Animal Health Research Journal Vol. 1 No. 2, June 2013 pp. 38– 49

Development of indirect ELISA for detection of Lumpy Skin Disease antibodies in cattle

By

Hassanin S.A.; Nahed K. A.; Wafaa A. A.; Eman M.B and Mervat M.M.

ELISA Research Unit and Viral Strain Bank

Animal Health Research Institute, Dokki, Giza.

Received in 10/6/2013
Accepted in 23/6/2013

Abstract

In this study 6, skin nodules and 100 sera samples were collected from affected and apparently healthy animals at different localities in El-Gharbia Governorate during 2010. Lumpy skin disease virus was detected in the samples using the indirect immunofluorescent antibody technique (IFAT). Isolation of the virus was done by inoculation onto the Chorioallantoic membrane of emberyonated chicken eggs (CAM, 9-11days old) showing pock lesions and a membrane thickening. Inoculation on the Madin Darby Bovine Kidney (MDBK) cell lines shows the specific cytopathic effect (CPE) of the virus, in the form of cell rounded and aggregated together in a separate manner, leaving large irregular holes in cell sheet and giant cells with several nuclei can be found . Identification of virus in the MDBK cell culture isolates was done by the agar gel immunodiffusion test and the Transmission electron microscopy (TEM) which reveals presence of the LS viral particles as roughly brick shaped particles with ridges covering them (x78000). The isolates were identified by using Agar Gel Immunodifffusion Test (AGID) and used for in-house LSDV antigen preparation. Detection of the antibodies against LSDV in the collected serum samples was done by Indirect ELISA using the in-house prepared antigen and the results were confirmed by using the virus neutralization test (VNT).

Key words: *klebsiella, chickens, polygalacturonase (pehX) gene, Antibiotic resistance.*

Introduction

Lumpy skin disease (LSD) (Neethling virus infection or “knopvelsiekte”), is a pox disease of cattle. It is an acute, sub-acute or inapparent infectious disease, caused by a single strain of capripox virus "genus Capripoxvirus of family Poxviridae". The virus is closely related to sheep and goats poxviruses by which there is a degree of homology between LSDV-DNA and that of other Capripox viruses so cattle could be protected against challenge with LSDV by vaccination with sheep pox virus (SPV) **(Abraham and Avive, 1991**).

The disease is usually more prevalent during the wet summer and autumn months and so more in low-lying areas along water courses **(Vorster & Mapham, 2008)**.Transmission of the LSD virus is primarily by biting of mosquitoes or by mechanical transmission by flies **(Carn and Kitching 1995).**

LSD is characterized by mild to severe signs including fever, enlarged lymph nodes, firm rounded nodules in the skin (1-4 cm) which may extend to mucous membranes and internal organs in severe cases, rapid eruption of multiple circumscribed skin nodules, skin edema, lymphadenitis, and may result in mastitis and sometimes death **(Davies, 1991& OIE, 2010).**

A considerable economic loss due to emaciation, damage to hides, infertility in males and females, mastitis and loss of milk production. Morbidity may reach up to (1-90%) and can reach to100% in natural outbreaks **(Salib and Osman, 2011)**, For these reasons, it is listed in

the Office International des Epizooties' 'List A' which identifies diseases with the potential for rapid spread and severe economic losses **(Tuppurainen and Oura, 2011).**

LSD is an infectious disease, endemic in Sub-Saharan Africa and Egypt with a marked increase in prevalence in southern Africa from 1990 to 1999**.** The clinical syndrome of lumpy skin disease was first described in Zambia in 1929, spreading into Botswana, then into South Africa **(Hunter and Wallace, 2001).** The disease was entered in different countries in the world, including Kenya, Sudan, Nigeria and Mauritania, Mali, Ghana and, Liberia, Tanzania, Kenya, Zimbabwe, Somalia, Cameroon and Bahrain **(OIE, 2010).**

In Egypt, LSD was firstly recognized clinically In May 1988 in the Suez Governorate, where it was arrived at the local quarantine station with cattle imported from Africa, then the disease spread locally in the summer of this year. It was reappeared in the summer of 1989 and spread to 22 of the 26 governorates of Egypt in a period of five to six months.. In Lower Egypt it was found that the incidence of LSD in 10 cattle herds from 6 different farms, between May 1988 and May 1989, was ranged from 21 to 71%. Two disease outbreaks' were reported in, 2005 & 2006 **(Aly *et al.,* 2006)**. The virus was isolated for the first time from cattle in Suez and Ismailia Governorate **(House *et al.,* 1990).** Sporadic cases of the diseasese with classical signs were observed in Minia governorate 1998. Another epizootic of LSD in 2005 and 2006 affected 5 different Governorates in Egypt **(OIE, 2010).**

A presumptive diagnosis of the disease can be based on clinical signs. Laboratory diagnosis of LSD can be performed either by isolation of the causative agent and its identification by different methods as TEM, IFAT, IP and AGPT or detection of specific antibody using conventional serological tests **(Tuppurainen *et al.,* 2005).** An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detecting serum which is raised against antigen of capripox virus was developed and also antibody detecting ELISA based on the purified whole viral antigen have been described by **(Babiuk *et al.,* 2009)** .Capripox antigen from biopsy suspensions or tissue culture supernatant can be trapped on an ELISA plate. The presence of the antigen can then be indicated using its specific antiserum, commercial horseradish-peroxidase- and a chromogen/ substrate solution **(OIE, 2010).**

The aim of the study:

Isolation and identification of LSDV by different serological methods.

Preparation of LSDV antigen and its use for development of indirect ELISA for detection of the specific antibodies against the virus in cattle.

Materials and Methods.

Animals.

A total of 100 cattle from different localities in El-Gharbia Governorate, were clinically examined during 2010. The clinical examination includes recording the temperature, skin lesions, mucous membrane and superficial lymph nodes.

Samples.

Nodular skin samples.

6 skin nodules were collected aseptically from infected animals with a typical clinical signs of lumpy skin disease. The samples were prepared for virological examination according to **Carn and Kitching (1995) (Table 1).** These samples were used for virus isolation, identification and antigen preparation.

Blood samples.

A total of 100 cattle serum samples were collected from different localities in El- Gharbia Governorate, from clinically infected and apparently healthy animals. Serum samples were stored at –20ºC until used for detection of LSDV antibodies by ELISA and VNT **(Table 1).**

Reference LSDV.

Ismailia strain of LSDV (local strain), kindly supplied by ELISA research unit and virus strain bank, Animal Health Research Institute. Dokki, Giza, and used for applying the VNT.

Reference LSDV antiserum.
Reference LSDV antiserum (USA) supplied by the ELISA research unit and virus strain bank, Animal Health Research Institute. Dokki, Giza, and used for applying the AGPT and ELISA techniques.

Cell culture.
Madin Darby Bovine Kidney (MDBK) cells, kindly supplied from the African horse sickness diseases department, Veterinary Vaccine and Sera Abbasia Institute.

Anti-bovine conjugate.
Antibovine conjugate (BDSL, England) was supplied by ELISA research unit and virus strain bank, Animal Health Research Institute. Dokki, Giza,

Fluorescence isothiocyanate conjugate.
Anti-Bovine fluorescence isothiocyanate conjugate was supplied by Sigma immune chemicals (USA) and used for applying indirect immunoflourescent antibody technique (IFAT**).**

Table (1). Samples collected from cattle suffered from cutaneous lesions and apparently healthy in El-Gharbia Governorate (2011).

Governorate	Type of samples		
	Skin nodules	Serum samples	Total
El-Gharbia	6	100	106

Identification of virus by indirect fluorescent antibody technique (IFAT).
Prepared skin nodules were inoculated in lighton tubes with cover slips having a confluent sheet of MDBK cell line. Indirect fluorescent antibody technique (IFAT) for detection of LSDV was applied according to **Davies et al., (1971**).

Virus isolation and identification:

Isolation on embryonated chicken eggs (ECE).
Six IFAT positive samples were inoculated on the chorioallantoic membrane of embryonated chicken eggs (ECE) 9-11 days old for two passages according to **House *et al.*, (1990).** The ECE were examined daily for 7 days after inoculation and incubation at 37ºC. CAMs that show lesions were collected from viable embryos, stored at -70 ºC for inoculation on cell culture.

Isolation on MDBK cell culture.
The harvested CAMs were prepared for inoculation into confluent sheet of MDBK cell line and observed daily (for 7 days) for the presence of cytopathic effect (CPE) according to **Sohair *et al.*, (2008) & OIE (2010).** The cells with clear (CPE) were frozen and thawed for 3 times, and then reinoculated on MDBK cells for three blind passages. Samples showing clear cytopathic effect were kept at -70C for LSDV antigen preparation..

Identification of LSDV isolate by Agar gel Immuno-precipitation test (AGPT):-
It was carried out according to **Albana (1978) & OIE, (2010).** Using reference LSDV positive serum & (1%) agarose, prepared in borate buffer.

Identification of LSDV isolate on Transmission Electron Microscope (TEM).
The method of examination of the positive MDBK cell culture isolates by the Electron Microscope was done according to (**Kitching and Smale, 1986 & Omyma, 2008)** in the VACCSERA institute.

Titration of the adapted tissue culture LSDV.
The isolated LSDV was titrated according to **(Frey and Liss 1971).** Virus titre was calculated according to **(Reed and Muench, 1938).**

Preparation of LSDV partial purified soluble viral antigen (PPSVA).
Partially purified soluble viral antigen was prepared according to the method described by

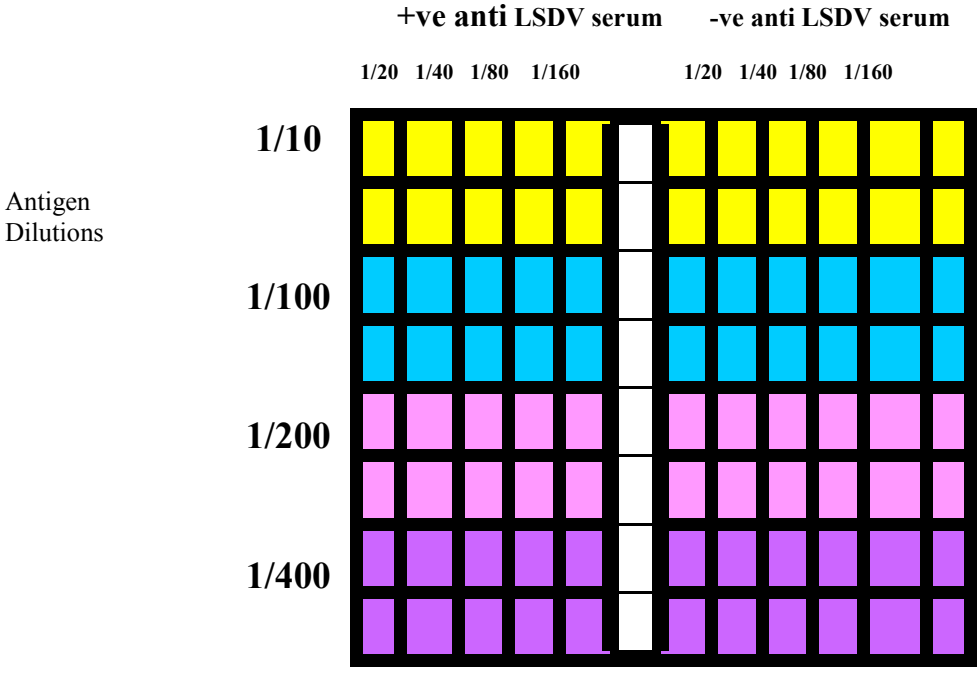
Capstick and Coakley (1962) & Amira (1996). Confluent monolayers of MDBK cells in roux bottles were washed once with sterile phosphate buffer saline (PBS), pH, 7.4 prior to inoculation of each with 1ml of isolated and identified lumpy skin disease virus (LSDV) suspension. The inoculated bottles were incubated at 37 ºC for one hour, followed by the addition of 100 ml maintainance minimal essential media per each bottle, reincubation at 37 ºC and examination daily for specific cytopathic effect (CPE). Bottles showing 80% of CPE were frozen at -20 ºC and thawed for three cycles. The fluid was harvested and clarified from cell debris by centrifugation at 2000 r.p.m. for 15 minutes at 4 ºC. The supernatants were collected. Add 0.5% of NonidetP_{40} (BDH chemical) to the supernatants, approximately (1ml/one roux flask) and kept at 4 ºC for one hour. The supernatant fluids were pooled and concentrated in dialysis bags against polyethylene glycol (M.Wt 6000) to 10X of the original volume. The concentrated fluid was centrifuged at 30,000 r.p.m. in cooling centrifuge for 3 hours. The supernatant fluid containing the soluble antigen was collected, dispensed into vials each of 1ml amount and kept at -20 ºC till used in solid phase ELISA.

Preparation of negative tissue culture control antigen.

Normal monolayers of non infected MDBK cell culture were treated with the same manner like that mentioned for the preparation of the soluble viral antigen (**Amira, 1996)**.

Titration of the prepared LSDV antigen.

Using check board titration against slandered positive and negative anti-LSDV antisera **Puran (1992) & Walid *et al.*, (2010)**.



(Plate layout in check board titration of the antigen)

Screening of LSDV antibodies in the serum samples by:

Indirect ELISA:

By using the prepared antigen, LSDV antibodies were detected in the collected serum samples by the ELISA technique .It was done according to the method described by **Bhanuprakash *et al.*, (2006).**

Briefly, 50 µl of partially purified LSDV soluble antigen (PPSA) (1/100) in coating buffer (carbonate bicarbonate buffer, pH9.6) was added /well in the ELISA plates. Alternative rows

of the plate are coated with viral antigen and control antigen. The plates were incubated at 4c^{o} overnight and then washed 3 times with washing buffer (phosphate buffer saline containing 0.05% Tween-20). The fixed dried wells were blocked with blocking buffer (5% skimmed milk powder dissolved in PBS containing 0.05% Tween-20), then incubated at 37 c^{o} for two hours. 50 µl of each of tested sera, LSDV Positive, negative control sera (diluted 1/140 in blocking buffer, according to check board titration) in two wells for each were added, incubated at 37 cº in a shaker for one hour, and then washed 3 times with washing buffer. 50 µl of antibovine IgG conjugated with peroxidase were added to each well at a dilution of 1/1000 in dilution buffer at 37 cº in a shaker for one hour. The conjugate was decanted and the plates were washed 3 times with washing buffer. 50 µl /well of the substrate (OPD) working dilution with the addition of 3% hydrogen peroxide (H202) . The plate was incubated at room temperature for 20 minutes and reaction was stopped by adding 50µl of 2M sulphuric acid. The absorbance values were measured at a wavelength of 492 nm using a titertek multiskan ELISA reader.

The ELISA results were expressed as corrected optimal density (COD) and calculated as a mean OD with control antigen subtracted from the mean OD with virus antigen for each serum sample. The cut off value used as higher than the mean of optical density values of control negative sera by 0.2 **(Martin 1977).**

Virus neutralization test.

The collected serum samples were examined by the Virus neutralization test for detection of antibodies against LSDV for confirming the results of the ELISA test, according to the method described by **OIE (2010)**.

Results.

The clinical findings.

The clinical observations among different cases of cattle revealed salivation, nasal and lacrimal discharges, fever (40-41 °C), depression and inappetance. Subcutaneous oedemas of different parts of the body accompanied by swelling of the superficial lymph nodes were observed. Most of the affected animals revealed obvious cutaneous nodules all over the body. Such nodules were firm, circumscribed, flat-tapped measuring 1-3 cm in diameter**.** The nodules may coalese forming large elevation measuring 10 cm in diameter or more. (**Fig.1a& b)**.

Detection of virus by indirect fluorescent antibody technique (IFAT).

Results of IFAT for detection LSDV on inoculated cover slips having a confluent sheet of MDBK cell line with the prepared nodules, revealed specific intracytoplasmic yellowish green immunofluorescent staining reactions in all samples **(Fig. 2).**

Virus isolation and identification:

Isolation on embryonated chicken eggs (ECE).

LSDV was successfully isolated through inoculation of prepared nodular samples on CAM of emberyonated chicken eggs (9-11) days old. Typical pock lesion and thickening were found on the harvested CAM at the 4th day post inoculation in 4 samples out of 6 as shown in **(Fig. 3) (Table 2).**

Isolation on MDBK cell culture.

2 positive CAM isolates out of 4, gave clear cytopathic effect on MDBK cell culture after 3 blind passages, showing, cell rounded and aggregated together in a separate manner, leaving large irregular holes in cell sheet and giant cells with several nuclei can be found .This occurred 3-5 days post inoculation and gradually increased till 70 – 80 % of the sheet was completely detached in some samples after 5-7 days **(Fig. 4a &b) (Table 2).**

Titration of the adapted tissue culture LSDV.

The results obtained from LSDV titration showed that the titre of the virus was log10^{5} $TCID_{50}$/ML This titre will be used in LSDSV antigen preparation and VNT.

Identification of LSDV isolates by Agar gel precipitation test (AGPT).

Examination of LSDV-MDBK cell culture iso-

lates by the agar gel immunoprecipitation test, revealed a visible precipitation lines within 48 hours using reference LSV antiserum.

Transmission Electron Microscope (TEM).

LSDV isolate in MDBK cell culture was identified by negative staining transmission electron microscope revealing presence of the viral particles which appeared as roughly brick shaped particles with ridges covering them (x 78000) **(Fig.5).**

Preparation of LSDV partial purified soluble viral antigen (PPSVA).

Partial purified soluble LSD viral antigen was prepared by inoculation and propagation in MDBK cell culture.

Titration of the prepared LSDV antigen.

The results of titration of the prepared LSDV antigen revealed that the best dilution of the antigen is 1:100 against serum dilution of 1:40 by the check board titration **(Fig.6).**

Screening of LSDV antibodies in the collected serum samples by:

Indirect ELISA:

LSDV antibodies were detected in 58 serum samples out of 100 samples by using indirect ELISA **(Table 3).**

virus neutralization test (VMT).

Results of Screening of LSDV antibodies in the collected serum samples by using VNT revealed presence of LSDV antibodies in 45sam-

Table (2). Isolation on CAM and MDBK cell line.

governorate	Type of samples	Isolation of LSDV					
		Inoculation of samples on CAM of ECE			Inoculation of +ve CAM on MDBK cell culture		
		No. of samples	No. of +ve	No. of -ve	No. of +ve CAM	No. of +ve	No. of -ve
El-Gharbia	Skin nodules	6	4	2	4	2	2

Table (3). Screening of LSDV antibodies in the serum samples by I-ELISA & VNT.

governorate	No of samples	Test used			
		ELISA		VNT	
El-Gharbia	100	No of +ve	No of -ve	No of +ve	No of -ve
		58	42	45	55

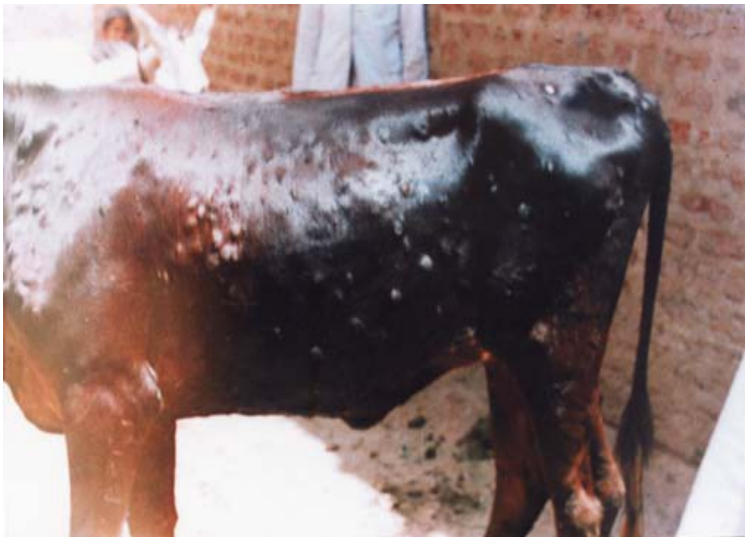


Fig (1) a. Clinical manifestations in the form of multiple nodules with variable size on the infected cattle with generalized swelling of lymph nodes and Lameness.

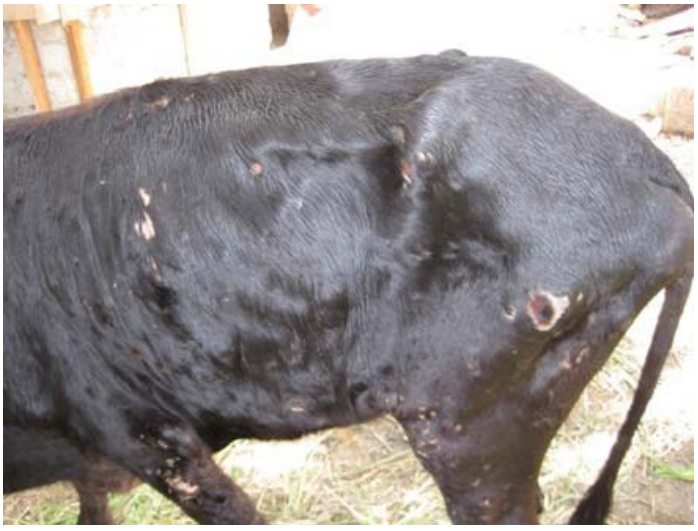


Fig (1) b. Progression of lumpy skin disease: note the necrotic nodules and deep scab formation.

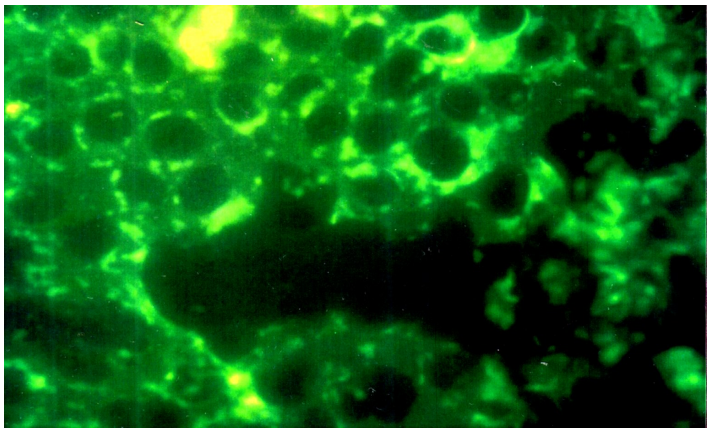


Fig. (2). LSDV antigen from LSD nodules showed green yellowish intracytoplasmic fluorescent granules(x 400) in MDBK cell line.

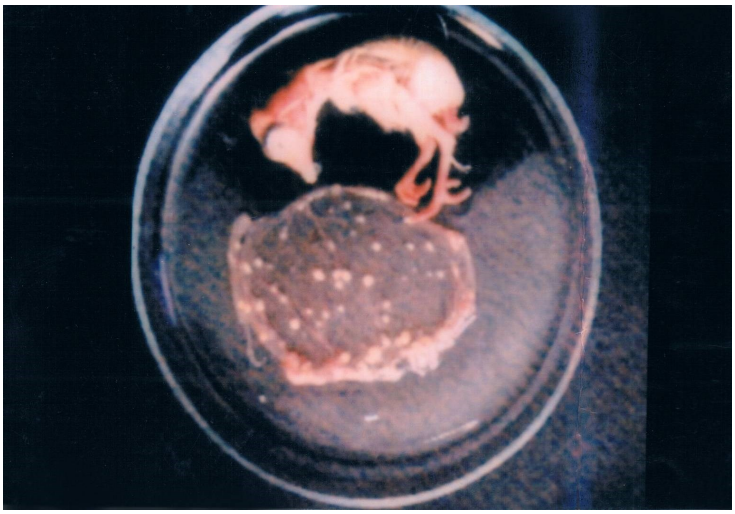
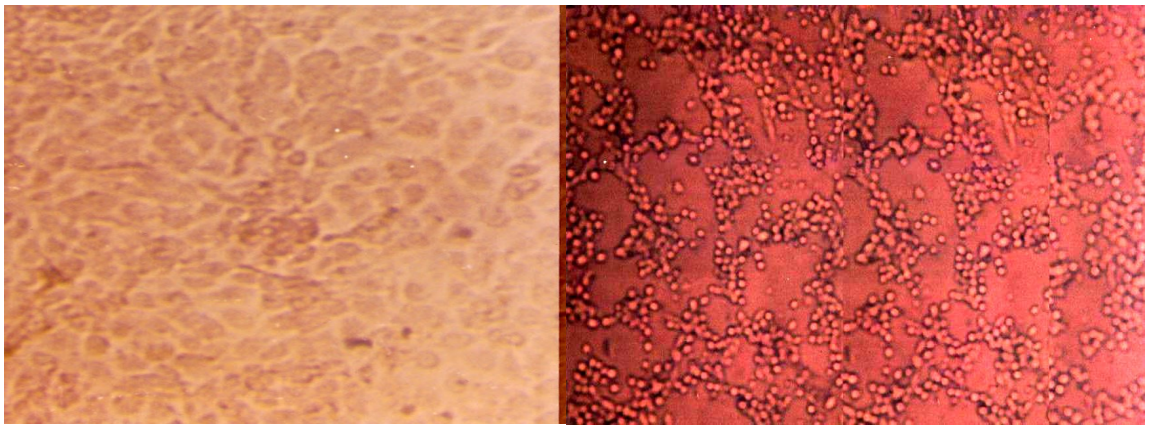


Fig. (3). Thickening and specific pock lesion of LSDVfrom inoculated nodule on CAM of ECE (9-11) days old.



(a) (B)

Fig. (4a &b). normal MDBK cell culture (a) / Characteristics CPE of LSDV isolates, 72 hours post inoculation on MDBK cell culture (b) (Mag.400x).

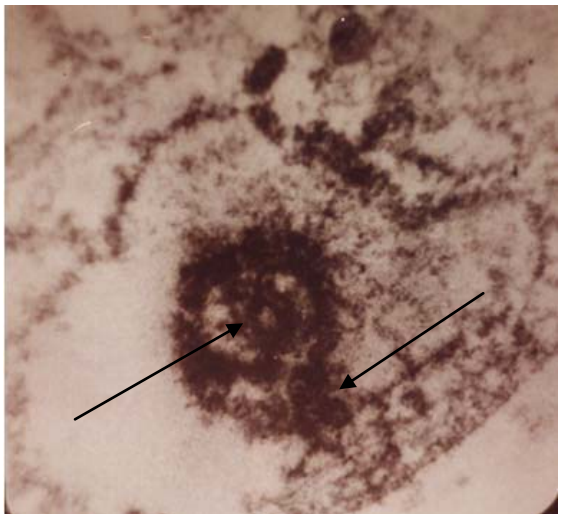


Fig. (5). Electron micrograph of LSDV showing presence of the viral particles which appeared as roughly brick shaped particles with ridges covering them (x 78000).

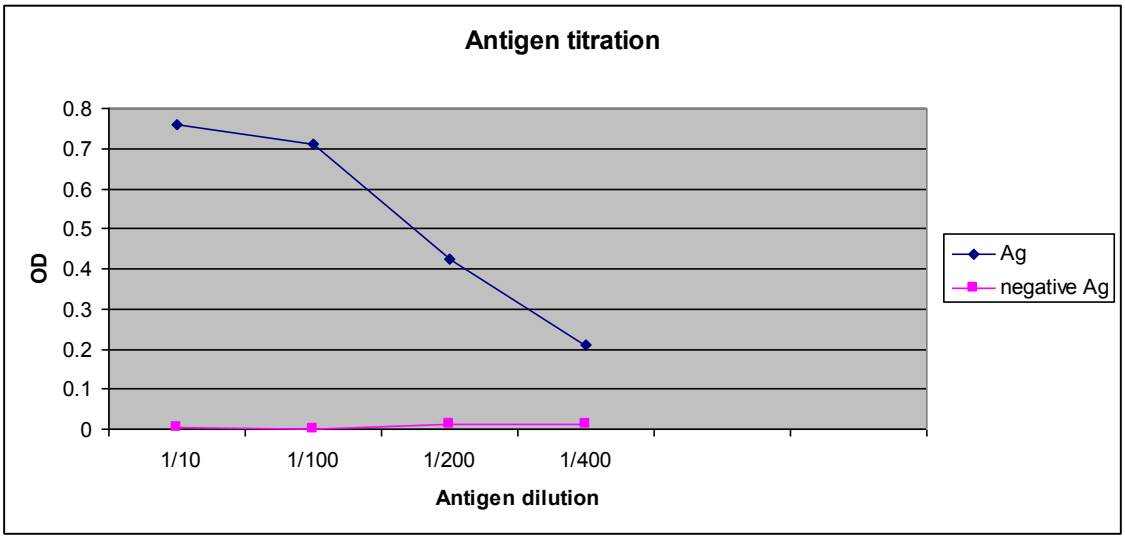


Fig. (6). Titration of the prepared LSDV antigen

Discussion.

Lumpy skin disease (LSD) is a serious skin disease of cattle. Accurate and rapid diagnosis of LSD is very important to control the spread of disease in Egypt.

In this study, a total of 6 skin nodules from infected cattle and 100 serum samples from diseased and apparently healthy cattle were collected from different localities in El-Gharbia Governorate during 2011. These nodules were used for isolation and identification of LSD virus, then preparation of LSD viral antigen while the serum to detect the neutralizing antibodies for LSD virus by indirect ELISA using the prepared LSD viral antigen then comparing the results with the VNT.

Diagnosis 0f LSD was mostly performed on the basis of clinical signs **(Reuma *et al.,* 2012).** The clinical observation of animals from different localities of El-Gharbia Governorate during 2011, revealed presence of fever as first clinical signs, few skin nodules in mild cases while whole body covered by nodules in severely affected skin. In advanced cases the nodules converted into scups and then leaving scars **(Fig. 1a& b).** Swelling of lymph nodes, congestion of oral, nasal and ocular mucous membranes were observed. These records agreed with the studies by (**Aly *et al.,* 2006**, **Ahmed and Kawther, 2008 and Amal *et al.,* 2010)** which stated that severely loss of weight and poor body condition with clear stiff gates or lameness are observed. Some animals revealed arthritis. The most pronounced signs were skin nodules which soon become erupted with clear ulcerations. These ulcers may be infected and become suppurated or showing myiasis. Sloughing of skin from a large area was seen in some cases .Also **Weiss (1968**) found that experimental infection of cattle with LSDV produce skin lesions on 40-50% of infected animals but the other produce localized lesion without any clinical signs except fever.

Definite diagnostic procedures in the laboratory are aimed for detection of the causative virus**.** IFAT in this study is used for Identification of virus on cover slips having a confluent sheet of MDBK cell line which inoculated with the prepared nodular tissues, revealing specific intracytoplasmic immunofluorescent staining reactions in all samples **(Fig. 2).** These results agreed with **(Hassanein *et al.,* 2008, Amal *et al.,* 2010 & El-Kenawy and El-Tholoth, 2011),** stating that the use of reference LSDV antiserum, a clear intracytoplasmic fluorescence was observed. Also, other studies stated that cultures are infected with suspensions of the early skin lesions, preferably in tubes or wells with cover slips which can be stained at 48 to 56 hours to show the intracytoplasmic inclusions **(Davies *et al.,* 1971 & Aly *et al.,* 2006).**

In current study, LSDV was successfully isolated through inoculation of prepared skin nodule onto the CAM of emberyonated chicken eggs (9-11) days old for two successive passages. Thickening and typical pock lesion was found on harvested CAM at the 4th day post inoculation in 4 samples out of 6 as shown in **(Table 2) (Fig. 3).** This result agreed with **(Omyma, 2008 & El-Nahas *et al.,* 2011)** who isolate LSDV by inoculation in CAM of ECE giving a characteristic pock lesions after 1st passage and become clear after 3rd passage with thickening and congestion of the membrane.

With regards to the results of isolation of the LSDV from the harvested CAM on monolayer MDBK Cell line for 3 blind passages, a characteristic CPE was observed as cell rounded and the cells are aggregated together in a separate manner around holes and giant cells with several nuclei can be found. This occurred 2-5 days post inoculation and gradually increased till 70 – 80 % of the sheet was completely detached in some samples after 5-7 days **(Fig. 4a &b) (Table 2).** This result agreed with (**House *et al.,* 1990**, **Hassanein *et al.,* 2008 & OIE, 2010**). Other studies stated that isolation of LSDV in culture need applying blind passages till clear appearance of C.P.E **(Prydie and Coackeley, 1959 & Aly *et al,* 2006).**

An agar gel immunodiffusion (AGID) test has been used for detecting the precipitating anti-

gen of capripoxvirus, but has the disadvantage that this antigen is shared by parapoxvirus **(OIE, 2010)**. In our study MDBK cell culture isolates of LSDV were successfully identified with the AGPT using the reference LSV antiserum, giving a clear line of precipitation within 48 hours. This result agreed with **(Tuppurainen 2004, Sohair *et al.,* 2008 & El-Nahas, 2011)**

Electron microscopy is used as rapid accurate diagnostic methods. It allows rapid morphological identification and differential diagnosis with other different agents **(Walid, *et al.,* 2010).** A study of **Madbouly *et al.,* (2005) & Aly *et al.,* (2006),** stated that Electron microscopy for the harvested positive culture suspension of LSDV was allowed showing roughly brick-shaped virus particIes. In our study, the identification of MDBK cell culture LSDV isolate on transmission electron microscope (TEM) by negative staining revealing presence of the viral particles which appeared as roughly brick shaped particles with ridges covering them **(Fig.5)**. These result was similar to those previously reported by **(Hazelton and Gelderblom, 2003, Iman *et al.,* 2007, Omyma, 2008 and El-Nahas, 2011).**

For serological diagnosis, the virus neutralization test is considered to be the most reliable serological test but it is expensive, laborious and time consuming so an indirect antibody ELISA has been developed as a rapid, sensitive and specific method. Inactivated partial purified soluble viral antigen (PPSVA) as coating antigen, for detection of antibodies against LSDV have been used but the viral antigen is difficult and expensive to produce in large quantities (**Babiuk *et al.,* 2008, Amal *et al.,* 2010** & **Hong *et al.,* 2010)**.

In our study LSDV antibodies in the collected serum samples were detected in 58 out of 100 samples by using indirect ELISA using prepared soluble partially purified antigen **(Table 3),** while on testing the serum samples by the VNT, 45samples were positive. These results agreed with the studies stated that antibodies against LSDV in serum samples were detected by using I-ELISA in clinically infected and fevered cows and could not be detected in those samples collected from incontacts **(Kitching and Hammond 1992).** Antibody levels are also related to sampling time relative to the onset of infection **(Davies 1991).** Other studies concluded that a very high incidence of LSDV antibodies in serum of tested animals by ELISA when compared with virus neutralization test (VNT), so the ELISA technique could be used for serosurvey as it is accurate, sensitive and specific for detection of LSDV antibodies **(Carn and kitching, 1995 and Walid *et al.,* 2010)**.

Conclusion.

Preparation of LSD viral antigen is important for development of indirect ELISA as a sensitive diagnostic method for LSD diagnosis in infected cattle .

The present study proposes such assay to be supplemented test for surveillance of LSDV among cattle population in Egypt for evaluation of the immune status of vaccinated animals.

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