MOLECULAR PROFILING AND GENETIC DIVERSITY OF SHEEP POX AND LUMBY SKIN DISEASE VIRUS

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Lumpy skin disease (LSD) is a highly contagious viral disease infecting cattle causing economic losses in milk production, abortion, infertility, loss of condition and damaged hides (Gusber *et al.,* 2004). LSD is considered an endemic disease in parts of Africa. It is a member of the Capripoxvirus genus within the Chordovirinae subfamily of poxviridae (Frank *et al*., 1993). It shares antigenic similarities with sheep and goat poxviruses, making differentiation challenging using routine serological tests (Youseif *et al*., 2017 and OIE, 2016). The LSDVirus (LSDV) genome consists of double-stranded DNA (Tulman *et al*., 2001 Gershon *et al.,* 1989). The mechanical transmission of LSDV occurs through biting insects (Carn, 1993; Hussein *et al*., 2017 and Chihota *et al*., 2001), and direct contact between cattle plays a limited role in the spread within affected herds (Magori-Cohen *et al*., 2012; Davies, 1981; Babiuk *et al*., 2008 and Black *et al*., 1986). Clinical symptoms of LSD include fever, increased salivation, and the formation of nodules covering the entire body of affected animals (Madzen and Bauer, 1970; Hanan, 2000 and House *et al*., 1990). LSDV outbreaks have been documented in Egypt since 1988, with notable occurrences in 1998 as recorded in El-Menia governorate among non vaccinated cattle and in 2006-2007 (Ahmed and Dessouki, 2013; Abdel-Rahim *et al*., 2002 and Brenner *et al*., 2009). Vaccination using a live attenuated freeze-dried vaccine derived from LSDV is the primary means of disease control in Egypt (Aboul Soud *et al*., 1998 and Fenner *et al*., 1987). To enhance protection, the General Organization of Veterinary Services in Egypt has implemented a sheep pox vaccine policy due to the antigenic relationship between the viruses (Ammar *et al*., 1999; Oguzolu

et al., 2006 and Rizkallah, 1994) along with the limited protection acuired using the vaccine prepared from LSDV (Somasundaram *et al*., 2011; Kasem *et al*., 2018 and Kitching, 2003). Genetic differentiation within the Capripoxvirus genus can be achieved by targeting the homolog of thgene (Tulman, 2002; Le-Goff, 2009; Tozzini *et al*., 1987; Zavala *et al*., 2001; Michael *et al*., 1996; El-Neweshy, *et al.*, 2013 and Tuppurainen and Oura 2014).

In this study, our aim is to isolate, characterize, and conduct an in-depth investigation into the molecular profiles of Lumpy Skin Disease Virus (LSDV) and Sheep Pox Virus (SPV). This was achieved through the following sequential steps: First, collection of skin lesion samples from cattle exhibiting symptoms of LSD and SPV. Followed by utilizing these samples for the isolation and propagation of suspected LSDV and SPV on MDBK (Madin Darby Bovine Kidney) and Vero (African Green Monkey Kidney) cell lines, respectively. Subsequently, the isolated viruses will be titrated on MDBK and Vero cell lines to determine their viral load. To further investigate the molecular genetics of LSDV and SPV, PCR amplification will be performed using specific primers targeting the viral attachment protein genes including G-protein-Coupled Chemokine receptor (GPCR) specific for LSDV and viral envelope protein gene (P32) specific for SPV. The PCR products were then sequenced, and a phylogenetic tree is constructed to analyze the genetic relationships between LSDV and SPV. These comprehensive steps will enable us to gain valuable insights into the isolation, characterization, and molecular genetics of LSDV and SPV.

MATERIALS AND METHODS

Sample collection

Samples were classified into two groups: for Lumby Skin Disease Virus (LSDV), and for Sheep Pox Virus (SPV). All samples for LSDV and SPV were taken from clinical cases having the skin nodules characteristic covering the entire skin surface of the affected animal (cattle and sheep) including head, neck, trunk, perineum, udder, and teats (Fig 1).

Lumby Skin Disease (LSD)

Biopsy specimens and scabs were collected from ten animals of dairy cattle on a vaccinated farm located in Ismailia Governorate, Egypt, exhibiting LSD lesions. The collected samples were preserved in transporting media with PBS, as recommended by OIE guidelines (OIE, 2016).

Sheep Pox Virus (SPV)

Samples were collected from ten animals selected from a thousand sheep of varying ages infected with sheep pox virus. The collection took place in El-Wadi El-Gedid Governorate, Egypt.

Cell culture

Two cell lines were devoted for our study; Madin Darby Bovine Kidney (MDBK) obtained from VACSERA and Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo and used in isolation, propagation, titration and SNT assays, and African Green Monkey Kidney cell line (VERO) which was obtained from Foreign Animal Disease Diagnostic Laboratory (FADDL). Propagation and maintenance of both cell lines was performed according to the method described by (Michael *et al*., 1994 and Zawa and Hazrat (1964). The MDBK was employed for isolation and propagation of LSDV, while the VERO was employed for production and titration of Sheep Pox vaccine.

DNA Extraction

Total DNA extraction of purified virus including LSDV and SPV was performed using VIVANTIS DNA Mini Kit (Vivantis Technologies, Malaysia) following the manufacturer protocol. The purified DNA was investigated for quantity and quality using Spectrophotometer and stored in -20ºC for further investigation.

PCR and primer design

Specific primers targeting the G-protein-Coupled Chemokine receptor (GPCR) gene and the envelope protein gene (P32) were designed according to (OIE, 2016 and Cao., *et al*., 1995) using perl primer software and the primers were manufactured by METABION® Company (Germany). The targeted segment was suspected to be 192 bp and 158 bp for *GPCR* and *P32* genes respectively (Alaa *et al*., 2008 and OIE, 2010) All designed primers were illustrated in Table (1). PCR was then performed based on the cycling protocol illustrated in Table (2) using Verso™ PCR kit. (ABgene). The PCR products thereafter were electrophoresed using 1.5% agarose gels stained with ethiduim bromide as mentioned by (Sambrook *et*

Sequencing of the amplified PCR product

After visualizing the PCR products, purification of products was carried out and the purified samples were sent to Animal Health Research Institute (AHRI) for Sanger sequencing in both directions (forward and reverse).

Sequence alignment and phylogenetic tree construction

Multiple sequence alignment for the obtained sequences was performed using Clustal W algorithm via BIOEDIT software version 7.0.4.1, which was also used for performing sequence editing, correction, frame adjustment, amino acid alignment and allocation of antigenic sites. The phylogenetic tree construction was conducted using neighbor joining method through employing MEGA software version 11 (Tamura *et al.*, 2021).

Propagation of LSDV and SPV in MDBK cells and VERO cells

The MDBK cells were used for the LSDV propagation. The harvested LSDV from the first passage was freeze–thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 g for 10 minutes. Dilution 1/10 from clarified virus was used for the second passage. LSDV remained 1hr's on MDBK cells as absorption time then Eagle's minimum essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS) was added. Cells were incubated at a CO2 incubator at 37ºC. Cytopathic effect (CPE) was observed through 5 days after infection (DPI: days post-infection). These steps were repeated extra 3 times and it was noted that CPE appeared faster according to El-Nahas *et al.* (2011).

On the other hand, the green monkey kidney cell line (Vero) was used for SPV propagation (Yogisharadhya, 2011), and the growth medium used was hank`s minimum essential medium (MEM) containing 5% inactivated sterile bovine serum and the CPE was observed for 9 days. In case of SPV, the same protocol used for the LSDV was employed for the propagation of the SPV using the appropriate growing media for each virus.

Titration of isolated viruses

Titration for LSDV and SPV was carried out according to Aboul Soud (1995) and the titer was calculated according to Reed and Muench (1938).

RESULTS AND DISCUSSION

Isolation and propagation of LSDV on MDBK cell line

After introducing the suspected sample onto MDBK cells (refer to Fig. (1), cytopathic effects (CPE) were monitored daily for 9 days. In the initial passage, CPE manifested on the 4^{th} day as cell rounding, and no further alterations were observed until the 9^{th} day when clusters of affected cells became apparent. During the second passage, cell rounding occurred on the 3^{rd} day, and by the 5^{th} day, cell clustering was evident (see Fig. 3). Cell aggregation was observed by the 9^{th} day. In the third passage, changes occurred more rapidly, with cell rounding and clustering observed by the 3^{rd} day, and cell aggregation observed by the 9^{th} day. The 4^{th} and 5^{th} passages exhibited even more pronounced changes in MDBK cells, with cell rounding observed as early as the 2^{nd} day, and by the 7^{th} day, cell detachment and degeneration were evident. The results of the titration for the LSDV virus, as shown in Table (3), indicated that as the dilution factor increased from 10^{-4} to 10^{-6}, the cumulative positive values decreased from 9 to 1, while the cumulative negative values increased from 0 to 6. Additionally, the occurrence of positive cytopathic effects (CPE) decreased with higher virus dilution, reducing from 5 to 1, while the occurrence of negative CPE increased from 1 to 4. Consequently, the percentage

of positive CPE decreased from 100% to 14%. Based on the obtained results the best dilution showing clear CPE was 10^{-4}.

Isolation and propagation of the selected sheep pox virus isolates on Vero cell line

The 5^{th} passage of the three selected sheep pox virus (SPV Elwadi, SPV-Kafr Elsheikh, and SPV- Giza) on Hank`s minimum essential medium was transmitted to grow on Vero cell line and examined daily for cytopathic effect (CPE)

SPV was inoculated in a confluent sheet of Vero cell culture and examined daily for detection of the cytopathic effect (CPE) **Fig (4) B, C, D, E)**.The results showed that,

The CPE of SPV characterized by the presence of intra cytoplasmic inclusion bodies in Vero cell culture begin to appear before the appearance of CPE by 24 hour it was small granular at the beginning, after that it became large dense and rounded masses which has a jute position to the nucleus at the 5^{th} DPI, the CPE characterized by rounding and aggregation of cell forming grape like appearance then the cell membrane was fused forming multinucleated giant cell . The infected giant cells were detached leaving holes in the cell sheet and some cell aggregates were detached forming foci of clear area by time a complete destruction of the cells was developed forming network. **Fig (2, F, G.H).**

These results agreed with those obtained by Mahmoud and Khafagi (2016); Tozzini *et al.*, (1987).

Cilli, (1961); Pandy and Singh (1970) reported that after aggregation of sheep pox virus on Vero cell, that the nuclei showed some enlargement, beside dense rod and granules appearing within the inclusion bodies which varied in size and shape and appeared to be fused together.

The cytopathic effects (CPE) observed in Vero cell culture were characterized by the presence of intracytoplasmic inclusion bodies. These inclusion bodies started appearing before the onset of CPE, typically within 24 hours. Initially, they appeared as small granular structures, but over time, they enlarged and became dense, rounded masses that are located close to the cell nucleus. By the 5^{th} day post-infection (DPI), the CPE is characterized by cell rounding and aggregation, giving rise to a grape-like appearance. Additionally, the cell membranes fuse, leading to the formation of multinucleated giant cells. As the infection progresses, these infected giant cells detached from the cell sheet, creating holes, and some cell aggregates detached, resulting in the formation of clear areas or foci. Eventually, destruction of the cells occurred, forming a network-like pattern.

Moreover, the titer calculation appeared in Table (5) showed that the highest dilution factor showing 0 % positivity was 10^{-5}, 10^{-6}, and 10^{-6} for Egyptian, Romanian, and Kenyan isolates, respectively.

Molecular characterization of the isolated virus

Polymerase chain reaction using GPCR and P32-specific primers

PCR was conducted using GPCR and P32-specific primers and **e**lectrophoretic patterns for the products were illustrated in Figs. (5 and 6) for GPCR and P32, respectively. Results revealed the presence of the targeted bands with molecular sizes of 192 and 158 bp for GPCR and P32 for all investigated samples (LSDV, SPV, and GPV). Purification of products was conducted for further investigation.

Partial sequence analysis, alignment, and phylogeny of SPV isolates based on the P32 gene

The partial P32 nucleotide of SPV Egyptian isolates and vaccinal strains (Romanian and Kenyan strains) were aligned (Fig. 7 and Table 6). The nucleotide sequences showed that SPV Elwadi, SPV-Kafr Elsheikh, and SPV-Menofeia shared identities of 90.4% to 95.5% with each other; SPV-Mattroh shared identities of 94.9% with SPV Romanian vaccinal strain; and SPV-Giza shared identities 99.1% with Kenyan vaccinal strain. The amino acid sequences showed that SPV-Elwadi, SPV Kafr Elsheikh, and SPV-Menofeia shared higher identities. In comparison, SPV-Mattroh and SPV-Giza shared higher identities with SPV Romanian and Kenyan vaccinal strains, respectively. The dendrogram was generated to determine the phylogenetic relation between SPV Egyptian strains and vaccinal strains as well as their position among other Capri pox viruses published strains on GenBank SP strains (Fig. 8). SPV-Giza and SPV-Mattroh were related to Kenyan and Romanian vaccinal strains, respectively. In contrast, the other three SPV Egyptian strains were clustered together. SPV-Elwadi, SPV-Kafrelsheikh, and SPV-Menofeia strains were related to a phylogenetic group of SPV strains of Asian (Al-Hassa, Abugharib, and Saudi Arabia) and turkey origin.

Partial Sequence analysis, alignment, and phylogeny of SPV isolates based on GPCR gene

The partial GPCR nucleotide sequences of SPV Egyptian isolates and the LSDV Ismailia isolate were aligned as illustrated in Fig. (9). the constructed phylogenetic tree showed that the two main groups all containing the LSDV isolates and there was no observation for SPV from the NCBI database. The nucleotide sequences showed that the SPV share similarity with LSDV KSGP 0240, LSDV Serbia 2016, and LSDV Russia all with 96.08%. However, the LSDV (Ismailia isolate) showed less similarity accounting 95 % with both LSDV pendik,

and LSDV Kubash, and 94 % with LSDV Kenya. The obtained result showed the effectiveness of the vaccination protocol of LSDV with Sheep pox vaccine to deduce the incidence of LSD among the Cattle and Calves population in Egypt.

SUMMARY

The objective of this research was to investigate the molecular genetic similarities between LSDV and SPV, which have spread extensively in Egypt. The aim was to identify these viruses through the propagation of LSDV on MDBK cells and SPV on VERO cells, as well as through PCR analysis and sequencing for G-protein-coupled chemokine receptor (GPCR) specific for LSDV and viral envelope protein gene (P32) specific for SPV of the isolated viruses. The obtained sequences were then compared using a phylogenetic tree to determine any immunogenic factors that could explain why cattle exhibit immunity when vaccinated with the sheep pox vaccine rather than the traditional vaccine prepared using LSDV. The study was conducted as follows:

1- LSDV and SPV were isolated from the clinical cases.
2- The isolated virus was propagated through five passages on MDBK cells for LSDV and VERO cells for SPV until cytopathic effects (CPE) were observed.
3- Determination of the viral titer for the isolated viruses.
4- Conducting antigenic characterization for the isolated viruses.

5- DNA extraction of the isolated viruses, followed by PCR amplification for genes GPCR and p32 and sequencing for the purified fragments using an automated sequencer.

6- Phylogenetic analysis using the partial sequences of the amplified genes.

The analysis of the sequences obtained and the antigenic characterization revealed the high degree of relatedness between LSDV and SPV. This information, along with the phylogenetic analysis, supports the possibility of vaccinating cattle with sheep pox vaccine.

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Table (1): Primers used in amplification of conserved sequence of viral attachment Gene of LSDV.

Target gene	Primer sequences (F and R)
P32	5'- TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT -3' 5'-TAT- GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC -3'
GPCR	5'- TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT -3' 5'-TAT- GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC -3'

Table (2): The cycling protocol of PCR for amplification of internal sequence of viral attachment protein gene of LSDV.

Step	Temperature	Time	Number of cycles
First step	95 ºC	2 minutes	1 cycle
Denaturation	95ºC	45 sec.	34 cycles
Primer annealing	50ºC	50 sec.	
Extension	72ºC	1 minute	
Final extension	72ºC	2 minute	1 cycle
Cooling	4ºC	∞	___

Table (3): Titration of LSDV.

Virus dilution	C.P.E.		Accumulated Values		Percentage Positive
	Positive	Negative	Positive	Negative	
10^{-4}	5	0	9	0	100%
10^{-5}	3	2	4	2	67%
10^{-6}	1	4	1	6	14%

Table (4): Titration of Egyptian, Romanian and Kenyan sheep pox virus strains.

Passage number	Titration in Log_{10} $TCID_{50}$/ml		
	Egyptian strain	Romanian strain	Kenyan strain
1	4.0	4.4	4.3
2	4.1	4.4	4.3
3	4.4	4.7	4.4
4	4.6	4.9	5.0
5	4.9	5.0	5.5
6	5.0	5.2	5.7
7	5.3	5.2	5.8
8	5.2	5.2	6.0
9	5.4		6.0
10	5.5		6.0
11	5.8		5.9
12	5.8		6.0
13	6.0		5.9
14	5.9		
15	6.1		
16	6.0		
17	6.1		
18	6.1		

Table (5): Calculation of the titration of SPV strains.

Egyptian		Romanian		Kenyan	
Dilution	Positive %	Dilution	Positive %	Dilution	Positive %
10^{-1}	100 %	10^{-1}	100 %	10^{-1}	100 %
10^{-2}	100 %	10^{-2}	100 %	10^{-2}	100 %
10^{-3}	100 %	10^{-3}	100 %	10^{-3}	100 %
10^{-4}	100 %	10^{-4}	100 %	10^{-4}	100 %
10^{-5}	0 %	10^{-5}	75 %	10^{-5}	75 %
10^{-6}	0 %	10^{-6}	0 %	10^{-6}	0 %

Table (6): Homology percentage of the gene *P32* PCR sequenced fragment compared to the published sequences in the GenBank (NCBI) by Blast analysis.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lumpy skin disease virus NI-2490 isolate Neethling 2490, complete genome	220	220	25%	9e-54	99%	AF325528.1
Lumpy skin disease virus NW-LW isolate Neethling Warm baths LW, complete genome	220	220	25%	9e-54	99%	AF409137.1
Sheep pox virus isolate RF envelop protein (P32) gene, complete	215	215	25%	4e-52	98%	KJ679574.1
Goat pox virus strain Qj/Cq envelope protein-like (p32) gene, complete sequence	215	215	25%	4e-52	98%	KJ026557.1
Sheep pox virus strain Lx/Gs envelope protein (p32) gene, complete cds	215	215	25%	4e-52	98%	KJ026555.1
Sheep pox virus strain Anyang/Henan envelope protein-like (p32) gene, complete sequence	215	215	25%	4e-52	98%	KJ026553.1
Sheep pox virus strain Jilin P32 gene, complete cds	215	215	25%	4e-52	98%	KF991005.1
Sheep pox virus strain GanSuGT/11/2012/China P32 gene, complete cds	215	215	25%	4e-52	98%	KF661974.1
Sheep pox virus strain SPPV/Ningx/2009/China envelope protein P32 (P32) gene, complete cds	215	215	25%	4e-52	98%	JN596274.1
Sheep pox virus strain Shanxi envelope protein (P32) gene, complete cds	215	215	25%	4e-52	98%	HM770955.1
Goat pox virus isolate GTPV Akola/2008 attachment protein (Att) gene, partial cds	215	215	25%	4e-52	98%	GQ442630.1
Sheep pox virus isolate SPPV Pune/2008 attachment protein (Att) gene, partial cds	215	215	25%	4e-52	98%	GQ442629.1

Table (6): Cont'

Sheep pox virus isolate SPPV-Srinagar attachment protein gene, partial cds	215	215	25%	4e-52	98%	GQ396156.1
Sheep pox virus isolate SPPV-Ranipet attachment protein gene, partial cds	215	215	25%	4e-52	98%	GQ396155.1
Sheep pox virus isolate SPPV-RF attachment protein gene, partial cds	215	215	25%	4e-52	98%	GQ396154.1
Sheep pox virus isolate Pune-08 P32 envelope protein (P32) gene, complete cds	215	215	25%	4e-52	98%	FJ882029.1
Sheep pox virus envelope protein (P32) gene, complete cds	215	215	25%	4e-52	98%	FJ748487.1
Sheep pox virus isolate Makhdoom-2007 envelope protein (P32) gene, complete cds	215	215	25%	4e-52	98%	EU314721.1

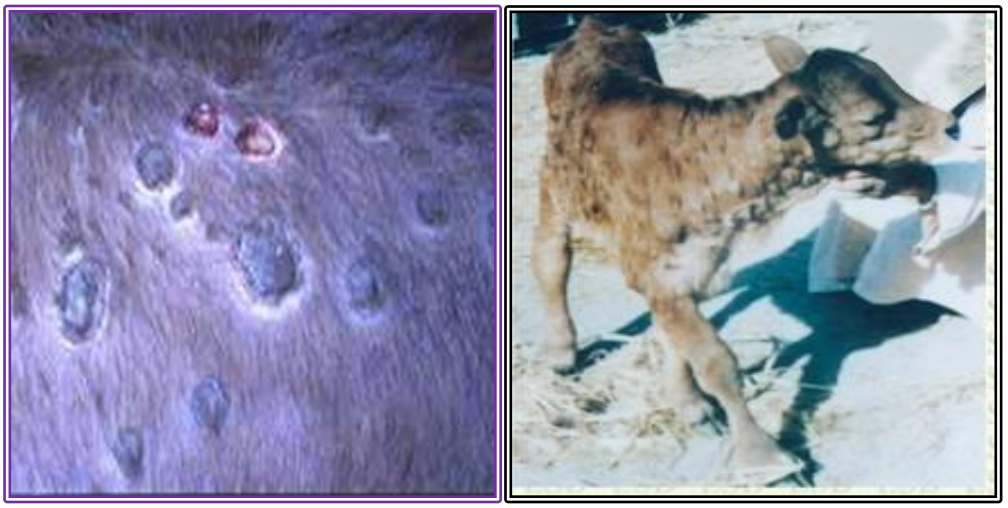

Fig. (1): Clinical cases of cattle and sheep which infected by pox disease.

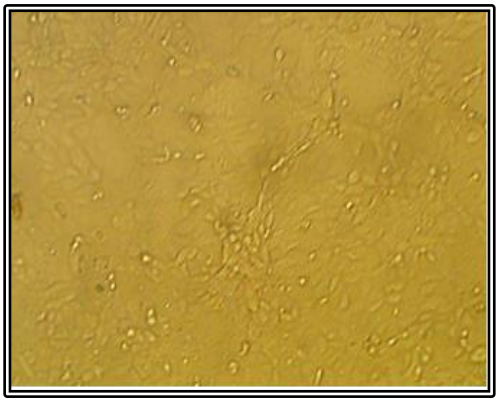

Fig. (2): MDBK Normal.

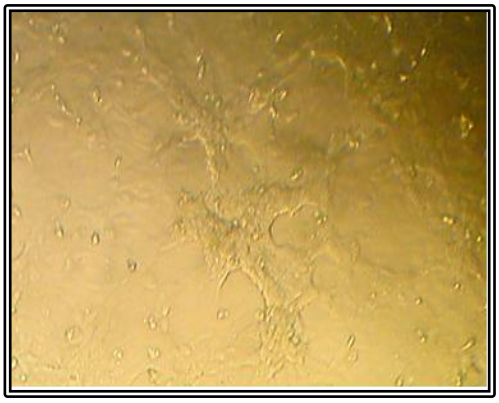

Fig. (3): Infected MDBK by LSDV.

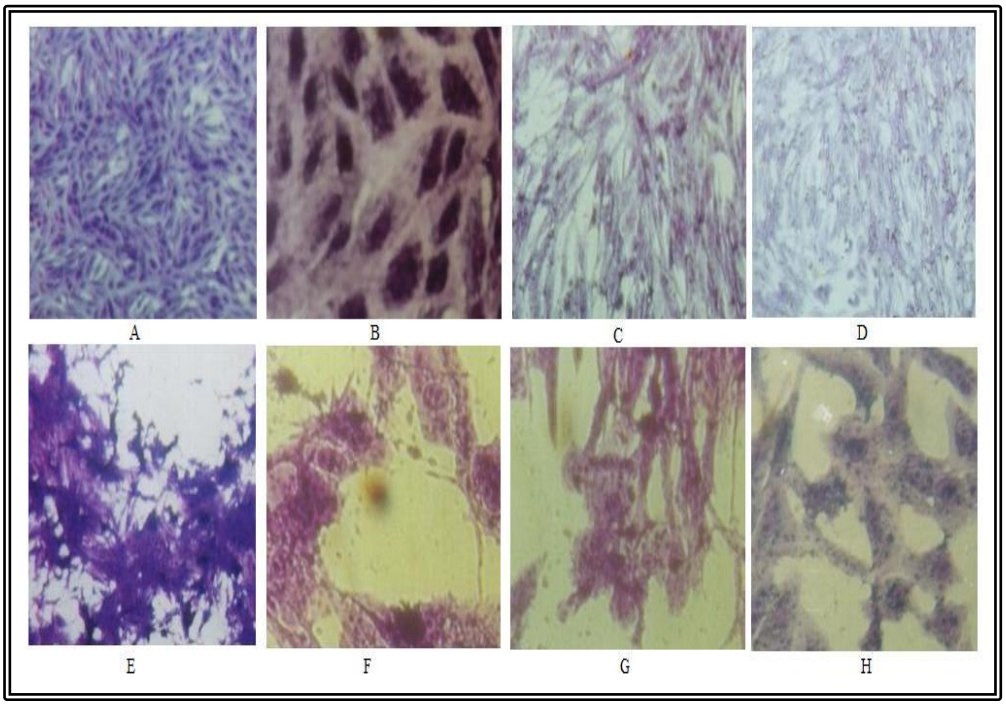

Fig. (4) A: Normal Vero cells showing no signs of abnormalit stained with crystal violet (low power 10x) .
B, C, D, E: Characteristic Cyto Pathic Effect (CPE) of selected developing early CPE in the form of cell aggregation and rounding (high power 32x).
F, G, H: The developing late (CPE) of SPV showing cell degradation and vaculation on Vero cell .

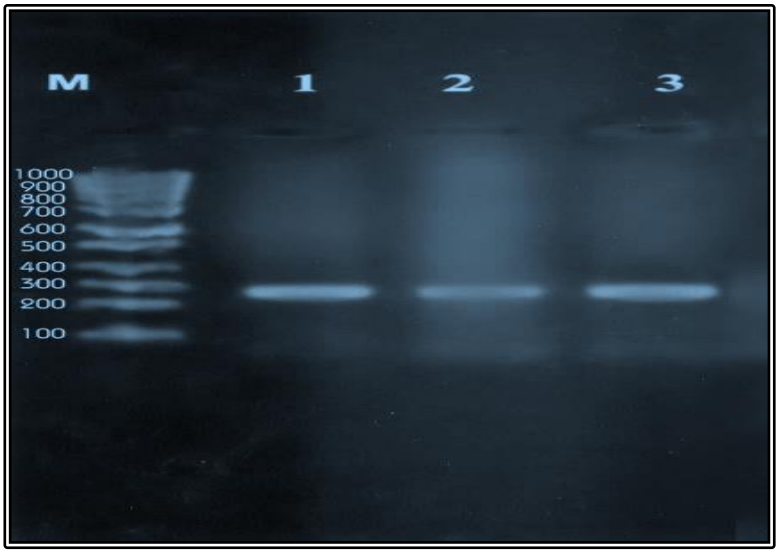

Fig. (5): Electrophoretic pattern of polymerase chain reaction (PCR) product, by using G-protein –coupled chemokine receptor (GPCR); Lane (M): molecular marker, Lane (1): Lumpy skin disease virus (LSDV), Lane (2): Sheep pox virus (SPV), and Lane (3): Goat pox virus strain (GPV).

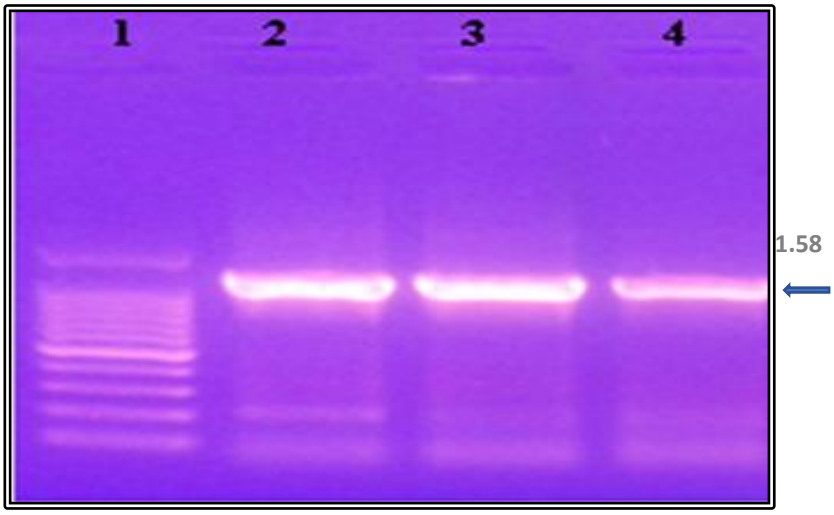

Fig. (6): Gel electrophoresis of P32 primer
Lane (1): High molecular weight nucleic marker,
Lane(2): Lumpy skin disease virus (LSDV)
Lane(3): Sheep pox virus (SPV),
Lane (4):Goat pox virus (GPV).

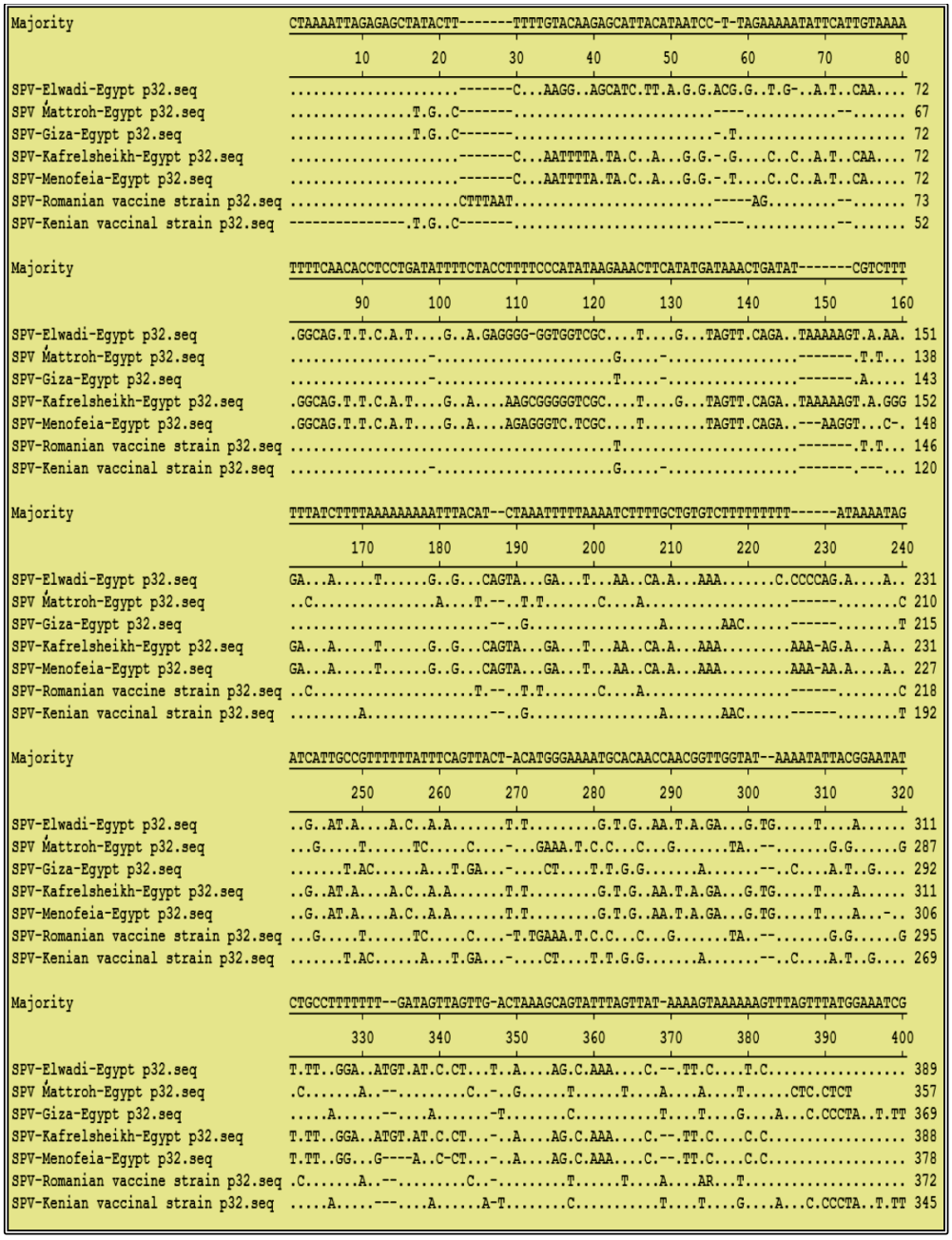

Fig. (7): Multiple nucleotides alignments of partial *P32* gene for SPV Egyptian strains compared to vaccinal strains in this study.

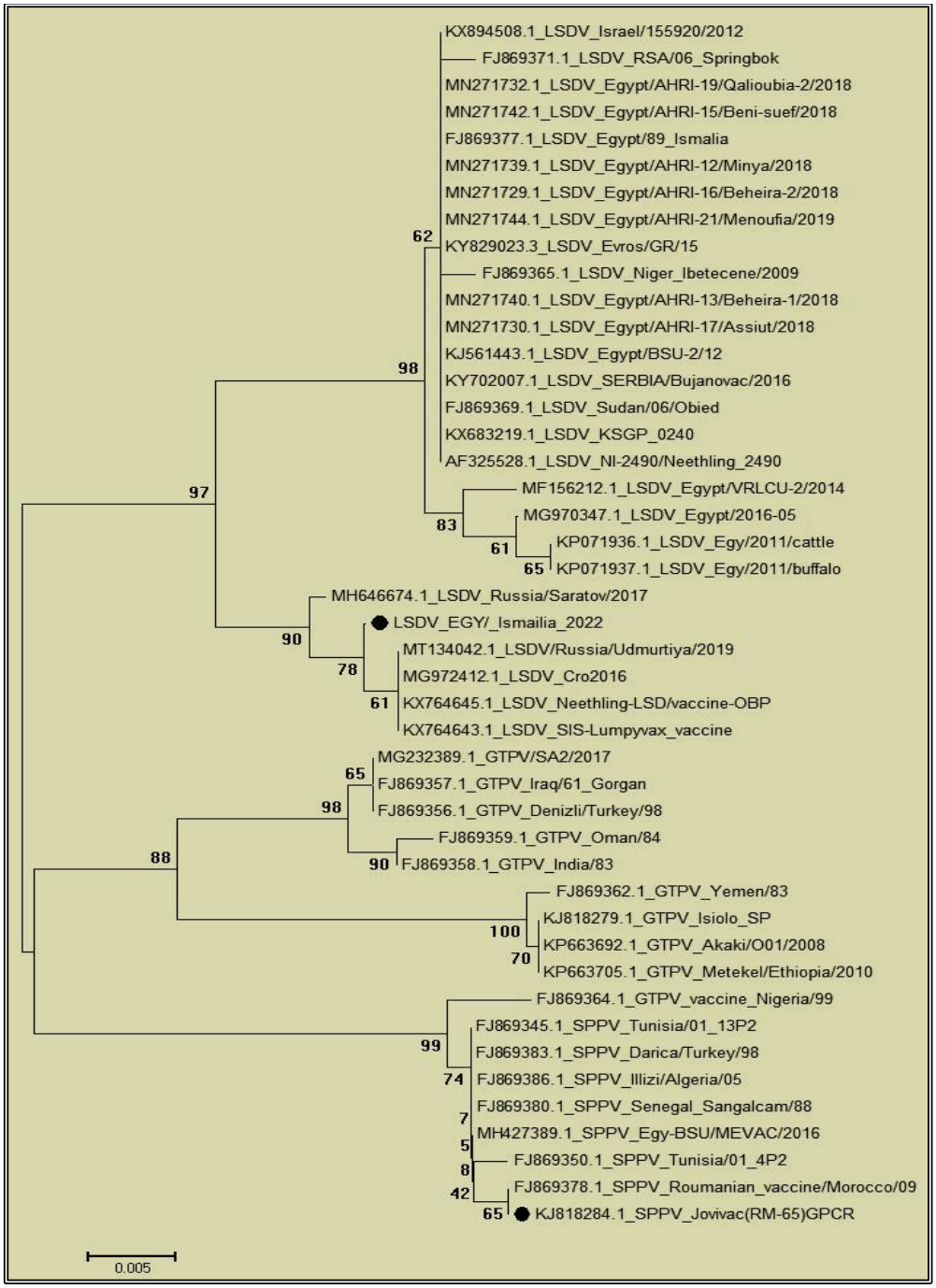

Fig. (8): Molecular phylogenetic analysis of the Capripox virus, Neighbor-Joining(N-J) tree depicting phylogenetic relationships of the LSDV and SPV isolated in this study and other capripoxvirus isolates based on *P32* nucleotides sequences.

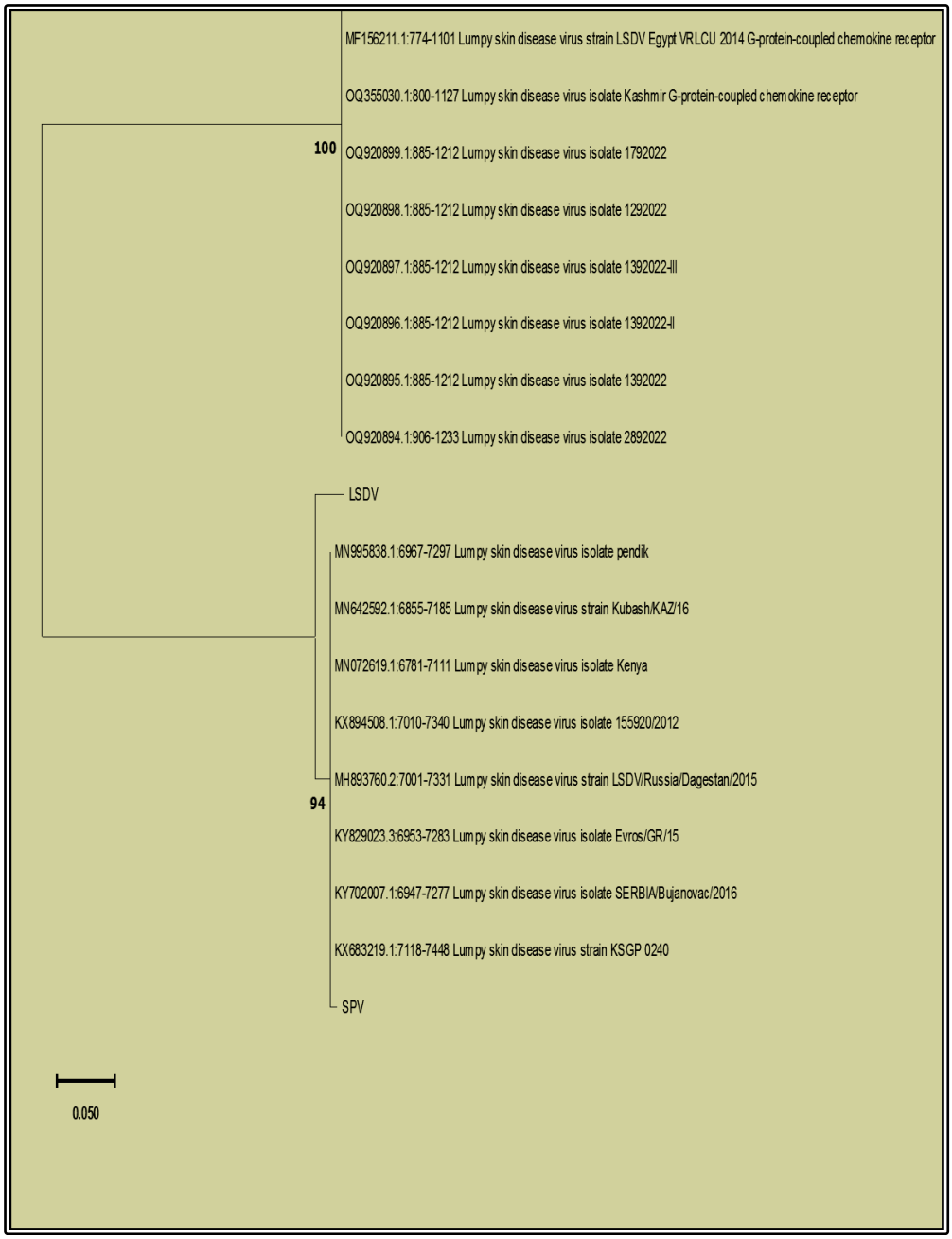

Fig. (9): Molecular phylogenetic analysis of the Capripox virus, Neighbor-Joining (N-J) tree depicting phylogenetic relationships of the LSDV and SPV isolated in this study and other capripoxvirus isolates based on GPCR nucleotides sequences.