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EPIDEMIOLOGY AND CHARACTERIZATION OF LUMBY SKIN DISEASE IN NORTH EASTERN OF EGYPT

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ABSTRACT
Lumby Skin Disease (LSD) is an insect born, notifiable, transboundary eruptive viral disease belonging *Capripox* viruses. Mostly affect cattle and causing a significant impact on cattle industry, trade and food security. Our work aimed to study some epidemiological data about LSD including the isolation serological and molecular characterization of its causative agent in the area of study. Samples were collected from some cow herds at the area of Gelbana & sahel al-Tina villages, North Sinai Governorate. The causative virus was isolated on chorioallantoic membrane (CAM) and MDBK cells. Polymerase Chain Reaction (PCR) identified the isolated virus as LSD. Protective viral neutralizing antibodies were found in 19/23 (82.60%) and 5/27 (18.51%) of previously vaccinated and non vaccinated animals respectively. The overall morbidity and mortality rates were 21.25% and 2.5% respectively. Other risk factors as age, gender and vaccination status were studied revealing that, clinically, the disease occurrence is more frequent in younger (27.5%), male (22.85%) and non-vaccinated animals (32.55%). In conclusion, proper extensive vaccination process using a local efficient LSD virus isolates, further education of herd owners about the etiology and transmission of LSD and Strict monitoring of animal in and out movements in the area of study including Quarantine should be employed by veterinary authorities in order to reducing the circulation of LSD.

INTRODUCTION

Lumpy skin disease (LSD) is an infectious viral disease usually affecting cattle with generally low mortality (less than10%) and varying (1-90%) morbidity rates. It is caused by a virus of the family Poxviridea and genus Capripox **(Salib and Osman, 2011)**. The disease is of significant economic importance to cattle industry due to reduction in milk production, abortion, temporary or permanent infertility, damaged hides and mortalities (**Tuppurainen and Oura, 2011).** Lumpy skin disease can be suspected whenever clinical signs indicate towards persistent fever, wide spread skin nodules (lumps), enlarged peripheral lymph nodes, conjunctivitis, keratitis, corneal opacity, edema in the brisket and legs (**Radostits et al., 2007)**. Animals recover slowly from the severe disease and may suffer from mastitis, pneumonia, formation of necrotic skin plugs leaving deep holes in the hide **(Tuppurainen et al., 2011).**

In Egypt, LSDV was first isolated and identified from cattle during two outbreaks in Suez and Ismalia governorates on 1989 **(House et al., 1990; Davies, 1991)**. The possible introduction of new strains of LSDV by the continuous movement of animals either in between different governorates and or across borders is a major constant threat. In early 2006, a severe LSD outbreak struck foreign (imported from Ethiopia) and local cattle in different Egyptian governorates, causing enormous economic losses (**OIE., 2006*).*** Since that time and the disease is recurrent and annually causes focal points of infection.

Viral isolation, polymerase chain reaction (PCR), dot blot hybridization (DBH), and indirect enzyme-linked immunosorbent assay (iELISA) were used for the diagnosis of lumpy skin disease in clinically infected, fevered, and apparently normal dairy cows **(Awad et al., 2010)**.

Biting flies (*Stomoxys calcitrans* and *Biomyia fasciata*) and mosquitoes (*Culex mirificens* and *Aedes natrionus*) act as a vector for transmission of the disease **(Chihota et al., 2001)**. Other risk factors associated with spread of LSD were found to be worm humid agroclimatic, communal grazing&watering and introduction of new animals in a herd **(Gari et al., 2010).**

To date, many vaccines had been tried to control the LSD and it is concluded that, in the wake of an outbreak cattle can be protected against LSD by using the strains of Capri poxvirus derived from sheep or goats **(Kitching, 2003)**. Although, commercial live attenuated LSDV vaccines are available but their use in countries previously free from disease is not recommended because of the potential safety issues as skin lesions containing high titers of virus can be developed in vaccinated animals that may disseminate the virus through vectors **(Tuppurainen and Oura, 2012).**

The aim of the present work is to provide some epidemiological data about lumpy skin disease, through examination of apparently healthy and other suspected animals showing skin lesions for virus isolation, serology and molecular characterization as well epidemiological description and clinical features of the disease at the study area.

MATERIALS AND METHODS

1- Area of Study:

El-Salam canal is that canal transport the Nile water into Sinai and extended through different localities in North Sinai. The areas of Gelbana and Sahel al-Tina around the branches of this canal are newly reclaimed villages used for different agricultural and animal production activities at which local animals of North Sinai and newly introduced animals from old Delta governorates lives closely and sharing the same environmental conditions.

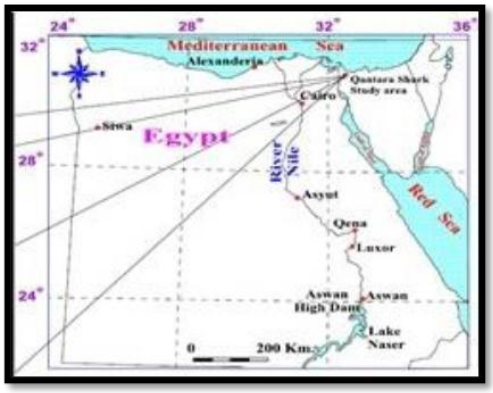


Figure (1): Location map of the study area.

2-Animals:

Eighty animals of different age & gender from some cattle herds at the area of study were examined clinically according to **(Rodostits, et al., 1995)** specially for the presence of characteristic skin lesions suspected to be LSD with the recording of the history of vaccination with sheep pox vaccine.

3- Samples:

3-1-clinical specimens:

Twelve representative skin nodules were collected aseptically from twelve acutely infected animals for virus isolation, identification and molecular characterization. Specimens were transported on ice to the lab. where preserved at −20 °C until processing according to **(OIE, 2014).** The skin samples were thawed at room temperature then washed with sterile phosphate buffer saline containing antibiotics and antifungal at a pH of 7.2. The samples were ground with sterile sand in a mortar. The homogenized suspension was frozen–thawed three times. Tissue homogenates (10% W/V) were centrifugated at, 4000 rpm/10 minutes. The supernatant was collected and stored at −20 °C till be used.

3-2 Serum samples:

Blood samples were randomly collected from twenty three previously vaccinated animals with sheep pox attenuated virus strain and twenty seven non vaccinated for serum separation. Serum samples were stored at -20 ºC until used for serum neutralization test.

4- Virus Isolation:

4-1 Specific Pathogen Free (SPF) Emberyonated Chicken Eggs (ECE):

Fertile SPF-ECE (11-12) days old were obtained from Newcastle disease Dept.Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. Emberyonated chicken eggs were inoculated by the supernatant fluid of tissue homogenate via chorio-allantoic membrane (CAM) route according to **(Van Rooyen et al., 1969),** incubated at 37°C for 5days and examined daily for the development of pock lesions.

4-2 Tissue culture:

0.2 ml from the supernatant fluid of tissue homogenates were inoculated for three passages into MDBK tissue culture cells after primary kidney tissue culture passage and examined daily for cytopathic effect (CPE) according to the method described by **(OIE, 2012).**

5- Molecular characterization:

DNA was extracted from collected skin lesions using GF-1 tissue DNA extraction kit (Vivantis Malaysia) following manufacture instructions for PCR, the sequence for the primer pair designed to amplify the ORF103 gene was illustrated in table (1).

Table 1: Specific primers sequence: according to (Zhu et al., 2013).

primer	Orientation	Sequence
1	Forward	5´ ATGTCTGATAAAAAATTATCTCG 3´
2	Reverse	3´ ATCCATACCATCGTCGATAG 5´

Table 2: Cycling protocol for amplification: according to (Zhu et al., 2013).

Steps	Temp.	time	No. of cycles
Initial denaturation	94°C	5 mint.	1
Denaturation	94°C	30sec.	35
Annealing	52°C	45sec.	35
Extension	72°C	45 sec.	35
Final extension	72°C	10 mint.	1
preservation	4°C		

PCR amplification was performed in 25X reaction volume and contained 5ul of DNA template, 100ul of each deoxynucleoside triphosphate (thermo scientific, USA), 2.5 units of Dream Taq Green DNA polymerase (therosentific, USA) 5ul of amplification buffer and 20 ul of primer. The PCR amplification was carried out using Gene Amp PCR system 9700 thermal cycler (Applied Biosystems, USA) the cycling condition for amplification of gene (P32) was illustrated in tables (2). PCR amplicons were analyzed by running 10 ul of PCR reaction mix in 1% agrose gel stained with Ethidium Bromide (0.5ug/ml). Therefore, gels were photographed under UV illumination using gel documentation and analysis system supplied with starlight express MX516 16-bit CCD camera and AAp-M5 software and amplification pattern was determined according to the molecular size of the amplified products.

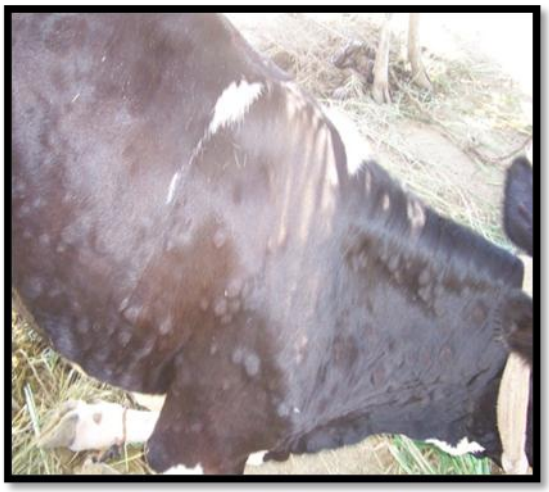
6- Serological Investigation by Neutralization Test (SNT) for LSDV antibodies:

Collected serum samples were used for detection of antibodies against LSDV using virus neutralization test according to **(OIE, 2012)**. The virus titer (VT) and the serum virus titer (SVT) were calculated as described by (**Reed and Meunch, 1938)** to determine the 50% pock forming infective dose end point (ID50). The neutralization index (NI) was subsequently calculated according to (**Pilchard et al., 1962)** as NI= VT – SVT, where NI ≥ 1.5 considered protective **(Cottral, 1978).**

RESULTS:

Clinical features:

Out of 80 cattle examined clinically, 17 showed the signs of infection with the development of clinical manifestations of the different stages of the disease which varied from fever, inappetence and low milk production to severe clinical signs characterized by generalized skin nodules of 2-3 cm in diameter mainly on neck and head, enlarged peripheral lymph nodes, edema of the dependent parts (brisket and forelegs) (photo. 1,2), lacrimation with enlarged lacrimal duct (photo, 3). With the progression of disease, the nodules became necrotic, and eventually a deep scab formed (photo.4). The small ruminants (sheep and goat) housed with disease affected cattle did not show any symptom of the disease.



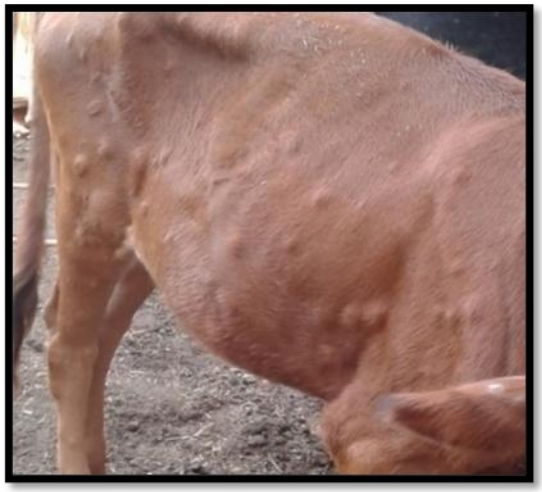


Photo (1, 2): showing enlarged lymph nodes and generalized skin nodules.

Table 3: Prevalence of lumpy skin virus Infection according to age, gender and history of vaccination.

Item		No. of examined animals	Morbidity		Mortality	
			No	%	No	%
Age	< 1 year	15	2	13.33	0	0
	1-3 years	40	11	27.5	2	5
	> 3 years	25	4	16	0	0
Total		80	17	21.25	2	2.5
Gender	Male	35	8	22.85	1	2.85
	Female	45	9	20	1	2.22
Total		80	17	21.25	2	2.5
History of vaccination	Vaccinated	37	3	8.10	0	0
	Non vaccinated	43	14	32.55	2	4.65
Total		80	17	21.25	2	2.5

Photo (3): ocular discharge.

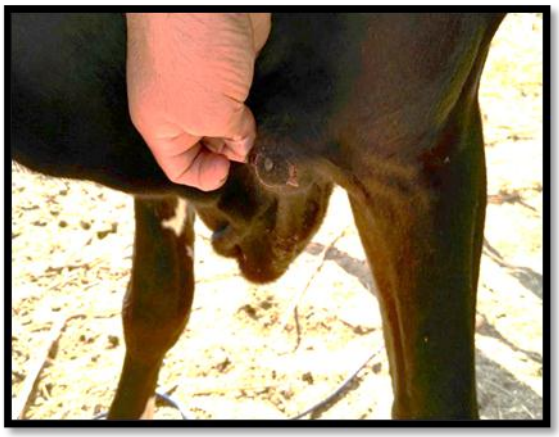


Photo (4): Necrotic skin nodules.

Propagation of the Virus on CAM of ECE and (MDBK) cells:

Isolated virus was inoculated on CAM of SPF-ECE 9-11day old resulting in the characteristic pock lesion of CPV (circular, opaque, enlarged white yellowish) as illustrated in (photo, 5,6). MDBK cell culture showed aggregation of cells with rapid progression and floating, few days post inoculation plaques with round degenerative cells are developed and massive detachments of cells were observed, (photo, 7).

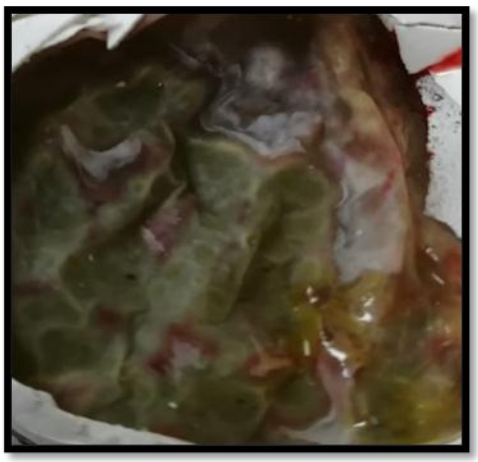


Photo (5): ECE after viral.

Photo (6): CAM showing bl.vessels congetion.

Inoculation. & white yellowish round opague foci**.**

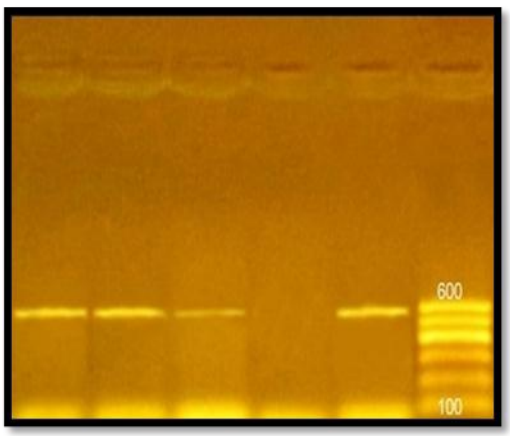


Photo (7) CPE of LSD virus on MDBK photo (8) electrophoresis showing the size of the Cells showing aggregation and detachment. amplicon (570bp) specific for ORF 103gene in 3 +Ve samples, marker & +Ve and -Ve control.

The ORF 103 gene was amplified by using PCR technique and amplification products consistent with the expected amplicon size (570bp) was obtained (photo, 8).

Table 4: Neutralization Index in vaccinated & nonvaccinated animals

Item	Neutralization Index (NI)				Total
	≥ 1.5		< 1.5		
	No.	%	No.	%	
Vaccinated	19	82.60	4	17.39	23
Non vaccinated	5	18.51	22	81.48	27
Total	24	48	26	52	50

DISCUSSION

Lumpy skin disease is a viral disease affecting cattle of all ages and breeds caused by a ***capripox*** virus belonging to ***poxviridea*** family **(Woods, 1988; Tuppurainen Oura, 2012)** characterized by high morbidity and low mortality rates **(Kitching and taylor, 1985; Barnard et al., 1994)** causing high economic losses through decreasing of animal weight and milk production, hides damage, mortalities and the cost of treatment and control. LSD was firstly recorded in Egypt in 1988 where it was diagnosed clinically in Suez Canal governorate then spread to the surrounding governorates in the summer of the same year as reported by **(Salem, 1989).** In the present study, clinically affected animals showed a mild form of the disease characterized by, the formation of a few numbers of skin nodules (lumps) specially in the neck, legs and back which are circumscribed, firm, raised and painful (photo, 1&2) appeared three to four days after the onset of systemic reactions including fever, anorexia, depression, enlargement of the superficial lymph nodes, conjunctivitis and or nasal discharges. Most of these nodules regressed while the others either sloughed or undergo necrosis forming hard necrotic lesions can be separated from the neighboring tissues (photo, 4). the form of the disease and its severity depends on the dose of the inoculum as well as the host susceptibility and the route of exposure as reported by **(Davies, 1981)**. The diagnosed mild form may be attributed also to the immunity status of the animals, previous exposure as well as routine vaccination.

As shown in (table, 1) the overall morbidity rate was 21.25% while the mortality was 2.5% in a relative agreement with the previously reports (morbidity 26% and mortality 1.9%) by (Abu tarbush et al., 2015) in Jordan. Higher morbidity rates (15.4%–26.3%) were reported in Oman with mortality rates ranged from (13.6%–29.7%) **(Tag eldin et al., 2014)**. In Egypt, 100% morbidity was recorded in Giza governorate with a mortality of 1.8% **(Salib and Osman, 2011)**. The variation in the prevalence of infection may be attribute to seasonal variation, entrance of newly purchased or imported animals, communal grazing and watering points **(Gari et al., 2011; Tuppurainen Oura, 2012**). Morbidity and mortality rates can also fluctuate according to agroclimatic conditions, disease management, the strain of virus involved and the immune status of the animals and their breeds (Tuppurainen and Oura 2012) which considered important risk factors causing the variation in the prevalence rate of LSD from area and time to another's. Concerning the age, it was found that, young animals in the age between 1-3 years are more affected with a prevalence rate 27.5% followed by the older animals more than 3 years old 16% then the lowest prevalence was in the newly born and less than one year old calves. These results were in agreement with that of **(Weiss, 1968; Tuppurainen et al., 2011)** who mentioned that, the disease can affect cattle of all ages and breeds but the young animals and cows in the peak of lactation are more severely affected. Also **(Salib and Osman, 2011; Ayelet et al., 2013; Sevik and Dogan, 2016)** were previously reported higher incidence of infection in younger animals than older while, **(Yousefi et al., 2017)** reported that, LSD occurred more frequently in older cattle over 5 years. Male animals were found to be infected to a little extend more than females (22.85%, 20%) respectively. It may result from the trading activities which depends mainly on males more than females subsequently they are more subjected to catch infection. On the other hand, **(Ayelet et al., 2013)** reported that, LSD was common in females due to stress factors related to pregnancy and lactation which decrease the immunity of the females and lead to increase susceptibility for the disease.

However, little is known about the immunological response and immune dynamics against this disease **(Christine et al., 2017)**, it is widely agreed that vaccination is the most effective way to control the spread of LSDV in endemic countries like Egypt **(Ayelet et al., 2013)**. Control of LSD among cattle in Egypt depends on mass vaccination programs, using a heterologous cross reacting sheep pox virus vaccine (Romanian strain) which is antigenically related to LSD and produce good immune response in cattle **(Michael et al., 1994)**. In our study, we found 3 out of 37 (8.10%) vaccinated animals showed mild symptoms of LSD revealing incomplete vaccination success or animal vaccination was done few days prior to examination and sampling as mentioned by **(Hunter and Wallace, 2001)** who reported that immunity to LSD starts developing 10 days after vaccination and reaches its peak after 21 days. However, Egyptian veterinary services performed an extensive vaccination programs to eradicate the disease, LSD and sheep pox are still recurrent and prevalent at different localities of Egypt **(Rouby et al., 2019)**. one of the causes of vaccination failure is that, sheep pox virus vaccine causes an incomplete protection and or adverse post vaccinal reactions against LSD among cattle in the Middle East as mentioned by **(Ayelet et al. 2013; Eeva et al., 2014)**. On the other hand, 14 out of 43 (32.55%) non vaccinated animals showed different clinical manifestations of the disease in agreement with the result reported by **(Yousefi et al., 2017)** who recorded 40.8% of vaccinated cattle with sheep and goat pox vaccines

and 71.3% of non-vaccinated cattle showed clinical signs revealing the success in decreasing the occurrence of clinical disease in the vaccinated animals by more than three times. This finding is in contrast to the results of **(Hailu et al., 2014)** which reported no association between vaccination and LSD occurrence.

In the present study, isolation of LSDV revealed characteristic pock lesions on the CAM of SPF-ECEs, which appeared as circular, opaque, small white yellowish distributed foci characteristic of LSD, in agreement with **(El-Tholoth and El-Kenawy, 2016; Allam et al., 2020)**. Moreover, MDBK cell line showed specific CPE appeared as cells aggregation and forming clusters scattering all over the monolayer after 3thday post inoculation. 5thday, a massive detachment of cells observed, in agreement with the findings of **(Fahmy, 2000)**.

PCR tends to be the test of choice for rapid detection and identification of LSD outbreak as serological methods are considered time-consuming to be used as primary diagnostic methods even though they are useful for confirming LSD retrospectively **(Heine et al. 1999; El-Kholy et al. 2008).** As well as serological assessment of antibodies to a capripox viruses may sometimes be difficult due to the crossreactivity and the low antibody titers produced after mild infection or vaccination **(Kitching and Hammond 1992)**. Although many other sources for virus detection such as blood, semen and milk were determined, skin biopsies were the best as they contain more viral particles for detection by PCR **(Tuppurainen et al. 2005)**. Clinically, affected animals from which skin specimens are collected were exhibited the typical signs of LSD, the collected specimens were used for viral genomic DNA extraction followed by a PCR assay. The PCR assay detected LSDV in (100 %) skin nodules from representative infected animals in agreement with **(El-Kholy et al., 2008)** who detected the LSDV in (100 %) of the samples they assayed by using PCR techniques. Therefore, PCR result was fully correlated to field diagnosis based on clinical symptoms. The high sensitivity of PCR (100%) in detecting the LSDV DNA in skin nodular samples may be attributed to the viral tropism to skin tissues and its persistence in high concentration up to 92 days post-infection while, LSD viremia is relatively short timed and blood samples were positive for PCR for 4–11 days **(Tuppurainen et al., 2005 & Zeynalova et al., 2016).**

Amplification of the extracted DNA with the specific primer for the open reading frame (ORF103) gene of Capvs revealed the positive amplicon of the gene at the of (570bp) in agreement with that previously reported by **(zhu et al., 2013; Eman, B., 2018 & Hala, A. et al., 2021)**.

Concerning the obtained result of neutralization test table (4), it was found that, 24out of 50 collected serum samples (48%) giving a neutralizing ≥ 1.5 revealing they are protected in agreement with **(Nashwa et al., 2017)**. while, the other 26 /50 (52%) are not (NI< 1.5) giving an idea about the immunity status against lumpy skin disease in the area of study and the need for improvement the restrictive control measure through health managements and or continuous massive vaccination programs. As shown in the same table, we found that, 4 out of 23 (17.39%) of vaccinated animals have NI < 1.5 (not protected) which may be attributed to animals themselves and their immunity and nutritional status or due to improper vaccination process (vaccination failure) in relation to the transportation, storage and or vaccine itself which may give incomplete protection as it is prepared from attenuated sheep pox virus. Although LSD virus is genetically and antigenically related to sheep pox virus **(Bhanuprakash et al., 2006)**, sheep pox virus vaccine could not completely protect vaccinated cattle against LSD which may be attributed to low dose, nonspecific immunity and or LSD virus is more virulent overcoming the immune response of the used vaccine **(Gapstic, 1959)**. On the other hand, **(Kitching, 1986)** reported that the immune status of a previously infected or vaccinated animal cannot be related to serum level of neutralizing antibody and **(Rao and Negi, 1997)** who concluded that, although the virus neutralization test is the most specific serological test, but because immunity to Capripox viruses infection is predominantly cell mediated, the test is not sufficient.

We also found that, 5 out of 27 non vaccinated animals (18.51%) have neutralizing index ≥ 1.5 revealing previous exposure and or cross reactivity as 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)**.

CONCLUSION

The recurrent occurrence of Lumpy skin disease in different localities in Egypt including the newly reclaimed areas increase the necessity for restricted control measures including early detection and reporting, continuous mass vaccination strategies, increasing the awareness of owners about the economic impact of the disease and its mode of transmission. In addition to more epidemiological studies about the risk factors of the disease including animal movements and seasonal variation with continuous monitoring and field evaluation of the used vaccines.

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