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Molecular diagnosis of Lumpy skin disease virus in cattle in some Egyptian governorates

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Abstract

Background: Lumpy skin disease virus (LSDV) is an important infectious skin viral disease of cattle which belongs to family poxviridae. In Egypt LSDV arrived with cattle imported from Somalia in May 1988 and caused Suez- Ismailia outbreaks. Infection was recorded in 2006 at Beni-Suef, Fayoum, Menofia, Sharquia, Qaliubiya, Alexandria and Damietta **(El-Kholy *et al.,* 2008).** At 2010, LSDV infection was diagnosed among cattle and buffaloes in Qaliubiya, Monoufia, Fayoum and Ismailia **(Sharawi and Abd El-Rahim, 2011).** At 2015, LSDV was identified among cattle at Ismailia, Qaliubiya and Sharquia governorate **(Aziza *et al.,* 2015).**

Objectives: This study was designed for isolation of LSD virus on CAM and MDBK cell culture and identification of isolated virus by FAT, PCR and sequencing.

Methods: A total 50 skin biopsies were collected from infected cattle with clinical signs of LSDV from some Egyptian governorates (Menofia, Kaluobia and Gharbia) during the period between 2018 - 2019 were used for virus isolation on chorio-allantoic membrane (CAM) of embryonated chicken eggs (ECE) and on MDBK cell culture. Isolated virus was identified by indirect fluorescent antibody technique (IFAT) using specific hyper immune serum against LSD virus. Further identifications were carried out by polymerase chain reaction (PCR) and sequencing of viral genome.

Results: The virus was isolated on CAM of ECE showed characteristic pock lesion on CAM and dead embryos which became more pronounced after 6 days of inoculation at 2nd and 3rd passages. isolation of LSD virus on MDBK cell culture showed rounding, cell aggregation, coalesce together to form clusters within 72 hr post inoculation and gradually increased till 70-80 % of sheet was completely detached. Isolated virus was identified by IFAT revealed specific intracytoplasmic yellowish green fluorescence emission. Further investigations were carried out using PCR for attachment protein gene confirmed LSDV isolate with specific amplified product 192 bp. Phylogenetic analysis of our published sequence with other published LSDV, sheep pox and goat pox on GenBank revealed that the recent local LSD virus strain showed high nucleotide similarities from 98 to 100%. Neutralizing antibodies against LSDV were (38) out of total 50 serum samples by serum neutralization test.

Conclusion: LSD virus is successfully isolated in CAM of ECE and MDBK cell culture. SNT is essential for identification of LSDV neutralizing antibodies for serological diagnosis. PCR is a very sensitive, specific and rapid confirmatory assay for rapid identification of LSD virus reflecting its importance in controlling the rapid spread of disease in Egypt.

Keywords: *LSDV; CAM; IFAT; MDBK; PCR*

Introduction

Lumpy skin Disease (LSD) is a skin disease of cattle caused by Neethling virus of genus capripoxvirus, chordopoxvirinae subfamily which belongs to family poxviridae **(Gari *et al,* 2010).**

The disease is characterized by fever, enlarged superficial lymph nodes and multiple nodules (measuring 2–5 cm in diameter) on the skin and mucous membranes (including those of the respiratory and gastrointestinal tracts **(Şevik *et al,* 2016).**

LSD was firstly recognized clinically in Egypt in the Suez Governorate, in May 1988, where it was arrived at the local quarantine station with cattle imported from Africa. The disease spread locally in the summer of 1988. It was reappeared in the summer of 1989 and in a period of five to six months spread to 22 of the 26 governorates of Egypt (**Salem, 1989).**

LSDV is closely related to sheep pox (SPPV) and Goat pox virus (GTPV) **(Babiuk *et al,* 2009).**

Although all three viruses are considered distinct species, it is not able to be differentiated serologically (**Magori *et al,* 2012).**

Molecular techniques are the only method to distinguish LSDV from SPPV and GTPV (**Fenner *et al*, 1996).**

LSDV is double-stranded DNA virus, has a 151-kbp genome, consisting of a central coding region which is bounded by identical 2.4 kbp-inverted terminal repeats and contains 156 genes. There are 146 conserved genes when comparing LSDV with chordopoxviruses of other genera **(Tulman, 2001).**

Diagnosis of LSD is often based on characteristic clinical signs. Laboratory diagnosis of LSD comprised either identification of the virus using: electron microscopy, egg inoculation, isolation in cell cultures, fluorescent antibody test; or detection of its specific antibody using serological tests. Several polymerase chain reaction (PCR) assays have been developed recently for more accurate and rapid detection of LSDV in suitable specimens **(Carn, 1993; Heine *et al.,* 1999).**

The PCR has been proved more sensitive and specific compared to immunoassays **(Ireland and Binepal, 1998).**

Multiple sequence alignments showed high homology percentage (≥ 99%) of the nucleotide sequences among local isolates of LSDV (**Fenner *et al.,* 1987 and Tulman *et al.,* 2001).**

Materials and Methods

Tissue Specimens: 50 Skin nodules were aseptically collected from infected cattle with a -typical clinical signs of lumpy skin disease from some Egyptian governorates (Menofia, Kaluobia and Gharbia) during the period between 2018- 2019. Each sample was prepared for virus isolation and identification by IFAT and PCR **(Carn *et al.,* 1995)** and stored at -70° C till used.

Serum samples: 50 serum samples were used for determination of antibodies against LSDV in cattle by serum neutralization test.

Reference Virus: LSDV Neethling strain was kindly obtained from the Department of virology, Animal health Research Institute, Dokki, Giza It was used in SNT and as positive control for IFAT, and PCR.

Antiserum: Reference antiserum to LSDV was supplied by the Department of virology, Animal health Research Institute, Dokki and Giza. It was used for viral identification by IFAT.

Cultures for Virus Isolation:

1-SPF Eight day old embryonated chicken eggs (ECE) were inoculated with the prepared samples via the chorioallantoic membrane (CAM) route.

2- Madin Darby bovine kidney (MDBK) cell line propagated with Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) were used for virus isolation **(OIE 2004)** and for serum neutralization test (**Carbery and Lee 1966).**

Conjugate: Anti-bovine IgG conjugated with

fluorescent Isothiocyanate was developed in rabbits and supplied by Sigma. It was used in IFAT.

Primers: The PCR primers were developed from the gene for viral attachment protein with the following sequences:
Forward primer 5'-TTTCCTGATTTTTCTTACTAT-3'
Reverse primer5'-AAATTATATACGTAAATAAC-3'.
The amplicon size of the PCR product is 192 bp. It was manufactured in the laboratories of the Midland Certified Reagent company Inc. of Midland, Texas.

Virus Isolation: Trials for LSDV isolation was carried out on CAM of ECE (**Babiuk *et al.;* 2008)** and also on MDBK cells **(Irons 2005)** for three blind passages.

Serological Identification of LSDV Isolates: IFAT were performed for identification of LSDV isolate. The techniques were performed according to (**Davies, 1991).**

Molecular Identification of Virus Isolates
Extraction of Viral DNA: Viral DNA was extracted from skin biopsy **(Markoulatos *et al.,* 2000)** and from infected CAM and MDBK cells (**Sambrook *et al*., 1989),** stored at -20°C until used in PCR.

Polymerase Chain Reaction (PCR) Assay: It was performed according to the procedures of Ireland and Binepal (**Sambrook *et al.;* 2000).** The PCR primers were developed from the gene for viral attachment protein was used. PCR reaction was applied in a total volume of 50 µl containing: 1 X PCR buffer (20 mMTrisHCl pH 8.4 and 50 mMKCl); 1.5 mM MgCl2; 0.2 mMdeoxynucleosides triphosphates mixture (dATP, dCTP, dGTP and dTTP); 20 pmol of each primer; 2.5 units (U) ThermusaquaticusTaq polymerase 0.1µg of extracted viral DNA and nuclease-free sterile double distilled water up to 50.0 µl. Then, the resulting mixture was subjected to precise thermal profile in a programmable thermocycler as follows: One cycle of: 94°C for 2 min; 40 cycles of: 94°C for 50 sec, 50°C for 50 sec and 72°C for 1 min; followed by one final cycle of 72°C for 10 min.

Analysis of PCR Amplification Products (Amplicons): The resulting PCR amplicons (10-15 µl) were analyzed by 2% agarose gel Electrophoresis **(Sambrook 2000).**

Material used for phylogenetic analysis of the sequence data:
1. Reference sequences of LSDV strains published on Gene Bank: Sequence data of LSDV strains were published on gene bank and were used for sequence alignments and phylogenetic analysis with the local LSDV isolated in this study.

2. Bioinformatics Programs:
2.1. BLAST and PSI-BLAST search programs: They were used for search of LSDV reference sequences in Gene Bank data base at National Center for Biotechnology Information (NCBI)**.**

2.2. Clustal W and DNA star Mega: It is program for sequence alignment (Clustal W) and phylogenetic analysis (DNA star mega) of the LSDV nucleotide sequence.
Serological Identification of LSDV antibodies: Serum neutralization test (SNT) both qualitative and quantitative tests were carried out on sera to identify and titrate LSDV neutralizing antibodies against 100 TCID50/ml of the reference LSDV on MDBK cells using the micro titer technique according to (**Florence *et al.* 1992).**

Results
Isolation of LSDV revealed the characteristic pock lesion on CAM of ECE **(Fig. 1)** and prominent CPE on MDBK cells started from third day post inoculation until complete destruction of cell sheet **(Fig. 2).**

Serological identification using IFAT demonstrated the specific intracytoplasmic yellowish green fluorescent granules characteristic for LSDV **(Fig. 3).**

The specific primers set amplified a DNA fragment of 192 bp equal to the expected amplification product size from Lumpy Skin Disease Virus. The reference strain of the LSDV and the local isolate from skin nodules, infected CAM and MDBK cells had the same size of

attachment protein gene fragment 192 bp, without significant differences between the strains **(Fig. 4)**

The purified PCR products for the local LSDV strain were sequenced using automatic DNA sequencer. Analyzed sample data is displayed as an electropherogram, a sequence of peaks in four colors. Each color represents the base called for that peak. **(Fig. 5).**

Antibodies against LSDV were detected by serum neutralization test. **Table (1)** revealed that neutralizing antibodies against LSDV were 76% from total serum samples.

Fig. (1): Pock lesion of LSDV on CAM numerous, small, scattered white foci.

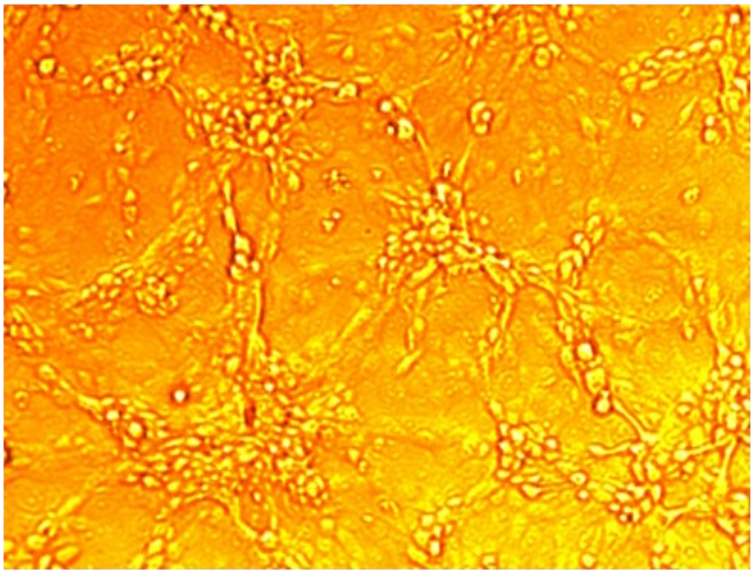
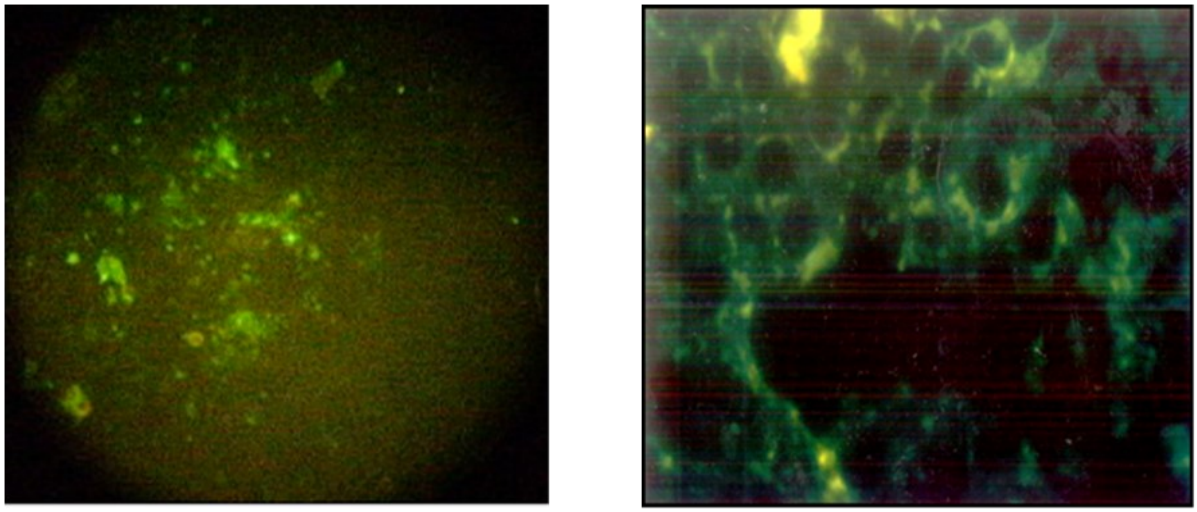


Fig. (2): Characteristic CPE of LSD in the form of clusters cell rounding, cell aggregations and vacuoles and cell beginning of detachment



(a) (b)

Fig. (3): CAM (a) and MDBK cells (b) infected by LSDV suspected local isolates and stained by fluoresce in isothiocynate showing the specific intracytoplasmic yellowish green fluorescent granules.

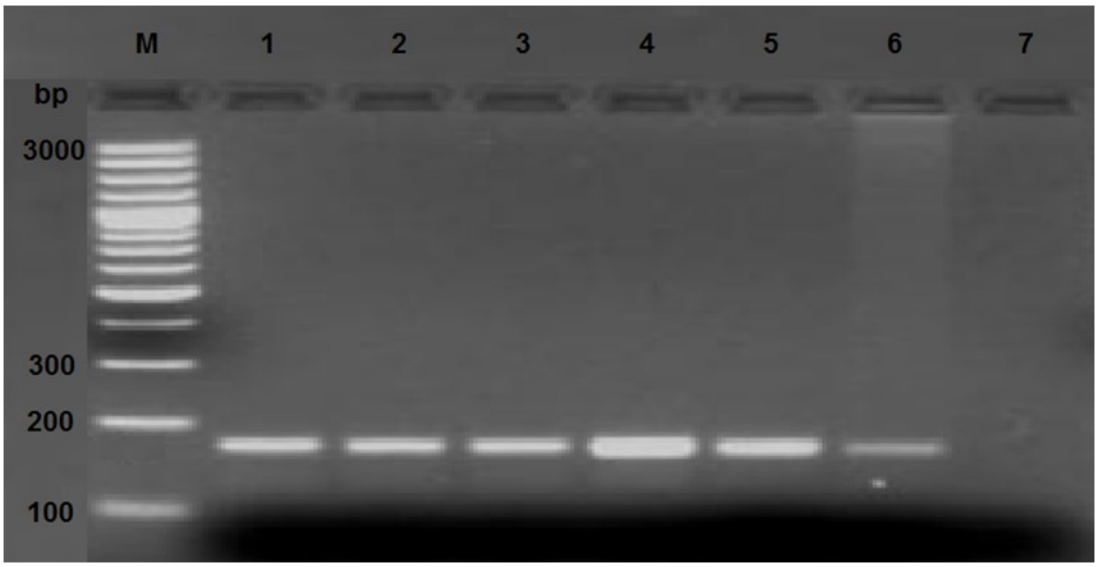
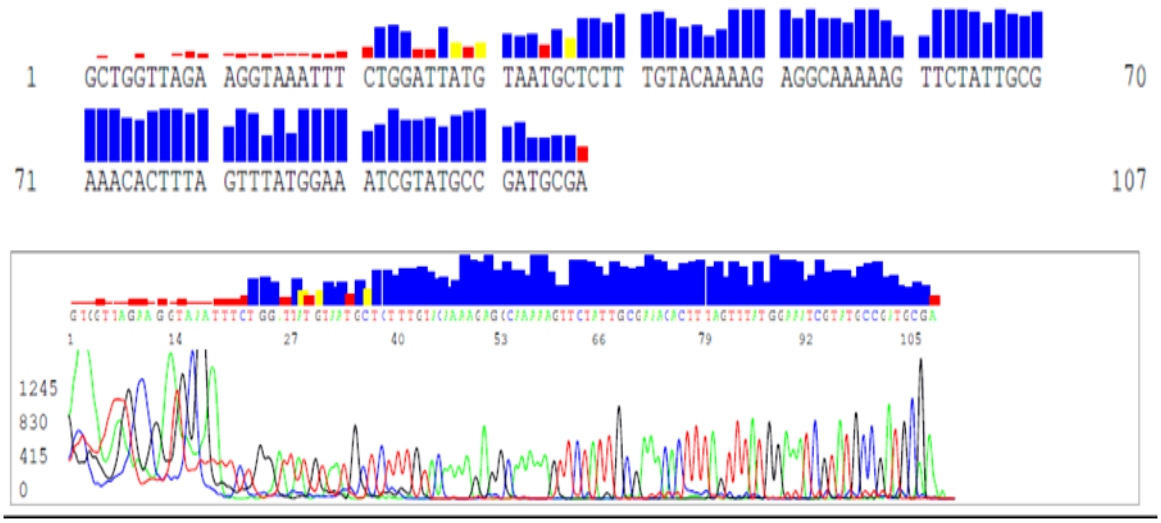


Fig. (4): Detection of DNA of LSDV by PCR (agarose gel electrophoresis of the polymerase chain reaction products) The specific primers set amplified a DNA fragment of 192 bp equivalent to the expected amplification product (amplicon) size from LSDV.
Lanes: (M) 100 bp DNA ladder; (1) Reference of LSDV "Neethling" strian; (7) -ve control (no primers); (2, 3, 4, 5, 6) +ve samples



GCTGGTTAGAAGGTAAATTTCTGGATTATGTAATGCTCTTTGTACAAAA-
GAGGCAAAAAGTT CTATTGCGAAACACTTTAGTTTATGGAAATCG-
TATGCCGATGCGA

Fig. (5): The partial sequence profile of LSDV envelope protein-like gene detected in CAM

Table (1): Antibody titre against LSD Virus in tested cattle sera by serum neutralization test.

No. of tested serum samples	No. of +ve samples	% of +ve samples	Antibody titre of LSDV					
			8		16		32	
50	38	76	No.	%	No.	%	No.	%
			6	15.8	13	34.2	19	50

Discussion

LSD is an economically important infectious disease of cattle caused by a virus of the family Poxviridae, also known as Neethling virus. Mortality rates as high as 40 % or more. The disease is characterized by fever, enlarged superficial lymph nodes and multiple nodules.The disease often results in chronic debility, reduced milk production, poor growth, infertility, abortion, and sometimes death (**Alkhamis & Vander Waal 2016 and Şevik *et al,* 2016).**

Massive outbreak of suspected LSD in cattle populations were observed during 2017 and 2019 in several Egyptians provinces.

Because, it is clear that there are many diseases causing similar signs (**Alexander *et al,* 1957 and Weiss, 1968),** it is important to obtain a definite diagnosis to ensure the best preventive and control measures.

The present study concerned with the trial for isolation of LSD virus from skin lesions of infected cattle on CAM of SPF-ECE and MDBK cell line with further identification by IFAT and molecular Identification of virus isolate using PCR.

LSDV can be propagated on the chorioallantoic membrane (CAM) of embryonated chicken eggs (ECE). After the first passage, the characteristic pock lesions were observed and become clear after the third passage (Fig. 1). These findings come in complete agreement with those of (**House *et al.* 1990 and Tamam, 2006).**

CPE on MDBK cells was characterized by cell rounding, cell aggregation, coalesce together to form clusters that scattered all over the monolayer within 72 hr post inoculation and gradually detached until complete destruction of cell sheet. These findings agree with those of (**Ibrahim, 1999 and Fahmy, 2000)** (Fig. 2).

Serological identification by IFAT for detection of LSD virus in infected CAM and MDBK monolayer using specific hyper immune serum against LSDV. It showed specific Intracytoplasmic yellowish green fluorescent granules characteristic for LSDV (Fig. 3). Our results came in agreement with those of (**Davies, 1991 and Ibrahim *et al.,* 1999).**

The specific primers set amplified a DNA fragment of 192 bp equivalent to the expected amplification product (amplicon) size from LSDV. The LSDV reference strain and the local isolate from skin nodules, infected CAM and MDBK cells had the same size of attachment protein gene fragment 192 bp, without significant differences between the strains (Fig. 4). Subsequently, it was certain that these specimens contained DNA of LSDV.

The partial sequence analysis and alignment report of the envelope protein-like gene for the recent local LSD virus strain using Clustal W showed high nucleotide similarity of the strain (CAM source) with identity percent 100%. (Fig 5 & 6).

Antibodies against LSDV were detected by serum neutralization test. Table (1) revealed that neutralizing antibodies against LSDV were 76% from total serum samples.

The partial sequence analysis and alignment report of the envelope protein-like gene for the recent local LSD virus strain using Clustal W showed high nucleotide similarity of the strain (CAM source) with identity percent 100%. (fig. 5).

Screened sera were titrated using SNT. A varied titer ranged from 8 to 32 was obtained. It was observed that 6 of samples show a titer of 8 while 13 and 19 of samples revealed a titer of 16 and 32 respectively. The protective titer were considered ≥ 16 **(Cottral, 1978).** These results are demonstrated in Table (1).

Conclusion

Lumpy Skin Disease is an important enzootic disease in Egypt. Lumpy Skin Disease virus (LSDV) is circulating among cattle in Egypt. Besides conventional techniques, PCR is a simple, rapid, sensitive and accurate method for the detection of LSDV DNA in skin nodules and in the CAM of inoculated chicken embryos.

References

Alexander, R.A.; Plowright, W. and Haig, D.A. (1957). "Cytopathogenic agents associated with LSD of cattle". Bull. Epiz. Dis. Afr., 5: 489-492.

Alkhamis, M.A. and VanderWaal, K. (2016). Spatia l and Temporal Epidemiology of Lumpy Skin Disease in the Middle East, 2012 - 2015. Front. Vet. Sci., 3-19.

Aziza, Amin; Ehab, El-Nahas and Abd-Elbaset, El-Mashed (2015). Pathological and Virological Studies on an Outbreak of Lumpy Skin Disease among Cattle in Kalubia Governorate-Egypt. Journal of Advanced Veterinary Research Volume 5, Issue 4, 165-175.

Babiuk, S.; Bowden, T.R.; Parkyn, G.; Dalman, B.; Manning, L; Neufeld, J.; Embury -Hyatt, C.; Copps, J. and Boyle, D.B. (2008). Quantification of Lumpy Skin Disease Virus Following Experimental Infection in Cattle. Transboundary and Emerging diseases. 55 :(7)299-307.

Babiuk, S.; D.B. Wallace; S.J. Smith; T.R. Bowden; B. Dalman; G. Parkyn; J. Copps and D.B. Boyle (2009). Detection of Antibodies against Capri poxviruses using an Inactivated Sheep pox Virus ELISA. Trans. and Emer. Dis., 10: 865-1682.

Carbery, E.A. and Lee, L.R. (1966). Serum neutralization test for BVD and IBR viruses culture cell line .Proc. 69-Annual Meet. U.S. Livestock Saint. Ass. 501.

Carn, V.M. (1993). "Control of Capri pox virus infectious vaccine." 11:1275-1279.

Carn, V.M. (1995). An antigen trapping ELISA for the detection of Capripoxvirus in tissue culture supernatant and biopsy samples. J. Virol. Meth. 51, 95 – 102.

Cottral, G.E (1978). "Pox viruses" In manual of standardization methods for veterinary microbiology, pp. 273-291.

Davies, F.G. (1991). Lumpy skin disease, an African capripoxvirus disease of cattle. Br. Vet. J., 147: 489-503.

El-Kholy, A.A.; Soliman, H.M.T. and Abdelrahman, K.A. (2008). Polymerase chain reaction for rapid diagnosis of a recent lumpy skin disease virus incursion to Egypt.Arab J. Biotech., Vol. 11, No. (2), pp.: 293-302.

Fahmy, H.A. (2000). Studies on intradermal allergic skin test of lumpy skin disease virus in cattle; Ph. D. Thesis (Virology). Fac. Vet. Med., Cairo University.

Fenner, F. (1996). Poxviruses. In: Fields BN, Knipe DM, Howley PM (eds), Fields virology third edn.,vol 2. Chapter 84. Lippincott-Raven, Philadelphia, PA.

Fenner, F.; P.A. Bachmann; E.P.J. Gibbs; F.A. Murphy; M.J. Studdert and D.O. White (1987). Poxviridae. Veterinary Virology. Academic Press, London. Fenner's Veterinary virology, fourth edition, 2011.

Florence, G. Burleson; Thomas, M. Chambers and Danny, L. Wiedbrauk (1992). Vi-

rology A Laboratory Manual. Academic press, New York.

Gari, G.; A. Waret-Szkuta; V. Grosbois; P. Jacquiet and F. Roger (2010). Risk factors associated with observed clinical lumpy skin disease in Ethiopia. Epidemiol. Infect. 138: 1657-1666.

Heine, H.G.; M.P. Stevens; A.J. Foord and D.B. Boyle (1999). A capripoxvirus detection PCR and antibody ELISA based on the major antigen P32, homologue of the vaccinia virus H3L gene. Journal of Journal of Immunological Methods, 227: 187-196. 21.

House, J.A.; T.M. Wilson; S. El Nakashly; I.A. Karim; I. El Ismail; N. Danaf; A.M. Moussa and N.N. Ayoub (1990). The isolation of lumpy skin disease virus. and bovine herpesvirus-4 from cattle in Egypt. J. Vet Diagn. Invest., 2(2): 111-115.

Ibrahim, M.I. (1999). Preliminary studies on inactivated lumpy skin disease virus vaccine. Ph. D. Thesis Virology), Fac. Vet. Med., Cairo University.

Irelnd, D.C. and Y.S. Binpal (1998). Improved detection of capripox virus in biopsy samples by PCR j.irol.Meth.74,1-7.

Irons, P.C.; Tuppurainen, E.S.M. and Venter, E.H. (2005). Excretion of lumpy skin disease virus in bull semen. Theriogenology, 63, 1290–1297.

Magori-Cohen, R.; Y. Louzoun; Y. Herziger; E. Oron; A. Arazi; E. Tuppurainen; N.Y. Shpige and E. Klement (2012). Mathematical modelling and evaluation of the different routes of transmission of lumpy skin disease virus. Veterinary Research, 43(1): 1297-1299.

Markoulatos, P.; O. Mangana-Vougiouka; G. Koptopoulos; K. Nomikou and O. Papadopoulos (2000). Detection of sheep poxvirus in skin biopsy samples by a multiplex polymerase chain reaction. Journal of Virological Methods, 84: 161-167.

OIE (2004). "Manual of diagnostic tests and vaccines for terre-strial animals."

Salem, A.S. (1989). Lumpy skin disease in Egypt. OIE Diseae Information. vol. 2. No.2.

Sambrook, J. and D.W. Russell (2000). Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, Plainview, New York, 6(13): 6-19.

Sambrook, J.; E.F. Fritsch and T. Maniatis (1989). Molecular cloningA laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Şevik, Murat; Avci, Oğuzhan; Doğan, Müge and İnce, ÖmerBarış (2016). "Serum Biochemistry of Lumpy Skin Disease Virus-Infected Cattle". BioMed Research International.

Sharawi, S.S.A. and Abd El-Rahim, I.H.A. (2011). The utility of polymerase chain reaction for diagnosis of lumpy skin disease in cattle and water buffaloes in Egypt, Rev. sci. tech. Off. int. Epiz., 30 (3), 821-830.

Tamam, S.M. (2006). Isolation of Lumpy skin diseas virus form naturally infected cattle previously vaccinated with live attenuated sheep poxvirus vaccine BS. VET. MED. J., 16(1): 27-31.

Tulman, E.R.; C.L. Afonso; Z. Lu; L. Zsak; G.F. Kutish and D.L. Rock (2001). Genome of Lumpy Skin Disease Virus. Journal of Virology, 75(15): 7122-7130

Weiss, K.E. (1968). Lumpy skin disease virus. Virol. Monograph, Vol. 3: pp. 111-31.