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Current situation of Lumpy Skin Disease Virus in some areas in El-Wady El-Gedid Governorate During 2020

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ABSTRACT

Lumpy skin disease virus (LSDV) was detected in skin biopsies which were collected from diseased animals surfing from LSD. in Eldakhla El-Wady El-Gedid Governorate in 2020.The virus was identified by electron microscopic (EM) examination and confirmed by molecular characterization through sequence and phylogenetic analysis. A phylogenetic analysis was performed using partial sequencing of the ORF103 gene and comparing with reference LSD viruses' isolates obtained from Gene Bank. The results of the sequence analysis were similar among themselves (99.4-100% identity), it was shown 100% nucleotide genetic similarity with Egyptian isolates of LSDV(CPD/Menofiya 1/2018 MK 342935) and LSD isolate (El Wadi El-Gedid 2018 MN792930)and identities were (99.8%) with LSD (Serbia/Bujanovac/2016/KY702007).and LSD (Russia/Dagestan/2015MH893760.) where identities were (99.4%) with LSD (Kenya/MN072619) and LSD (KZ-Kostanay MT992618) .and they were found different from the other LSD viruses around the world. Further investigation, ELISA test was applied and the specific antibodies of LSDV were detected in serum samples of diseased animals (93.33%) and some of contact animals (10%).

INTRODUCTION

Lumpy skin disease (LSD) is an infectious viral disease of cattle of all ages and breeds **(Tageldin et al. 2014).** It is a vector-borne disease transmitted mainly by different biting and biting blood-feeding arthropods **(Magori-Cohen et al.2012).** It is caused by lumpy skin disease virus (LSDV) in the family Poxviridae, sub-family Chordopoxvirinae, genus Capri poxvirus **(Buller et al. 2005).** LSDV is enveloped double-stranded DNA virus, closely related antigenic ally to sheep and goat pox virus **(Bhanuprakish et al. 2006**). LSD. causes considerable economic losses in the livestock industry. The economic losses of this disease are due to reduced milk production, emaciation, poor growth, abortion, temporary or permanent infertility, permanent damage to hides.

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and pneumonia especially in young animals and respiratory tract lesions and mortality up to 20%. **(OIE. 2010, OIE. 2018, Abera et al. 2015)** it results in serious restrictions to international trade **.**Morbidity and mortality vary greatly depending on the activity of insects, susceptibility and the immune status of cattle. Morbidity ranging from 2% to 85% and even higher has been recorded. However, mortality is low (1 – 5%) but can be as high as 40% in some cases **(Davies 1991, Tuppurainen and Oura 2012).**

LSD signs range from in apparent to severe disease. There is no current evidence of variation in virulence regarding the different LSDV **(Kumar et al. 2011).** The incubation period in the field is believed to be 2 to 5 weeks, and lesions first appear at the inoculation site in 4 to 20 days. Fever is the initial sign that is followed by the development of nodules within 48 hour son the skin particularly on the head, neck, limbs, udder, genitalia and perineum. and mucous membranes of the mouth and alimentary tract and in the trachea and lungs and enlargement of superficial lymph nodes (**Brenner et al. 2006, Elhaige *et al*. 2017, Tuppurainen and Oura 2012).** Edema of one or more Limbs and other ventral parts of the body, such as the dewlap, brisket, scrotum and vulva, may be edematous, causing the animal to be reluctant to move (**Coetzer et al. 2005**).

The first record of LSD in Zambia in 1929. the disease is still spread to different parts of Africa until 1983 **(Davies 1991)**. LSD was limited to countries in sub-Saharan Africa; from 1984 to 1988, the disease has extended to the surrounding states. it is considered as an endemic disease in the African continent. **(Tuppurainen et al. 2011**). However, the disease is moved outside Africa to Madagascar and the Middle East. Recently, the disease is reported in LSD free countries (Jordan, Syria, Lebanon, Iraq,) and Iran and Turkey inOctober 2013 **(Sherrilyn *et al*. 2013, Calistri *et al*. 2019)**. with serious economic loss to the livestock industry. Since 2012 it has spread rapidly through the Middle East, South-east Europe, and West Asian regions (**Tageldin et al. 2014).** In 2015 and 2016 the disease spread to southeast Europe, the Balkans and Caucasus, Russia and Kazakhstan (**Alkhamis & Vander Waal. 2016 and OIE. 2017).** and since 2016, LSDV has been rapidly spreading into six Balkan countries (Bulgaria, FYR Macedonia, Serbia, Kosovo, Montenegro, and Albania) **(Spryginet al. 2019** and **Biswas et al. 2020)** and in parts of Asia (Azerbaijan, Armenia, Georgia, Kazakhstan and Russia **(Toplak et al. 2017),** causing serious challenges to the implementation of successful control measures .in Egypt there was an outbreak, for the first time between 1988 and1989, **(El-Nahas, *et al*. 2011, OIE. 2017)** and was reported again in 2006, 2011, 2014 ,2017and 2018 **(Brenneret al.2006 and Elhaig et al.2017, Abdallah et al. 2018, Hala et al. 2021)**. In 2017, outbreaks of LSDV in Egyptre-introduced of LSDV to Egypt through imported cattle from Ethiopia or other endemic countries and unrestricted animals, movement across country borders is a major and constant threat for LSD **(Hussein et al. 2017).**

Diagnosis of LSD is depending upon the basis of the characteristic clinical signs. A confirmed diagnosis is based on laboratory investigations including biopsy material or crust through routine diagnostic techniques either identification of the virus by using the transmission electron microscope (TEM), **(OlE. 2010, Gari *et al*. 2008, Elkenway and EL-Tholoth 2011)** Immunoperoxidase (IP) staining, Or detection of its specific antibody using an antigen trapping enzyme-linked immunosorbent assay (ELISA) **(Tuppurainen, et al. 2005)** and a polymerase chain reaction (PCR) test **(Balinsky *et al*. 2008, Bowden *et al*. 2009 and OIE 2011)** that is the superior test in detecting LSD virus where it detects viral nucleic acid in skin lesions.

This study aimed to investigate the LSDV infection in cattle in El-Wady El-Gedid governorate in 2020based on electron microscopic examination and polymerase chain reaction (PCR) assay and to analyze their phylogenetic against reference genome sequences. ELISA test was applied to detect the specific antibodies of LSDV seroconversion in serum samples of diseased on vaccinated animals showing clinical symptom which suspect LSD and apparently healthy (contact) animals.

MATERIAL and METHODS.

1.Samples:

Symptoms of lumpy skin disease appears on cattle in different localities of Eldakhla El-Wady El-Gedid Governorate from August , 2020 to early 2021**.**

1.1 fifteen biopsies of Skin nodules were collected from diseased animals of native, Frisian and mixed (Frisian with native) breeds for detection of LSDV by using of electron microscopic (EM) examination and Polymerase chain reaction (PCR). Samples were collected under aseptic conditions, transported to the laboratory, and stored at −70°C until use

1.2 A total of 80 Serum Samples were collected for ELISA test from cattle of different ages and sex (30 non vaccinated ,natural infected animals which showed clinical signs, paired serum samples were collected, one in initial stages of disease and the other after3 weeks and 20 serum samples were collected from (10) apparently healthy (contact) animals in the same time of diseased animals to determine the presence of the specific antibodies of LSDV

2.Electron microscope examination:

Transmission electron microscopy (TEM): Negative staining of skin biopsy for TEM was done according to (**OIE. 2004, Tuppurainen, et al. 2005**)

3.Polymerase chain reaction (PCR)

3.1-DNA extraction.

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 180 µl of ATL buffer was added to 25 mg of the sample and 20 µl QIAGEN protease. For homogenization of samples, tubes were placed into the adaptor sets, which are fixed into the clamps of the Qiagen tissue Lyser. Disruption was performed in 2 minutes high-speed (30 Hz) shaking step. Then samples were incubated at 56˚C. After lysis, 200 µl of the lysate was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 72^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit **(Zhuet al. 2013).**

3.2-Oligonucleotide Primer. Primers used were supplied from **Mutation (Germany)** are listed in table (1)

3.3-PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler

Table (1) Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified Segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
ORF 103	ATGTCTGATA AAAAATTAT CTCG ATCCATACCA TCGTCGATAG	570	94˚C 5 min.	94˚C 30 sec.	52˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Zhu *et al.* 2013**

3.4-Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

4. Purification and sequence of PCR product.

The 570 bp of the ORF gene were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul *et al.*1990)** was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of Laser gene DNA Star version 12.1 (**Thompson *et al.* 1994)** and Phylogenetic analyses was done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (**Tamura *et al.* 2013).**

4.1-Analysis of sequenced.PCR product:

Nucleotide sequences were analyzed with **DNA Star program** (version 7.0, CA, USA) and Phylogenetic tree based on 570bp nucleotide sequences of ORF gene constructed by the neighbor-joining method of **DNA Star software**.

5.ELISAtest: was applied on all collected serum samples to detect specific antibodies of LSDV correlate and confirm each otherusing ID Screen Capri-pox Double Antigen Multispecie skits (CPVDA-5P, ID vet innovative Diagnostics, France). (indirect ELISA) was carried according to manufacturer's instruction.

5.1-ELISATest kits: ID Screen® Capripox Double Antigen Multi-species for the detection of antibodies against capripox viruses in serum or plasma.

5.2-Validation:

The mean value of the positive control OD (OD_{PC}) is greater than 0.350

$OD_{PC} > 0.350$

the ratio of the mean values of the positive and negative control (OD_{PC} and OD_{NC}) is greater than 3

$$OD_{PC}/OD_{NC} > 3$$

5.3- Interpretation**.**

For each sample, calculate the S/P percentage (S/P%)

$$S/P\% = \frac{OD_{sample}-OD_{NC} \quad X\ 100}{OD_{PC}-OD_{NC}}$$

Sample presenting a S/P %:

-Less than 30% considered negative. S/P % <30% negative

greater than or equal 30% are considered positive S/P % ≥ 30% positive

Results:

1.Clinical investigation:

The clinical investigation of diseased animals under the present study revealed that the clinical signs started with high fever (40-41.5 c^0**),** depression, in appetence then appearance of skin nodules on the head, neck, limbs, udder, genitalia and perineum. and mucous membranes of the mouth, These nodules are circumscribed, firm, round and raised, and involve the skin, subcutaneous tissue and sometimes even the underlying muscles, enlargement of superficial lymph nodes**,** Rhinitis, conjunctivitis and corneal opacity excessive salivation, Pregnant cows aborted in some cases ,Edema in one or more Limbs and dewlap, causing lameness. As show in **Photo(1, 2, 3, 4**)

Photo (1) (2) (3) (4)

Photo (1, 2) showing skin nodules on the head, neck, limbs and Rhinitis the nose of calf
Photo (3) showing conjunctivitis and corneal opacity
Photo (4) enlargement of superficial lymph nodes and edema in Limbs and dewlap.
2.Electron microscopy Examination of skin biopsies (**figures 1, 2, 3)**

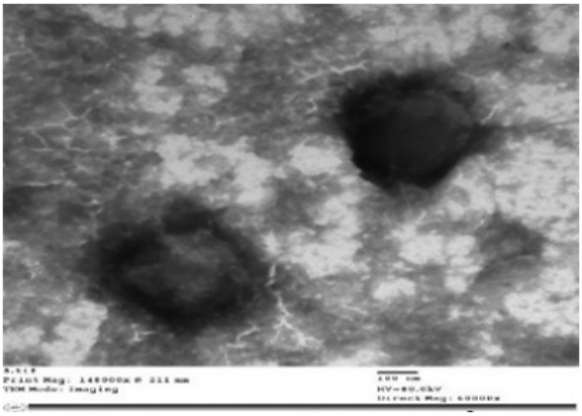


Figure (1) Ultra structure of LSD visions shown by negative staining: roughly brick shaped particles with ridges covering them.(x60.000)

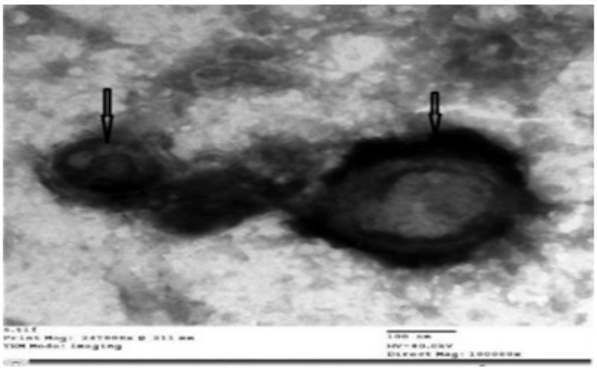
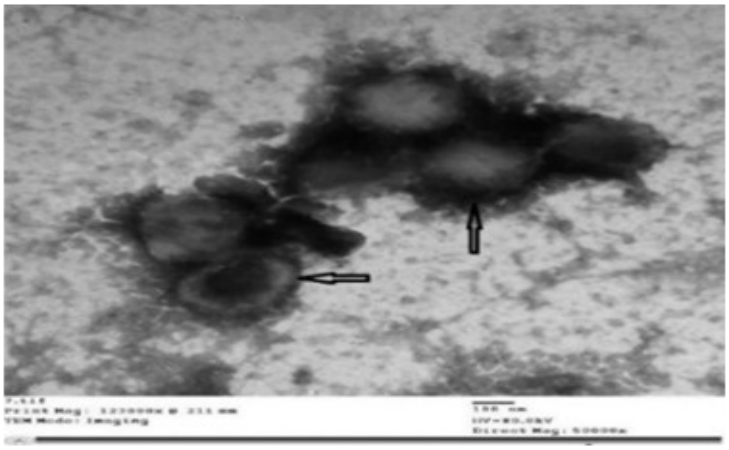
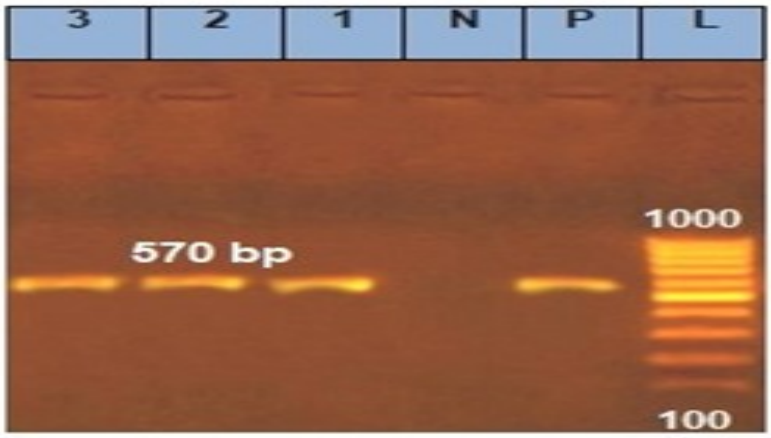


Figure (2) Ultra structure of LSD virions shown by negative staining**:** viral particles appeared oval shaped and surrounded by multilayered enveloped and present on surface of cells before their release (x100.000).



Figure(3) Ultrastructure of LSD virions shown by negative staining**:** viral particles at different steps of maturation which were circular with brush like membrane and dense nucleoprotein mass embedded in granular matrix (x50.000)



3. Detection of LSDV DNA by PCR

Figure (4)3.1 agarose gel (1.5%) electrophoresis separation of PCR. amplification products (570 bp length DNA band) of three selected samples LSD viral DNA of. El dakhla, EL Wadi/ El-gedid
Lane L: Ladder, Lane P: positive control, Lane N: negative control, Lane 1,2,3: LSD DNA in three suspected skin biopsies of different localities.

3.2-Analysis of the PCR Products

To confirm the causative agent, we amplified of ORF 103 gene via PCR (**Figure 4).** specific products with the expected size **(Figure 4),** including the ORF 103 (570 bp) gene, were amplified from the samples extracted from the skin biopsies. The purified of ORF 103 genes via PCR 570 bps Specific product size isolate was sequenced, subjected to similarity analysis, analyzed phylogenetically & sequence analysis was submitted to gene bank and identified under number of (S_1S_2 and $S_3$2020), subjected to similarity analysis, analyzed phylogenetically (**Figure 5).**

4.-Phylogenetic trees

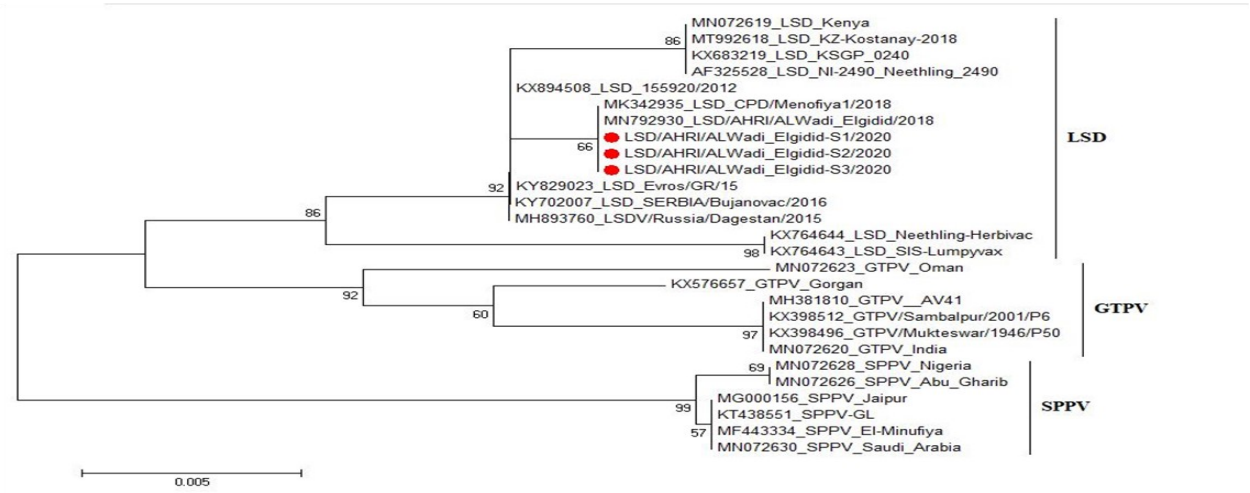


Figure (5) Phylogenetic tree of LSD virus detected (LSD/ AHRI. ALWadi/ El-GedidS1,S2and S3/2020) based on 570 nucleotide sequences of ORF 103 genes drawn by (DNA-Star version 7.0,CA, USA) & Sequences of other strains were obtained from GenBank**.**

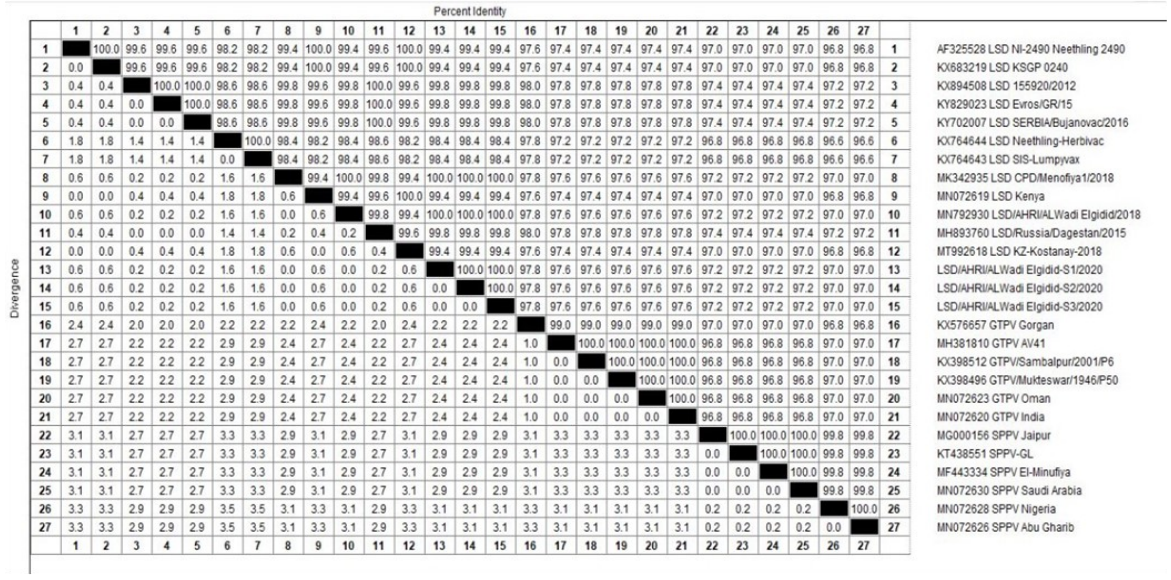


Figure (6) Identity % of nucleotide sequence.

The nucleotide sequence identities of three selective samples of different localities (LSD/ AHRI. AL Wadi/ El-Gedid S1, S2and S3/2020) were 100% with each other and shared very close relationships with Egyptian isolates of LSDV CPD/Menofiya 1/2018 MK 342935and LSDV isolate El Wadi El-Gedid 2018 (MN792930). and identities were (99.8%) with LSDV isolates of (Serbia/ Bujanovac /2016/KY702007) and LSDV isolates(Russia/Dagestan/2015MH893760.)
where identities were (99.4%) with LSDV isolates (Kenya/MN072619) and LSD (KZ-Kostanay MT992618). ..

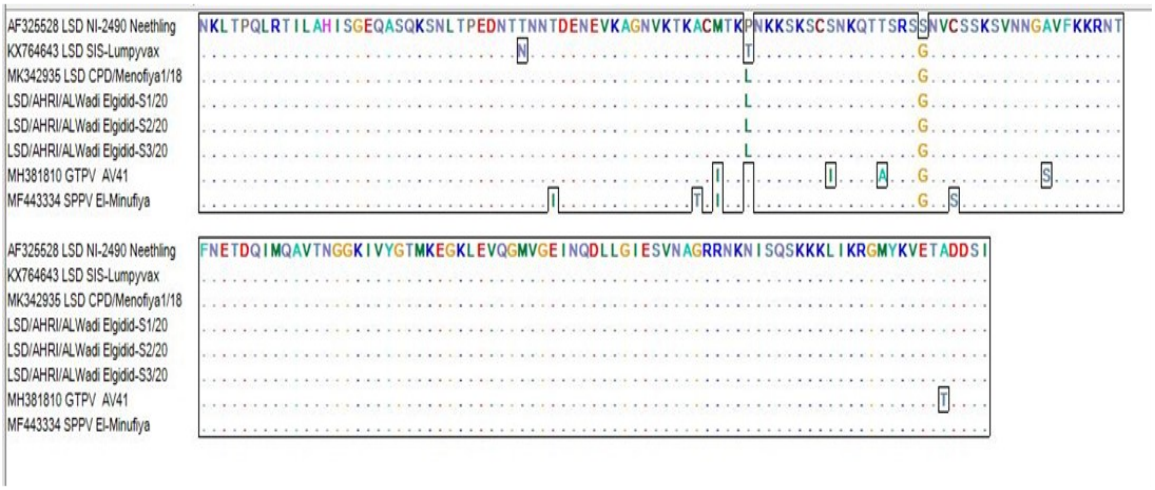


Figure (7) Protein alignment
Only 2 Amino acid changes were reported in comparison with Neethling strain (AF 325528)

5- ELISA test results

The results of serological survey for LSDV are shown in **table** (**2**). In viremic stage of diseased animals there were no positive samples, but after (21) days, (28) samples out of (30) (93.3%) were positive for LSDV antibodies, where In contact animals there were no positive samples in initial stage of disease and, (2) samples only out of (10) (20%) were positive after (21) days. **table** (**3**)

Table (2) ELISA test results of serum samples from diseased animals

State of animals	No. of examine samples	+ve %	-ve %
in viremic stages	30	0/30 (0%)	30/30 (100%)
After 21 days	30	28/30 (93.3%)	2/30 (6.6%)
Total no. of tested samples	60	28	32

There were (28) samples out of (30) (93.3%) were positive for LSDV antibodies after 21 days

Table (3)ELISA test results of serum samples from Contact animals

State of animals	No. of examine samples	+ve %	-ve %
Contact animals in initial stages	10	0/10 (0%)	10/10 (100%)
After 21 days	10	2/10 (20%)	8/10 (80%)
Total no. of tested samples	20	2 (10%)	18 (90%)

Only 2 samples (10%)were positive for LSDV antibodies after (21) days.

DISCUSSION

In the present study A typical clinical signs of lumpy skin disease were observed on cattle of native, Frisian, and mixture between them breeds from August 2020 till to early 2021 in El- Wady El-gedid governorate, where the disease appeared with a vigorous clinical pictures shown in the photo (1,2,3,4)with high mortality and subsequent abortion of pregnant animals and decrease milk production, which causes high loses, some Infected cattle also develop edematous swelling in their limbs and exhibit lameness these agreement with previous records **(Davies 1991**,**Tuppurainen and Oura 2012, Abutarbush et al. 2015, Elhaige *et al.* 2017, Spryin *et al.*, 2018**).and electron microscopic examination of skin biopsies in negatively stained preparations (figures1,2,3) showed characteristic brick, ovoid shaped virion surrounded by multilayered of enveloped and different steps of maturation, the same results were recorded by **OIE. 2010)**. which records TEM diagnosis of LSD can be confirmed within a few hours of receipt of specimens. and (**Babiuk, et al. 2008)** who reported that the virus persists in skin nodules or scabs for long periods of time, so it is the most important source of infection to healthy animals.

In the current study ,the results of identified and confirmed detected genes by conventional PCR. The results of PCR certified infection of the cattle by LSDV through observation of PCR positive band at the expected size of 570 bp fragment. These findings were consistent with the results of **El-Nahas et al. 2011);** With further identification by molecular characterization of virus isolate using PCR with primers specific to ORF103 gene (570bp) such that the PCR product could be used to detect LSDV in skin biopsy, and agree with the previous studies of **Al-Salihi 2014, Awad et al. 2010)** who reported that PCR is a fast and accurate method to detect LSDV during emergencies and it can detect low genome copies of the pathogen even in the dead state ,

Sequencing analysis is a very effective method to help understand the genomic nature of the virus. Gene Sequence analysis can be used in differentiating SPPV, GTPV and LSD genetic relationship among different virus isolates **(Lamien et al. 2011, and Zhou et al. 2012)** obtained results revealed that the Sequence and phylogenetic analysis was a close relationship among 570 nucleotide sequence of ORF 103 genes of three localities, (LSD/AHRI. AL-Wadi/El-Gedid S1, S2and S3/2020 Egypt) and it was shown nucleotide genetic similarity (the nucleotide sequence identities were100%) shared very close relationships with Egyptian isolates of LSDVCPD/Menofiya 1/2018 MK 342935andLSDV isolate El Wadi El-Gedid 2018 (MN792930) based on ORF 103 genes of LSD/AHRI. ALWadi/El-Gedid2020 Egypt was fully identical to that in Egypt.

The circulating virus in El wadi El gedid 2020 were clustered with previous Egyptian isolates circulating since 2017 (GenBank accession number MK342935 , MN792930) shown in (Fig5) which coincided with previous report (**Hala et al. 2021**) and identities were 99.8% with LSD (Serbia/Bujanovac /2016/ KY702007). and LSD (Russia/Dagestan/2015 MH893760.) where identities were 99.4% with LSD (Kenya/MN072619) and LSD(KZ-Kostanay MT992618) On other hand the isolates that recently affect northern hemisphere Russia , Serbia and Greese area were clustered together as the virus spread from Africa to middle east and then to Turkey, Greece and ASIA Gen Bank accession number (KY702007. MH893760. KY829023) which share less nucleotide identity with Egyptian isolates 99.8% and 99.4%with each other (Fig 6) which agree with previous report **(Sprygin et al. 2019).** Only 2 Amino acid changes were reported in comparison with Neethling strain (AF 325528) (Fig 7).All results mentioned above indicates that there was less nucleotide exchange through the years between the outbreaks.

Results of ELISA test kit revealed that the specific antibodies of LSDV were detected in serum samples of diseased animals after 21 days in percentage of (93.3%) where there was no antibodies detected inviremic stages of disease ,this indicate that paired serum samples at 21 day interval can be used as diagnostic tool in mild cases where good quality samples is not available, These results agree with **(Babiuk et al. 2008)** who detected Antibodies of LSDV after 21 days of natural infected animals, and there was no antibodies detected in

initial stages of contact animals except in 2 samples after 21 days These results agree with (**Tuppurain et al. 2017**) who reported that, direct or indirect contact between infected and susceptible animals is an inefficient method of transmission **(Ali et al. 2012, AU-IBAR. 2013**) also mentioned that, Mechanical transmission of LSD mainly related to flying insects, the scourge of LSD occurs during the peak period of the effectiveness of the mosquitoes so that the warm and humid weather play essential roles in increasing morbidities.

ELISA test was the confirmatory, specific and accurate test in detection of specific antibodies of LSDV and other caprypox viruses (**Tuppurainen, et al. 2005**) and can be used as diagnostic tool in mild cases where good quality samples is not available or when there were no other samples.

Conclusion:

We concluded that the present study indicated LSDV was the cause of the disease in cattle and circulating of it in this area from August 2020 to early 2021 and still important disease in cattle, which is still the main natural host, and PCR assay is sensitive and accurate to identify LSDV. peri-odic viral sequence comparisons and phylogenetic analyses of the current LSDVs are necessary for iden-tifying new circulating strains, observing the spread of viruses, and choosing suitable vaccines as a prophy-lactic measure against viral infection.

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