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Phylogenetic and sequence analysis of Lumpy skin disease virus circulating in some governorates of Egypt during 2019/2020

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ABSTRACT

Between July 2019 and September 2020, cases of lumpy skin disease (LSD) were reported in Egypt. 15 skin nodule samples, one blood sample and one nasal swab sample were collected from clinically infected cattle from eight governorates in Egypt in addition to one skin scab sample was collected from sheep clinically suspected for sheep pox virus (SPV) from El-Menia governorate. Samples were examined by real time polymerase chain reaction (PCR) where they were positive for lumpy skin disease virus (LSDV) and the SPV sample was positive. Then four samples were isolated for three passages on chorio-allantoic membrane (CAM) of specific pathogen free eggs (SPF-ECEs) and confirmed by presence of pock lesion. Finally, partial gene sequencing was applied for three strains based on GPCR gene. The results showed that the strains belong to the LSDV group. Two of the strains were identical, while one was almost the same with a 99.78% identity. The three strains had 12 nucleotides deletion in return for the Indian field strains of the 2019 outbreak in India, (NI-2490/Kenya) and (KSGP O-240, Kenyavac, KS-1) from Kenya. One strain was partially sequenced for three genes (P32, ORF 95 and ORF 103) to identify it more, and only ORF 103 was partially sequenced for the SPV sample for comparison. The ORF 103 comparison between LSDV strain and SPV sample showed 97.17% identity and 13 nucleotides dissimilarities. Results consequently points to a close relationship between the study strains and the circulating LSDV in Egypt, Africa, and the Middle East.

1. INTRODUCTION

Lumpy skin disease (LSD) is a serious skin disease of cattle caused by single strain of Capripoxvirus, it belongs to family *Poxviridae* and antigenically related to sheep and goat poxvirus (El-Desawy, 2008). The Lumpy skin disease virus (LSDV) is characterized by high morbidity and low mortality rates and affect all ages and breeds of cattle (Hailu, 2015). LSD is listed as a notifiable disease under the OIE guidelines (Möller et al., 2019).

The first presence of LSDV in Egypt was in 1988 in Suez governorate after cattle were imported from Somalia (Abera et al., 2015). Severe LSD outbreak occur in 2006 among foreign and local cattle in different Egyptian governorates (El-Kholy et al., 2008).

The genome of LSDV is a 150Kbp long double-stranded DNA, covalently cross-linked at the ends. Capripoxvirus genomes is highly conserved and there is homology more than 95% amongst LSDV, SPV and goat pox virus (Stram et al., 2008). G-protein-coupled chemokine receptor (GPCR) gene of capripoxviruses (CaPVs) plays a role as host discriminative gene (El-Tholoth and El-Kenawy, 2016). Characterization of CaPVs needs molecular detection targeting Capripoxviruses specific genes such as RPO30 and P32 genes (Yan et al., 2012). ORF103 gene can be used for genotyping and phylogenetic analysis of CaPVs (Zhu et al., 2013). ORF95 encodes the virion protein which form a great part of protein content of the virion and is important during the assembly of virion (Zhao et al., 2012)

LSDV transmitted mechanically by Aedes aegypti female mosquitoes and ticks (Tuppurainen and Oura, 2014), or by ingestion of feed and water contaminated with infected saliva (OIE, 2017). It is characterized by skin nodules about 0.5-5 cm in diameter in whole skin or subcutaneous tissue and superficial lymph nodes are swollen and cattle infected with LSDV suffered biphasic fever (40°C-41.5°C), depression, inappetence, salivation, and ocular-nasal discharges (Abdulqa et al., 2016; El-Kholy et al., 2008).

LSDV could be isolated on CAM of embryonated chicken egg of 7 – 9 days old or on tissue culture of bovine, ovine or caprine origin (Sharawi and Abd El-Rahim 2011), and identified using transmission electron microscope or Histopathological examination (Haftu, 2012; Bakar et al., 2021). It can be detected serologically by serum neutralization test (SNT), Enzyme Linked Immunosorbent Assay (ELISA), Agar gel precipitation test (AGPT) and fluorescent antibody technique (FAT) (Möller et al., 2019; Helmy et al., 2017). Polymerase chain reaction (PCR) is the least expensive and quickest method for detection of LSDV in different samples as skin nodules and scabs, saliva, nasal secretions, and blood (OIE, 2017). Real time PCR assays are quantitative, more sensitive, simpler and faster if compared with conventional PCR (Vidanović et al, 2016).

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In view of the numerous reports of LSDV cases in Egypt in 2019 and 2020, the purpose of this study was to facilitate rapid detection, isolation, and molecular analysis of the LSDV that caused these cases, and also to compare it with SPV.

2. MATERIAL AND METHODS

2.1 Virological Samples

Fifteen skin nodule samples, one blood sample and one nasal swab sample were collected from suspected animals showed fever and skin nodules as clinical signs of LSD from eight governorates in Egypt during the period between July2019 and September 2020 (Table 1). One skin scab sample was collected form sheep from El-Menia governorate suspected for SPV. These samples were labeled, transported and stored at -80 according to OIE (2010) until be used for real time PCR, isolation and sequencing.

Table 1 Details of samples collected from different Egyptian governorates in the period between July 2019 and September 2020.

Year	Governorates	Sample type			
		Skin nodule	blood	Nasal swab	Total
2019	Beheira	3	0	0	3
	Menofia	2	0	0	2
	Kafr El-Shikh	1	0	0	1
	Gharbia	1	0	0	1
	Total	7	0	0	7
2020	Beheira	0	1	1	2
	Menofia	4	0	0	4
	Elwadi Elgadid	1	0	0	1
	Dakahlia	1	0	0	1
	Sharkia	1	0	0	1
	Kalyobiya	1	0	0	1
	Total	8	1	1	10
	Total	15	1	1	17

2.2. Taqman Real time PCR

Preparation of collected samples and extraction of viral DNA was performed using (G-SPIN™ Total DNA Extraction Kit, Korea) according to the manufacturer instructions, then the DNA was stored at -20 until be used. Mixing step was performed using commercial kit (LSDV dtec-qPCR test, GPS, Spain). The content of the mix kit was rehydrated and used according to the manufacturer. The reaction was prepared in a sterile 0.2 ml tube with final volume of 20 µl prepared and mixed thoroughly by pipetting **(**4µl mix stable qPCR 5x, 1µl specific primer/probe, 10 µl DNase/RNase free water and 5µl template (sample, positive, or negative). The mix was then applied to the Applied Biosystems™ Step One real time PCR system, with the following cycling conditions as per the manufacturer: 1 cycle at 95°C for 15 min for (activation), followed by 40 cycles at 95°C for 15 sec for (denaturation) and 60°C for 1 min for (hybridization/extension and data collection). The cut off was determined by the manufacturer at 35 cycle threshold (Ct) value. The positive control was among the content of the commercial kit.

2.3. Isolation of suspected LSDV on CAM

Four LSDV real time PCR positive skin nodule samples were isolated on chorio allantoic membrane (CAM) of SPF-ECEs for three passages. The SPF-ECEs were obtained from the specific pathogen free egg project, Koshim, El-Fayoum governorate. They were incubated at 37°C and 70% humidity until being inoculated at 9 days of age via CAM route according to House et al. (1990). They were examined daily for 7 days post inoculation. Eggs with died embryo within the first 24 hours were considered as non-specific death. The samples were compared each passage with non-inoculated negative control.

2.4. Sequencing and sequence analysis

2.4.1. Conventional PCR for GPCR gene, P32, ORF 95 and ORF 103

Conventional PCR was utilized to detect nucleic acids in three of the harvested CAMs. The PCR amplification reaction was performed according to the manufacturer instructions using AmpliTaq Gold™ 360 Master Mix (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA). Primers used for amplification of partial sequence of GPCR gene, P32 gene, ORF95 and ORF103 was manufactured by Bio Basic, Canada Inc. (Markham, Ontario, Canada) (Table 2).

The PCR reaction of 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 according to the target PCR products (Table 3). Positive and negative controls were included. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis according to Sambrook et al. (1989). The DNA bands of predicted sizes visualized and detected in comparison with 100 bp DNA molecular weight marker.

Table 2 Primers sequences for GPCR gene, P32 gene, ORF 95 and ORF 103.

Gene name	Forward primer	Reverse primer	Product size	References
GPCR	5'-AGT ACA GTT AGT AGC GCA ACC-3'	5'-GGG TGA ACT ACA GCT AGG TAT C-3'	554 bp	(Hussein *et al.*, 2017)
P32	5'-CTA AAA TTA GAG AGC TAT ACT TCT T-3'	5'-CGA TTT CCA TAA ACT AAA GTA C -3'	390 bp	(Heine *et al.*, 1999)
ORF 95	5'-ATG GAC TTC ATG AAA AAA TAT ACT -3'	5'-TTT GCT GTT ATT ATC ATC CAG -3'	483 bp	(Zhu *et al.*, 2013)
ORF 103	5'- ATG TCT GAT AAA AAA TTA TCT CG -3'	5'- ATC CAT ACC ATC GTC GAT AG -3'	570 bp	(Zhu *et al.*, 2013)

Table 3 Cycling conditions and temperatures of PCR.

Step		Temperature	Time	Number of cycles
Initial denaturation		95 ºC	2 minutes	1 cycle
Denaturation		95 ºC	15 seconds	40 cycles
Primer annealing	GPCR gene	50 ºC	60 seconds	
	P32 gene	50 ºC		
	ORF 95	52 ºC		
	ORF 103	50 ºC		
Extension		72 ºC	60 seconds	
Final extension		72 ºC	10 minutes	1 cycle
Holding		4 ºC	For ever	stop

2.4.2. Viral DNA sequencing

QIA quick® Gel Extraction Kit (Qiagen, Germany) was used to purify PCR amplicons, and then the partial sequence of each target gene was directly determined by sequencing. Sequencing was performed on Applied Biosystems™ 3500 Series genetic analyzer system through Sanger sequencing method using Big Dye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and CENTRI-SEP Spin Columns for Removal of Dye terminators (PRINCETON SEPARATIONS, Inc., Adelphia, NJ, USA). The primers used for sequencing were

as in Table (2) and the cycling protocol to each target gene was as in Table (3).

2.4.3. Sequence alignment and phylogenetic analysis

The partial sequences of GPCR gene, P32 gene, ORF95 and ORF103 were submitted to Gene Bank and assigned accession numbers including (OQ448186, OQ448187 and OQ448188) for GPCR, (OQ448191) for P32, (OQ448192) for ORF95, (OQ448189) for ORF103 of LSDV isolate and (OQ448190) for ORF103 of SPV sample. Then they were searched in the GenBank database (National Centre for Biotechnology Information, Rockville Pike, Bethesda, MD) using BLAST similarity search option (available at http://www.ncbi.nlm. nih.gov).

GPCR, P32, ORF95 andORF103 coding domain sequences chosen from GenBank data, including sequences reported in this study for phylogenetic analysis. Multiple nucleotide sequences alignments were performed by BioEdit with Clustal W method (Hall, 1999). MEGA 7 was used to perform phylogenetic analysis with Neighbor-Joining method (NJ) (Kumar et al., 2016)

3. RESULTS

3.1. Molecular detection of LSDV and SPV by Real time PCR

Fifteen skin nodule samples, one blood sample and one nasal sample from clinically suspected animals from different governorates in Egypt between 2019 and 2020 were positive for LSDV using real time PCR with different Ct values (Table 4). The Ct values of nodular samples are between 16-30 (Figure 1; blot A and B), while the Ct values of blood and nasal swab samples are 28 and 31 respectively (Figure 1; blot C). The suspected skin scab sample for sheep pox was positive for SPV at Ct value of 30 (Figure 1; blot D).

Table 4 Samples details and Ct values of real time PCR

Number of samples	Type of sample	Governorate	Ct value	year
1	Nodule	Beheira	18	2019
2	Nodule	Beheira	21	2019
3	Nodule	Beheira	23	2019
4	Nodule	Menofia	16	2019
5	Nodule	Menofia	21	2019
6	Nodule	Kafr El-Shikh	30	2019
7	Nodule	Gharbia	27	2019
8	Nodule	Elwadi Elgadid	20	2020
9	Nodule	Dakahlia	29	2020
10	Nodule	Sharkia	26	2020
11	Nodule	Kalyobiya	21	2020
12	Nodule	Menofia	23	2020
13	Nodule	Menofia	21	2020
14	Nodule	Menofia	24	2020
15	Nodule	Menofia	20	2020
16	Blood	Beheira	28	2020
17	Nasal swab	Beheira	31	2020
18	Skin scab from sheep	Menia	30	2020

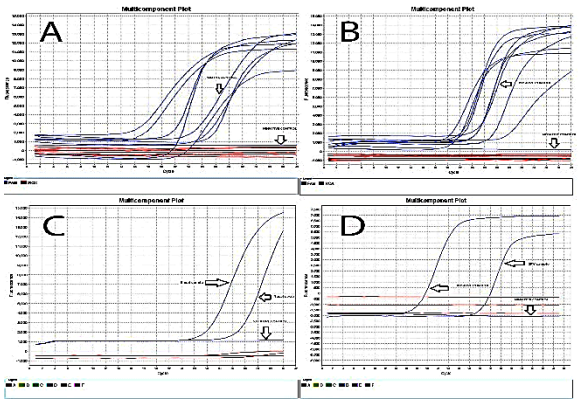

Fig (1). Multicomponent plot of real time PCR with Ct values 16-27 (A) and Ct values 20-29 (B) for skin nodules, blood and nasal swap samples with Ct values 28 and 30 (C), SPV sample with Ct value 30 (D).

3.2. Isolation of suspected LSDV on CAM of SPF-ECEs

The samples induced lesion on the CAM from first passage till third passage. The lesion characterized by congestion of CAM Blood vessels, presence of pock lesion in the form of small, scattered, numerous white foci and turbidity (Figure 2). The results then verified by conventional PCR and sequencing.

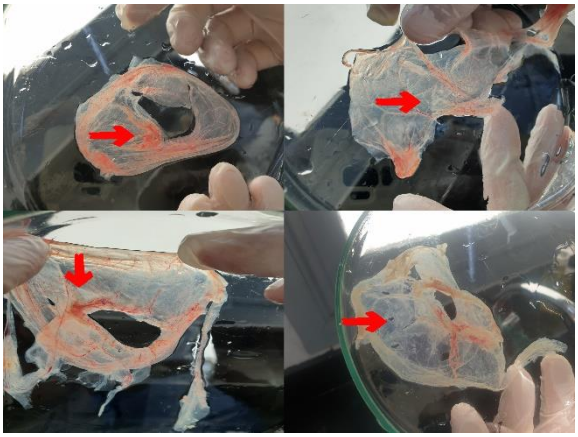

Fig (2). Lesion of LSDV on CAM of SPF-ECEs in the form of small numerous scattered opaque white foci, congestion and turbidity (Red arrows).

3.3. Sequencing and sequence analysis of LSDV isolates

The multiple alignment showed that two strains LS201/EGY/ELWADI-ELGADID/2020 (OQ448186) and LS202/EGY/MENOFIA/2020 (OQ448187) had 100% identity while LS203/EGY/MENOFIA/2020 (OQ448188) showed 99.78% identity to them. The three LSDV strains were closely related to LSDV group.

The two strains (OQ448186) and (OQ448187) showed identity more than 99% to other LSDV strains available on Gene bank with 100% identity to more than 70 of these sequences.

The two sequences showed 100% identity for example with LSDV strain (MN831843.1), LSDV isolate (MN381843), LSDV isolate (MK736888) from Egypt, LSDV strain (KP176638.1) From Turkey and LSDV NW-LW isolate Neethling Warmbaths LW (AF409137.1). The strain LS203/EGY/MENOFIA/2020 (OQ448188) showed 99.78% identity to the previously mentioned sequences (Figure 3).

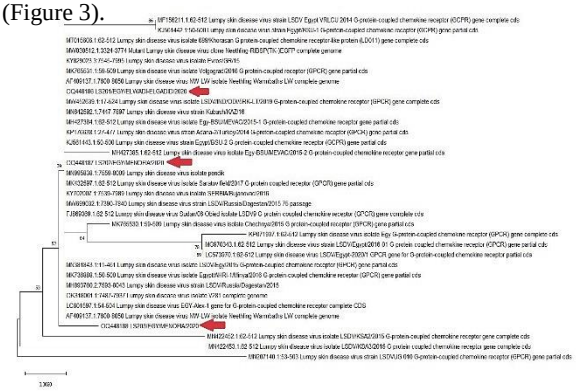

Fig (3). Phylogenetic tree based on LSDV GPCR gene sequence, of LSDV strains of this study (red arrows) with LSDV strains in GeneBank database using MEGA 7 software using Neighbor-joining (ML) statistical method.

The three sequences were compared with the Indian strain (MW452639), which similar to the historical field strains (NI-2490/Kenya) and KSGP-like strains (KSGP O-240,

Kenyavac, KS-1) from Kenya, to figure out any genetic differences. The result revealed 12 nucleotide deletions spanning from nucleotide 94 to nucleotide 105 of the entire gene (Figure 4).

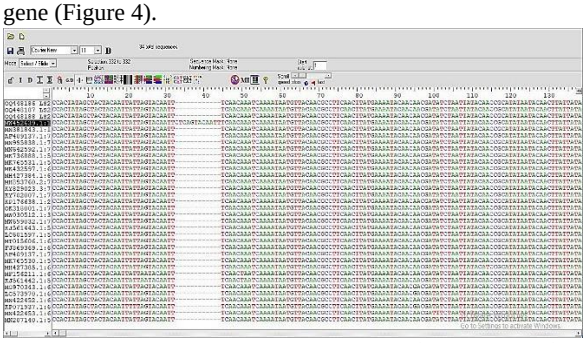

Fig (4). Multiple alignment between LSDV strains of this study (the first three strains) and LSDV strains available in GeneBank database using ClustalW toll implemented in BioEdit software showing the 12 nucleotides deletion.

The strain LS32/EGY/MENOFIA/2020/P32 (OQ448191) showed more than 98 %, identity with other LSDV strains available in GeneBank database for P32 gene. It showed 100% identity for example with LSDV isolate (KU298638) from Egypt, LSDV isolate (MN072619.1)from Kenya, and LSDV NW-LW isolate Neethling Warmbaths LW (AF409137.1). It showed more than 98 % and 97% identity with GPV and SPV strains respectively (Figure 5).

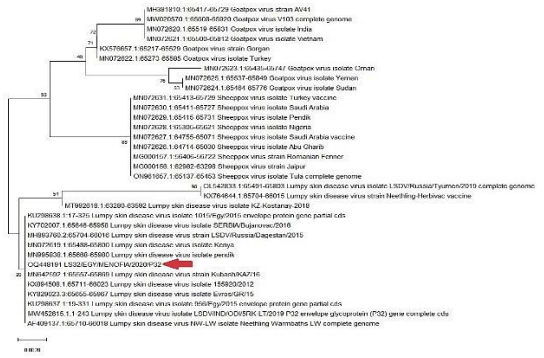

Fig (5). Phylogenetic tree based on LSDV P32 gene sequence, showing the relationship between LSDV strain of this study (red arrow) with LSDV-SPV-GPV strains in GeneBank database using MEGA 7 software using Neighbor-joining (ML) statistical method.

The strain LS95/EGY/MENOFIA/2020/ORF95 (OQ448192) showed more than 98 %, identity with other LSDV strains available in GeneBank database for ORF95. It showed 100% identity with LSDV strains such as LSDV isolate (MN072619.1) from Kenya, LSDV NW-LW isolate Neethling Warmbaths LW (AF409137.1) and LSDV isolate pendik (MN995838.1). It showed more than 98 % and 97.40% identity with SPV strains GPV strains respectively (Figure 6).

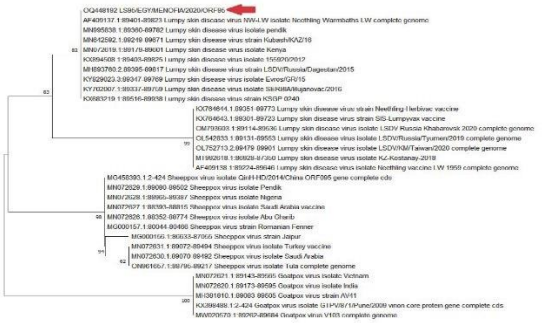

Fig (6). Phylogenetic tree based on LSDV ORF 95 sequence, showing the relationship between LSDV strain of this study (red arrow) with LSDV-SPV-GPV strains in GeneBank database using MEGA 7 software using Neighbor-joining (ML) statistical method.

The alignment of LS103/EGY/MENOFIA/2020/ORF103 (OQ448189) with SP103/EGY/EL-MENIA/2020/ORF103 (OQ448190) showed that they have identity of 97.17% with differences in 13 nucleotides occurring at positions 34, 142, 226, 233, 244, 250, 268, 293, 301, 309, 343, 350 and 418 of the sequences.

The LS103/EGY/MENOFIA/2020/ORF103 (OQ448189) showed more than 99% identity for with other LSDV strains available in GeneBank database for ORF103. It showed for example 100% identity with LSDV strain (MK342935.1) from Egypt, 99.78% identity with LSDV NW-LW isolate Neethling Warmbaths LW (AF409137.1) and 99.35% identity with LSDV isolate (MN072619.1) from Kenya. It showed more than 97% identity with GPV and SPV sequences (Figure 7).

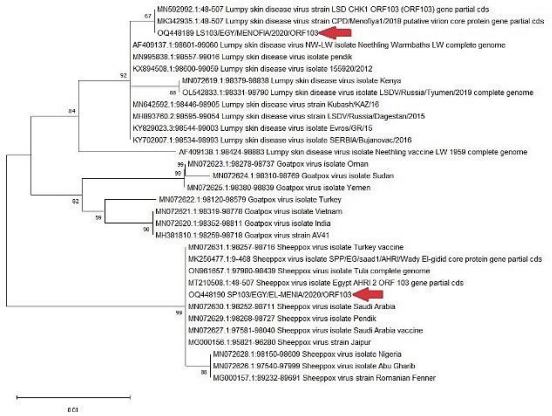

Fig (7). Phylogenetic tree based on LSDV ORF 103 sequence, showing the relation between LSDV strain and SPV sample of this study (red arrows) with LSDV-SPV-GPV strains in GeneBank database using MEGA 7 software using Neighbor-joining (ML) statistical method.

4- DISCUSSION

LSD is a serious skin disease that infects cattle of all ages, caused by a single strain of Capripoxvirus with DNA genome 150 kbp coding for 156 putative genes, and cannot be distinguished serologically from SPV or GPV (Tulman et al., 2001). It is of economic importance as it can cause a temporary reduction in milk production, temporary or permanent sterility in bulls, damage to hides and death (OIE, 2010). LSD can be misdiagnosed with many infections and diseases (Abdulqa et al., 2016), so it is significant to depend on tools for rapid detection and characterization of LSDV and to differentiate it from other infections.

In this study, real time PCR was used as a tool for rapid, sensitive and specific diagnosis of LSDV. The results showed that all collected samples were positive. These results agreed with Helmy et al. (2017). The presence of LSDV in nasal swab samples may be attributed to the ability of LSDV to affect the internal organs and respiratory systems as described by Vidanović et al. (2016) who detected LSDV in nasal swab samples and blood samples by qPCR. Variability in Ct values may be related to differences in viral tropism across sample type and different concentrations of the virus in samples. Additionally, this study was concerned with isolation of LSDV on CAM of SPF-ECEs and the results were confirmed by the presence of pock lesion as well as congestion of blood vessels, thickening and turbidity of CAM. These results were similar to those reported by El-Nahas et al. (2011) and El-Bagoury et al. (2018). To verify the results, conventional PCR and sequencing were then performed.

Several studies have shown that capripoxviruses are not exactly host-specific and can infect different animal species as El-Habbaa et al. (2021). Consequently, we depended on GPCR gene sequencing to distinguish LSDV from other members of capripoxviruses. The analysis of aligned sequences of amplified GPCR gene for the three LSDV strains of the study together with other strains of capripoxviruses available in GeneBank database showed that the three strains segregated into LSDV group.

The result of multiple alignment and phylogenetic analysis showed the ability of GPCR gene sequencing to differentiate LSDV from SPV and GPV and that GPCR gene can be used as a host-range gene suitable for virus animal origin discrimination. The ability of GPCR gene to discriminate the origin of the LSDV may be attributed to a 21-nucleotide insertion and a 12-nucleotide deletion in the GPCR gene identified by El-Tholoth and El-Kenawy (2016).

The genome of LSDV is highly conserved and this explains the similarity between the genomic sequence of GPCR gene of the strains of this study and other strains of LSDV in agreement with Stram et al. (2008) who demonstrate the inability to distinguish between the various LSDV, even though they were isolated from different locations at different times as LSDV has highly conserved nature.

On comparing the three sequences of this study with the Indian strain (MW452639), which was nearly identical to the historical field strains (NI-2490/Kenya) and KSGP-like strains (KSGP O-240, Kenyavac, KS-1) from Kenya, we found 12 nucleotide deletions in the sequences of the study that can be explain as the strains of this study may be recombinant LSDV strains as described by Sudhakar et al. (2022).

Further multigenic sequencing was applied on one strain depending on three different genes (P32, ORF 95 and ORF 103) for adequate characterization of the field strain and comparing the ORF 103 sequence with that of a SPV field sample. The results revealed that the strain of the study located into LSDV group and that the three capripoxviruses members had the same common ancestor in agreement with El-Kholy et al. (2008). The ORF 103 multiple alignments of the LSDV field strain with the SPV field sample showed that they have identity of 97.17% in agreement with Yanni et al. (2021) whose results showed 97.2% identity between LSDV and SPV. This result agreed also with El-Habbaa et al. (2021) who amplified and sequenced the ORF 103 and P32 gene and found that the field isolate of LSDV from cattle were found to have more than 98% identity with Egyptian LSDV isolates.

For P32 the results of this study opposite the results of Sudhakar et al. (2022) who found that the phylogenetic analyses of four complete genes (GPCR, RPO30, P32 and EEV) revealed that LSDVs from 2019 outbreaks in India is different from the currently circulating LSDV field strains in Africa and Middle East

5. CONCLUSION

From this study, we concluded that the strains of the study are closely related to the circulating LSDV strains in Egypt, middle east and Africa and differ from those found in India in the outbreak of 2019, and those strains of this study are recombinant field strains, we suggest further screening and sequencing of LSDV for detection of any mutations or conversion that may affect the result of vaccination protocol in the future.

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