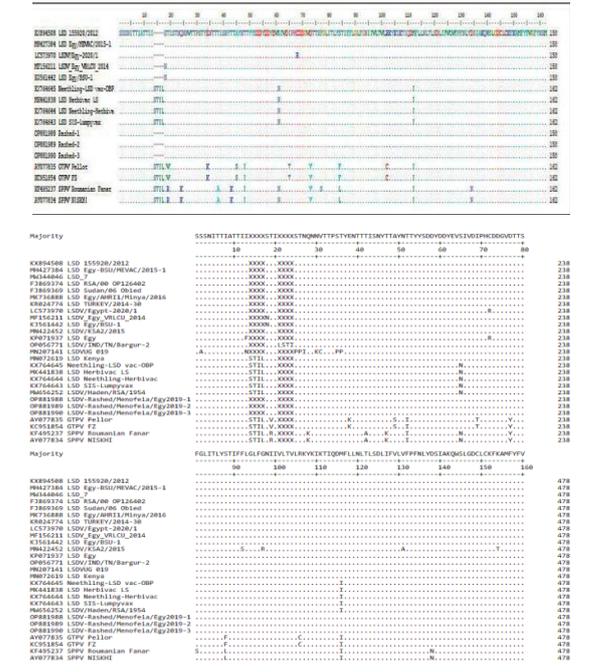


Fig. 6 Multiple alignments of the amino acids for GPCR gene of local LSDV isolates with sequences of reference LSDVs from GenBank

4. DISCUSSION

In Egypt, where LSD is prevalent, vaccination with the LSDV Neethling vaccine is used. Numerous epidemics were recorded at various time intervals, there were clearly some flaws. During the summer of 2019, a new LSD epizootic was observed, affecting cattle of various ages and both sexes in various locations throughout Menofeia governorate. The current study is aimed at molecularly identifying and characterizing the LSDV strains responsible for such an outbreak.

The suspected cattle in this study showed clinical signs of LSD and its complications including sharp fever, loss of appetite, and decreases in milk production with the presence of characteristic hard nodular lesions and some of these nodules were necrotic and sloughed. Additionally opacity of the cornea, lymphadenopathy, edema of the forelegs, and tarry-like diarrhea were also recorded. These signs were also mentioned by Tasioudi et al. (2016), Tassew et al. (2018) and Rouby et al. (2019).

In this study, trials for LSDV isolation from skin nodules specimens from diseased cattle on CAM of SPF-ECEs, identification of the virus isolates by molecular characterization using PCR and Studying homology and diversity of LSDV isolates in comparison to reference and vaccinal LSDV strains by sequencing and phylogenetic analysis based on GPCR gene. Isolation of LSDV from 35 skin nodule samples through inoculation on the CAM of SPF-ECE, positive results were detected in 26 samples (74.3%) out of them. After the first passage, small, scattered white foci were detected, after the 3rd passage, they became numerous, clear, and distinct. (Fig. 1). These results concur with those of El-Kenawy and El-Tholoth (2011) and Hodhod (2020).

Viral DNAs were noticed in the 26 isolates by PCR. PCR results established that the cause of this outbreak is LSDV. This indicates that PCR could aid as a fast, real, and specific method for lab validation of CaPVs. This finding was in accordance with prior studies by El-Kenawy and El-Tholoth (2011) that identify LSDV by PCR in CAMs of ECEs. According to various studies, LSDV was the main cause for numerous epidemics of the disease in various governorates in Egypt over the earlier years. (El-Bagoury et al., 2009; Abdallah et al., 2018; Hodhod et al., 2020).

The outbreak occurred in the Menofeya governorate in summer 2019, with high temperature and relative humidity, resulting in rise in insect population, which is significantly increased by humidity and temperature (Ayelet et al., 2014; Zeynalova et al., 2016; Kasem et al., 2018). As a result of constant import of live cattle, particularly from Africa, the molecular study of the origin of LSDV is very significant in epidemiology of the disease. The genetic characteristics of detected LSDV, from examined cattle in Menofeya governorate, were assessed by interpretation of the sequence identity and phylogenetic tree. The achieved sequence and all field isolates in Egypt were recorded in the similar cluster, which indicated that the circulating strain in various Egyptian governorates is the same and constant in nature of LSD. Despite its distribution in various geographical regions, the LSDV genome is highly conserved (Yousefi et al., 2018; Adedeji et al., 2019).

Database searches and blast analyses suggest that the sequence similarity between this outbreak LSDV sequence and the Egyptian virulent isolates stored in Genbank is quite high (> 97.5-100%). The phylogenetic analysis of the obtained partial GPCR gene sequence was able to classify capripoxviruses into four different clusters (LSDV field isolates, SPPV, GTPV, and LSDV vaccinal strains); this is strongly confirmed by 1000 bootstrap replicates confidence. These results were also reported by Adedeji et al. (2019) and Ochwo et al. (2020). Our results showed higher homology to LSDV reference strains specially reference strain number 2 in pairwisw table MH427384 (vaccinal strain of MEVAC Egypt) but lower homology to sheep pox and goat pox viruses.

The field LSDV lineage, which includes the current outbreak strains, is more related to GTPV than SPPV. Similar findings were reported by Le Goff et al. (2009), who mentioned that LSDV was closely associated with

GTPV rather than SPPV, whereas Beard et al. (2010), El-Kenawy and El-Tholoth (2011), and Rashid et al. (2017) reported that LSDV is more closely associated with SPPV than GTPV.

Despite this, genomic investigations found that members of the Capripoxvirus genus developed from a single common ancestor (Tulman et al., 2002; Le Goff et al., 2009).

Phylogenetic analysis depends on the GPCR gene in this study revealed that entirely field isolates of LSDV were situated in a separate cluster away from the vaccinal strains, which appears to be useful in distinguishing the field isolates from the vaccinal strains.

5. CONCLUSION

The first step toward controlling infectious diseases appears to be an accurate and timely diagnosis. GPCR-based PCR is an extremely useful technique for diagnosing LSD in susceptible animals. According to our findings, the Egyptian LSDV strains and our isolates share a high degree of homology. The sequencing of the GPCR gene is useful not only in distinguishing between different Capripoxvirus members, but also in distinguishing between field and vaccinal strains of LSDV.

6. REFERENCES

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