



Virological, Molecular and Immuno-Biochemical Studies of Lumpy Skin Disease in Naturally Infected Cattle

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ABSTRACT

This study aimed to diagnose Lumpy skin disease by isolation and identification of LSD from suspected naturally infected cattle and to study its effect on immuno-biochemical parameters, inflammatory and oxidative stress markers. The study was conducted on 25 cattle, 1-3 years age from two cities in the Sharkia governorate. Ten clinically healthy cattle represented the control group and fifteen suspected diseased cases by LSD represented the infected group. The clinical signs of suspected cases showed anorexia, skin nodules all over the body, fever above 40°C, edema in forelimbs and dewlap, enlarged superficial lymph nodes, retarded milk production, and abortion in some cases. The virological examination of LSDV from the samples of skin nodules was done. Isolation from skin nodules was done on MDBK cell line and embryonated chicken egg through chorioallantoic membrane route followed by identification using fluorescence technique . Molecular studies including phylogenetic analysis for three positive isolates showed similarity to previously locally isolated strains in Egypt and close relation to strains of SPPV. Immuno-biochemical results revealed a significant increase in aspartate aminotransferase , alanine aminotransferase , alkaline phosphatase , Malondialdehyde , Tumor necrosis factor , Interleukin4, immunoglobulin M and immunoglobulin G . Meanwhile, a significant decrease in total protein, albumin, globulin, Glutathione peroxidase, phagocytic assay, calcium, inorganic phosphorous, sodium, and potassium with a non-significant change in magnesium was seen when compared to control ones. It could be concluded that the infection with LSD in cattle induce severe effects on immuno-biochemical, antioxidant parameters, and inflammatory markers causing great economic loss

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INTRODUCTION

Lumpy skin disease virus (LSDV), sheep pox and goat poxviruses are members of the genus Capripoxvirus, within the chordo-poxvirinae subfamily of Poxviridae. The genomic sequence of LSDV is about 151-kbp in length. It consists of a central coding region bounded by identical 2.4 kbp-inverted terminal repeats. It contains 156 genes. Genomic comparisons revealed that LSDV is closely related to other members of the

Chordopoxvirinae, it, however, contains a unique gene responsible for viral host range and virulence (Tulman *et al.*, 2001). LSDV is the etiologic agent of an economically important disease of cattle in the Middle East and Africa (Fenner, 1996).

Lumpy skin disease (LSD) is an acute, subacute, or unapparent viral disease of cattle and occasionally buffaloes. The acute disease is

characterized by pyrexia, generalized skin and internal pox lesions, and generalized lymphadenopathy, orchitis, and mastitis (**Hamouda et al., 2002, Brenner et al., 2006 and Tuppurainen and Oura, 2012**). It causes high economic losses, characteristic skin nodules, fever, and emaciation (**Ebtsam et al., 2012**). The disease considered a (list A) diseases by the office international des Epizooties (OIE) due to its potential for rapid spread and ability to cause severe significant economic losses, as to hide damage, Loss of milk production, mastitis, infertility, and death (**Younis and Aboul souad, 2005 and OIE 2006**). In Egypt, LSDV was first isolated and identified from cattle during two outbreaks in Suez and Ismalia governorates during 1989 (**House et al 1990 and Davies, 1991**).

LSDV exists in skin nodules, crusts of skin lesions, blood, saliva, nasal discharge, semen, and milk of the affected cattle (**Babiuk et al., 2008**). LSD is primarily spread among animals by biting insects such as mosquitoes and biting flies (**Iubinga et al., 2014**). Less commonly, the virus may be spread by direct contact to the skin lesion, saliva, nasal discharge, milk, or semen of infected animals (**Coetzer, 2004**).

In Sharkia province during the summer of 2016 , cases of LSD have been observed in cattle previously vaccinated with the Romanian sheep poxvirus (SPPV) vaccine. Phylogenetic analysis revealed that the LSDV isolates were clustered together with other African and European LSDV strains. Also, the LSDV isolates have a unique signature of LSDVs (A11, T12, T34, S99, and P199).(**Fatma et al., 2018**).

LSD control through vaccination may be endangered by improper use of vaccines and partial protection of some LSD vaccines(**Gelaye et al., 2015**) . So the need for genetic characterization of LSDV during outbreaks is important to understand the genetic variation of field isolates(**Sylvester ochwo et al., 2020**) .On the other hand, abused vaccines bringing may lead to co-infection and recombination of vaccine strains with virulent strains , resulting in virulent reversal of vaccine strains, which may lead to more outbreaks (**Sprygin et al., 2018**).

This study aimed to diagnose Lumpy skin disease at the molecular level and to study its effect on some immuno-biochemical, oxidative stress parameters, and inflammatory markers in naturally infected cattle.

MATERIALS AND METHODS

1. Animal

Twenty five cattle from Belbes & Dyarb Ngm cities in Sharkia governorate at Arab Republic of Egypt(ARE)was used. All cattles aged between 1-3

years old not vaccinated against LSD and were free from external, internal, and blood parasites. The animals were divided into ten clinically healthy cattle without LSD reports used as a control group. And Fifteen suspected infected cattle showing clinical signs were used as an infected group.

Samples

Fifteen skin nodules were collected from suspected infected cases with LSDV from farms during the period between 2018-2019 for virus isolation and identification. Whole blood samples with and without anticoagulants were taken from the jugular vein of healthy and ill cattle for serum separation for immunobiochemical study and phagocytic activity .

3. Isolation and identification

3.1. Skin nodules preparation

The nodules were incised using a sterile scalpel and the lesions were ground with sterile sand and PBS containing 1000IU/ml penicillin;1mg /ml streptomycin and 100IU\ml Mycostatin in 10% suspension.

3.2. Virus isolation

3.2.1. Tissue culture

Samples from Skin nodules prepared in 10% suspension of maintenance media(Dulbecco' s Modified Eagle' s Medium (DMEM)) supplemented with antibiotics) were inoculated in tissue culture flask 25ml of MDBK cell line. The media used for the growth of MDBK cell line was Dulbecco' s Modified Eagle' s Medium (DMEM) supplemented with antibiotics and 10% foetal calf serum.

The flask is examined daily for 7–14 days for evidence of cytopathic effect (CPE). Contaminated flasks should be discarded. If no CPE is apparent by day 14, the culture should be freeze-thawed three times, and clarified supernatant inoculated on to fresh culture. (**OIE terrestrial manual 2010**).

3.2. Chorioallantoic membrane(CAM)

Skin nodules sample suspension was inoculated in fertile egg via chorioallantoic membrane according to (**Van Rooyen et al., 1969**). After incubation CAM was harvested, washed in PBS and examined for pock lesion. The positive sample was stored in freeze to be used in Indirect florescent antibodies technique LSDV antiserum: locally prepared in rabbit. Antisera was prepared from previously isolated identified virus. Inactivated virus mixture with incomplete Freund's adjuvant was inoculated into rabbits at 0,15,30,45,60 and 90 days respectively according to the protocol of (**Hussein., 2004**)

3.3. Indirect fluorescent antibody technique

MDBK was grown in Leighton tube containing coverslip. After 24h, the media was discarded and 50µl was inoculated on the coverslip. The inoculated tube was incubated for 1h at 37°C for adsorption time. The maintenance media was added and incubation for farther 24-36h at Co2 incubator (nuaire). The coverslip was taken, air-dried, and fixed in cold acetone for 1h. Also frozen sections of the pock lesion of the CAM were cut using a cryostat, then fixed on a slide using cold acetone. The samples were treated with lumpy skin virus hyperimmune serum prepared in a rabbit and incubate at 37°C for 1h, then wash 3 times with PBS and leave to dry. Add the second anti-rabbit serum conjugate. And further incubation at 37°C for 1h followed by washing 3 times with PBS and leave to dry. Then all samples including negative control were examined using a fluorescent microscope (optika) (Walid *et al.*, 2010 ; OIE terrestrial manual 2010)

4. Molecular studies

4.1. Nucleic acid extraction

Whole nucleic acid extraction from samples was performed using the QIAamp mini elute virus spin kit (Qiagen, Germany, GmbH). Briefly, 200 µl of the sample suspension was incubated with 25 µl of Qiagen protease and 200 µl of AL lysis buffer at 56°C for 15 min. After incubation, 250 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 50 µl of elution buffer. Oligonucleotide Primers. Supplied from (Metabion Germany) are listed in the table (1).

Table 1: Primers sequences, target genes, amplicon sizes

Target agent	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
Capripox	ORF 103	F ATGCTGATAAAAAATTATCTCG	570 bp	Zhu <i>et al.</i> , 2013
		R TCCATACCATCGTCGATAG		

4.2. PCR

4.2.1. PCR amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied

bio-system 2720 thermal cycler. The primary denaturation step was done at 95 OC for 5 min, followed by 35 cycles of 94°C for 30 sec., (annealing 52C⁰ for 40 sec.) and extension on 72C⁰ for 40 sec. A final extension step was done at 72°C for 10 min.

4.2.2. Analysis of the PCR Products

The PCR products 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 were loaded in each gel slot. A gel pilot 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.

4.2.3. Nucleotide sequencing

The sequencing of the product was done by genetic analyzer 3500 (life technology) using a big dye terminator V3.1 sequencing kit. The analysis is done by Bioedit and CLC's main workbench 6 software.

5. Biochemical parameters

Serum biochemical parameters ALT, AST, ALP were Analysed by procedure used by (Reitman & Frankel 1975 and Kind and King 1954) , also total protein & albumin were measured in serum samples as described by Dumas *et al.* (1981) and Drupt (1974), respectively. Serum globulin concentration was calculated by subtracting the measured albumin level from the total protein level.

-Calcium, inorganic phosphorous, Sodium, Potassium & Magnesium were measured in serum samples as described by (Gindler and King 1972; Drek and Jung 1970; and Terri and Sesin 1958).

- Oxidative stress markers plasma malondialdehyde (MDA) & glutathione peroxidase (GPX) (Ohkawa *et al.*, 1979 ; Paglia and Valentine 1967). Inflammatory cytokines TNF -α, IL-4 was measured by using commercial ELISA kits used by method described in standard kits from bio source and ALPCO diagnosis. Further more immunoglobulins titer of both IgG & IgM measured by sandwich ELISA (Erhard *et al.*, 1992).

-The phagocytic activity was measured in peripheral blood monocytes using candida Albicans according to Anthony *et al.*, (1985) and Chu and Dietert (1989). Separation of peripheral blood mononuclear cell (PBMC) using a ficall-hypoque density gradient was carried out as described by Boyum (1986); Goddeeris *et al.*, (1986) and Wilikinson, (1976).

Statistical analysis

The obtained data were statistically analyzed by using the T-test (Tamhan and Dunlop, 2000).

RESULTS

The cattle affected by Lumpy skin disease show signs of anorexia, skin nodules, fever above 40°C, edema in forelimbs, and dewlap with a decrease in the milk production, lacrimation, nasal discharge, and enlarged superficial lymph node (Figs. 1-4). Tissue culture MDBK Infected cells develop a characteristic cytopathic effect CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin . At first, only small areas of CPE can be seen, sometimes as soon as 2 days after infection; over the following 4–6 days, these expand to involve the whole-cell sheet . CPE appeared on inoculated MDBK cell line after 72 h of inoculation(figs.5-6) . Isolated LSDV on tissue culture and frozen sections from positive CAM(fig.9) were identified by the fluorescent antibody technique (Figs.7-8-10-11). Molecular studies included PCR . Analysis of PCR products in the 2% gel as shown in Figure 12 .Results were visualized under ultraviolet (UV) light and images documented using a gel documentation system. Phylogenetic analysis for three positive isolates (Figs.13-14) showed similarity to previously locally isolated strains in Egypt and close relation to strains of SPPV.



Fig.1: Enlargement of superficial L.N



Fig.2: Skin nodules all over the body



Fig.3: Nodules on the perineal area and all over the body



Fig.4: Edema in forelimbs and dewlap



Fig.5: Infected MDBK cell line showing CPE(rounding.granulion,clustering)

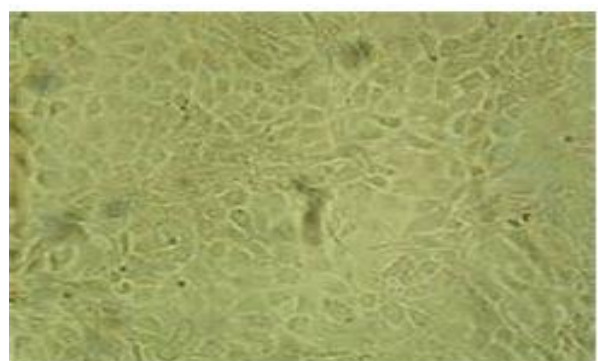


Fig.6: Normal non infected MDBK cell line with no CPE

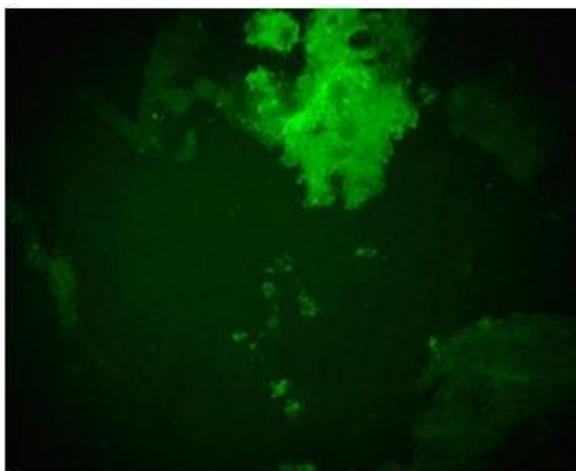


Fig.7: Specific intracytoplasmic yellowish green fluorescent granules emitted from the infected CAM

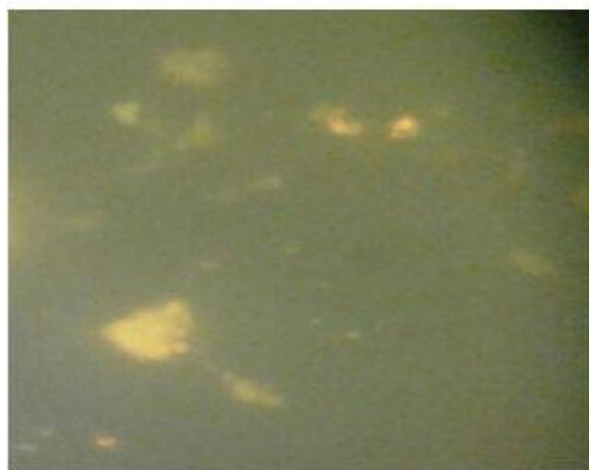


Fig.8: Non infected CAM stained with fluorescein didn't show any fluorescence



Fig.9: Infected CAM showed thickening and pock lesions

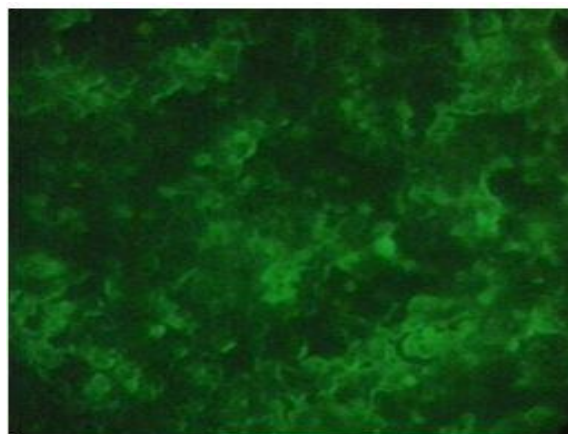


Fig.10: Infected stained MDBK cell line showed specific intracytoplasmic green fluorescence

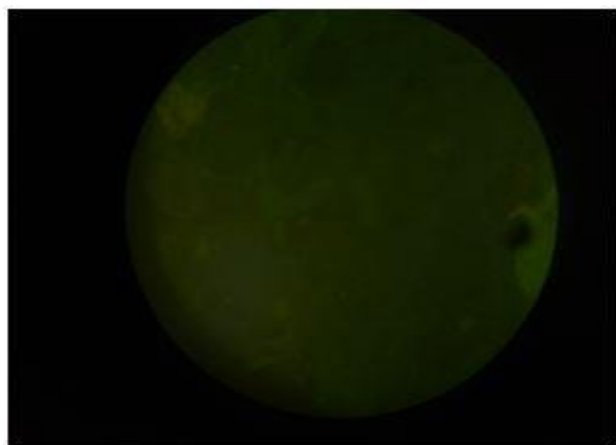


Fig.11: Stained normal non infected MDBK cell line didn't show any fluorescence

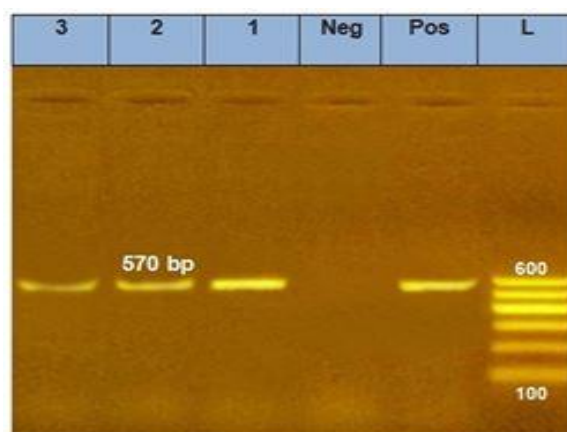


Fig.12: Electrophoretic pattern for LSDV-PCR- Lane L is 600 bp ladder.Lane1,2,3 are Positive (amplicon size is 570 bp)

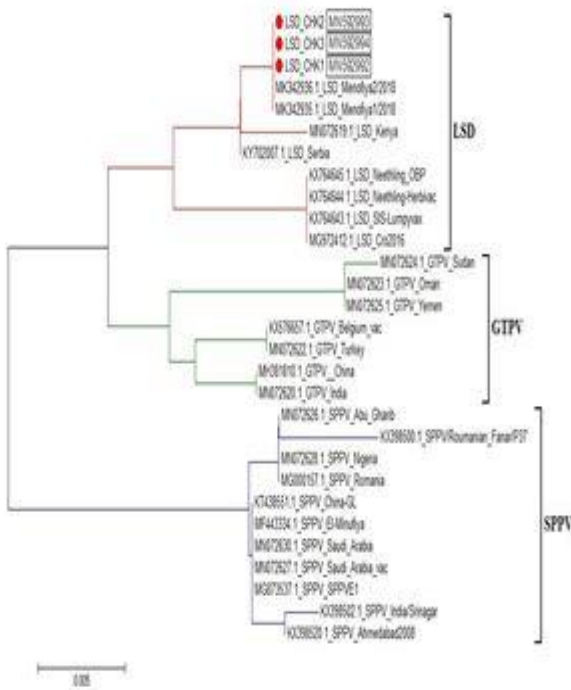


Fig.13: Phylogenetic analysis of the 3 sequenced LSD specimens isolated from Sharkia governorate in 2019 and previously isolated egyptian samples, universal LSD isolates, sheep pox, goat pox, and

DISCUSSION

The clinical signs in the affected cattle agree with **Ismail and Youssef (2006)** and **Neamat-Allah (2015)**. **Tulman et al., (2001)** and **Ebtisam et al., (2012)** who stated that LSDV infected cattle characterized by fever, nodules on the skin, edema, and enlargement of superficial lymph nodes. LSDV contains 156 ORFs which have been annotated here as putative genes. These genes represent a 95% coding density and encode proteins of 53 to 2,025 amino acids (**Tulman et al., 2001**).

We concluded from the analysis of NCBI result and the phylogenetic tree that, our isolated strains of LSDV has identity similar to other LSDV with % of identity range from 100% as El-Monufiya ORF 103 gene (MK342936.1 and MK342935.1) to 99.4%; 99.8% as LSD (MN072619.1) from Kenya; The isolated strains was closely related to GTPV with percentage 97.6% as GTPV (MN072624.1) from Sudan; The isolated strains was closely related to SPPV with a percentage of 97.2% with SPPV (MG873537.1) from El-Sharkia 2017and SPPV (MF443334.1) El-Monofeya (**Ashraf et al., 2018**).

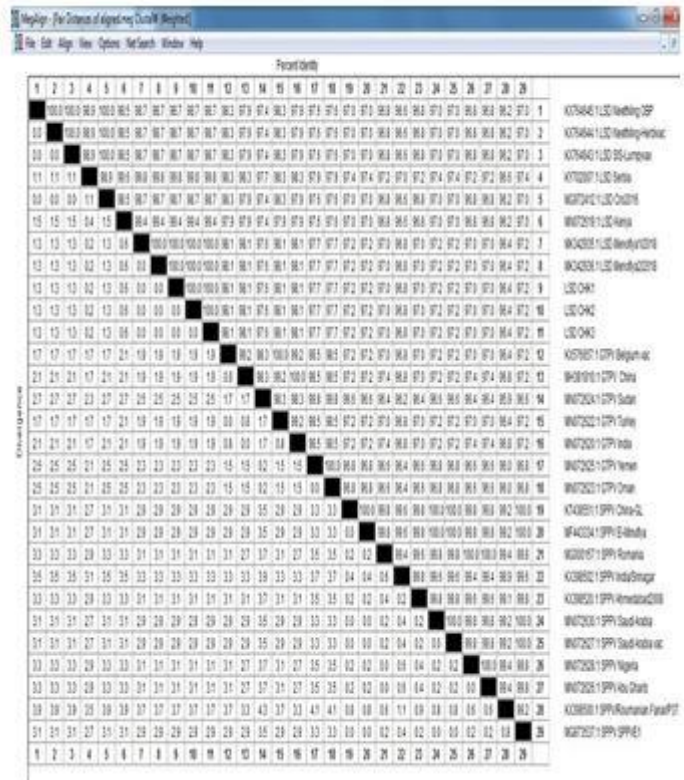


Fig. 14: Sequence distance of the 3sequenced LSD samples in comparison with Egyptian LSD isolates, universal LSD isolates, sheep pox, goat pox, and vaccinal LSD strains.

The analysis gave a good image to illustrate that our newly isolated strains were similar to previously locally isolated strains in Egypt (accession number MK342936.1 and MK342935.1).Those two previously mentioned registered isolates at gene bank were isolated in 2018 from sheep.

The great similarity between the LSDV isolates and the field SPV isolates indicates that field skin isolate of SPV may be LSDV that changed its host to infect sheep. (**El-Kenawy and El-Tholoth 2010**). Their results agree with an earlier report (**Tulman, et al., 2002**) that found sheep and goat poxvirus is probably derived from a common ancestor LSDV. This point needs further study on the whole virus genome. Although there is some strong evidence suggesting that these viruses emerged thousands of years ago, their genome has evolved through the gain and loss of genes, especially through gene duplication and horizontal gene transfer (HGT) (**Hughes, and Friedman 2005; Bratke, and McLysaght, et al., 2008**).

Many of the genes present in the poxvirus genome are not essential to viral replication in cell culture but are important to the modulation of the host antiviral response, and thus are considered virulence genes (McFadden, 2005, and Haller, *et al.*, 2014). The evolution of virulence genes for a certain host is probably influenced by the effectiveness and route of virus transmission, the immune status of the host species, the availability of additional reservoir hosts, intra- and inter-species competition with other viruses. And positive selection for hosts that are more resistant to virus infection (Hughes, *et al.*, 2010; Ebtisam *et al.*, 2012 and Haller, *et al.*, 2014) and according to that our study may give a prediction for the identification of lumpy skin disease virus in Egypt.

LSD cause changes in biochemical parameters in infected cattle . They elevated AST&ALP in LSDV infected cattle may be related to viremia induced hepatic injury (Sevik *et al.*, 2016). Serum Alkaline phosphatase was increased significantly may be due to inflammation of cell lining and surrounding the biliary duct and may have been induced by intrahepatic cholestasis and biliary disease (Stockham and Scott 2008). The significant increase in liver enzymes may be attributed to liver injuries. The increase in AST may be due to heart and general tissue break down (Agag *et al.*, 1989). Our results agree with Aly *et al.*, (2006); Jalali *et al.*, (2017), and Helal *et al.*, (2019).

The significant decrease in T.P and albumin in the infected group may be due to anorexia, off food, and damage of the liver by the virus as the synthesis of albumin occurs in the liver. These results agree with (Hassan *et al.*, 2011) who reported that Hypoproteinemia and hypoalbuminemia result in LSD cattle may be due to decreased synthesis and higher catabolic rate of protein as well as damaged of liver parenchyma.

The infected LSD cattle showed a significant decrease in serum calcium, inorganic phosphorous, sodium, and potassium. This adverse effect may be from decrease food intake and malnutrition and fever with subsequent disturbance of all metabolic processes (Rosby *et al.*, 1991, Ahmed 2007 and Hassan *et al.*, 2011). Hypocalcemia could be attributed to hypoproteinemia which result in a decrease of protein bounded calcium (Stockholm and Scott, 2008). Furthermore mentioned that changes in the trace elements may be related to a decrease in food consumption or to hypoproteinaemia which hinders its absorption (Ahmed, 2007). This results agree with Helal *et al.*, (2019) who stated that serum calcium and sodium concentrations were significantly decreased in LSD infected cattle. Another explanation of significant decrease in mineral concentration could be attributed to

two main factors, decreased synthesis and higher catabolic rate as well as damaged liver parenchyma. Moreover, the infection was considered as a sort of stress on animals and is associated with the increased level of disturbed oxidant/antioxidant status in the body (Ahmed 2007 and Essayed *et al.*, 2016).

The effect of LSD on both antioxidant and inflammatory markers in cattle revealed a significant increase in MDA with a significant decrease in GPx in infected animals compared to the control group. These changes may be attributed to increasing the oxidative stress due to free radical production and lipid peroxidation with the exhaustion of antioxidants in the blood resulting from tissue injury Nashwa *et al.*, (2017) ; Shefaa and Rasha (2018). Gpx enzyme plays a critical role in protecting the cell from free radical damage particularly lipid peroxidation. The significant decrease in GPx in affected cattle indicated its use in an attempt to reduce oxidative stress (Kataria *et al.*, 2010 and Nashwa *et al.*, 2017).

there was a significant increase in IL4and TNF- α concentration and this may be due to the stimulation of macrophages and lymphocytes to release cytokines that initiate inflammation in different tissue during the viraemic stage of the disease(Shefaa and Rasha 2018).

Our immunological results revealed a significant increase in IgG and IgM with a significant decrease in phagocytic % and phagocytic index in infected cattle compared to the control group. The increase in immunoglobulin may be due to the immune response of the body following viral infection. These results agree with Agag *et al.*, (1989), Estes *et al.*, (1990), Hassan *et al.*, (2011) and Neamat- Allah (2015). On the other hand, Serological assessment of antibodies to a capripox virus may sometimes be difficult due to the cross-reactivity encountered with other poxviruses as well as to the low antibody titers elicited in some animals following mild infection or vaccination Kitching and Hammond, (1992).

Also there was significant decrease in phagocytic % and index in this study may be due to the stress of infection that decreases cellular immunity as mentioned by Neamat- Allah (2015). Also due to viral infection inhibits macrophage phagocytosis as lowering the mediator's factors such as opsonins and cytokines, which attract and activate leukocytes. The degree of phagocytic activity of macrophages and neutrophils is impaired led to decrease phagocytic uptake of particles, their phagocytic rates, and intracellular killing activities (Murphy *et al.*, 1999).

CONCLUSION

It can be concluded that, lumpy skin disease in cattle induces adverse effects on immuno-biochemical, antioxidant parameters, and inflammatory markers. That causes great economic loss in the animal industry.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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