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RESEARCH

ISOLATION AND IDENTIFICATION OF LUMPY SKIN DISEASE VIRUS IN CATTLE IN KALUBEYA GOVERNORATE

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ABSTRACT

Background: Lumpy skin disease virus (LSDV) is an infectious viral disease which is an important serious skin disease of cattle. LSDV was isolated from skin biopsies collected from cattle which are clinically infected at Kaluobia governorate.
Objectives: This study was designed for isolation and identification of LSD virus on CAM and MDBK cell culture and identification of isolated virus by FAT and PCR.
Methods: A total 23 skin biopsies were used for virus isolation on chorio-allantoic membrane (CAM) of embryonated chicken eggs SPF at 9-11 day which showed pock lesions and CPE on MDBK cell culture. Isolated virus was identified by indirect fluorescent antibody technique (IFAT) using specific hyper immune serum against Lumpy Skin Disease Virus. Further identifications were carried out by polymerase chain reaction (PCR).
Results: 15 samples were +ve for LSDV by conventional PCR.The results showed that 11/23 biopsies were positive by IFAT. Molecular identification of LSD virus by using RT-PCR, revealed positive for amplification of bands at the predicted molecular size (1926bp). Neutralizing antibodies against LSDV were (55) out of total 100 serum samples by serum neutralization test.
Conclusion: Selection and processing of clinical specimens, viral isolation and PCR assay applied, for LSDV are much sensitive and rapid diagnostic tool of LSD reflecting their importance in controlling the rapid spread of disease in Egypt.

Key words: CAM; IFAT; LSDV; MDBK; PCR.

BACKGROUND

Lumpy skin disease along with goat pox and sheep pox are the most highly risk poxvirus diseases that affects all ages and breeds of cattle and are caused by viruses that belong to the genus Capripoxvirus within the subfamily Chordopoxvirinae, family Poxviridae **(Carter and wise2006 and Tulman et al.;2001).** LSDV is usually associated with high morbidity and low mortality causes economic losses because of decreased weight gain, permanent damage to hides, decreased milk production and infertility**(OIE 2010)** The Lumpy Skin Disease is range from acute and severe to subclinical and is characterized by pyrexia, lymphadenopathy, skin nodules and subsequent sitfasts. Pox lesions can affect internal organs such as the stomach **(Wallace & Viljoen;. 2005).** Substantial outbreaks of lumpy skin disease (LSD) have recently occurred in Egypt and Israel **(Bowdent et al. ;2009 and goffc et al., 2009).** *Aedes aegypti* female mosquitoes are capable of the mechanical transmission of lumpy skin disease virus (LSDV) from infected to susceptible cattle **(Kitching & Mellor, 1986.; Chihota et al., 2001)**. Mosquitoes fed upon lesions of cattle infected with LSDV were able to transmit virus to susceptible cattle over a period of 2–6 days post-infective feeding. (**Wallace et al. ; 2006).** The outbreaks depend on animal movements, immune status, wind and rainfall patterns affecting vector populations **(Brenner et al., 2006)**. The incubation period is ranged from six to nine days until the onset of

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fever. There is initial pyrexia, which may exceed 41°C and persist for 1 week in the acutely infected animal **(Coetzer, 2004)**. The limbs may be edematous and the animal is reluctant to move. Pregnant cattle may abort and bulls may become permanently or temporarily infertile and the virus excreted in the semen for long periods **(Tulmaner et al.;2001, Irons et al., 2005).** Conventional PCR is a fast, simple and sensitive method for the detection of viral genome **(Bowden et al., 2008; Le Goff et al., 2009; Balinsky et al., 2009).** Cattle developing anti-capripoxvirus antibodies starting at 21 day post infection was detected by serum neutralization (SNT). **The objective** of this study was to diagnose lumpy skin disease virus by isolation of lumpy skin disease virus on fertile egg and MDBK cell culture, identification of isolated virus by PCR and IFA and detection of antibodies against LSDV in cattle in Kalubeya Governorate.

MATERIALS AND METHODS

Samples

A- Skin biopsies:

23 skin biopsies from cutaneous nodules were obtained from infected and apparently healthy non vaccinated cattle at different stages during the course of the disease for virus isolation and identification by IFAT and PCR **(Burleson et al.,1997)** .

B-Serum samples:

100 serum samples were used for determination of antibodies against LSDV in cattle at Kaliobeia Governorate by serum neutralization test.

Viral strain and antisera:

Standard reference LSDV (Neethling strain) and standard reference LSDV antiserum was supplied from department of virology, Animal Health Research Institute, Dokki, Giza for application of IFAT, PCR and SNT.

Cultures for Virus Isolation:

1- embryonated chicken eggs 9-10 (SPF) Eight-day-old (ECE) were inoculated with the prepared samples via the chorioallantoic membrane (CAM) route.

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

Virus Isolation:

LSDV isolation was carried out on CAM of ECE **(House et al**.; **1990)** and also on MDBK cells **(Irons 2009)** for three blind passages.

Serological Identification of LSDV Isolates:

IFAT were performed for identification of LSDV isolate. The techniques were performed according to the method described by **(OIE, 2010).**

Molecular Identification of Virus Isolates by polymerase chain reaction:

Viral DNA extraction

Viral DNA was extracted from skin biopsy **(Markoulatos et al.; 2000)** and from infected CAM **(Sambrook et al.; 1989)** and stored at -20°C till used for PCR.

PCR Assay:

The listed primer sets for Capripoxvirus and contagious ecthyma (Orf) virus manufactured by TIB – MOL Biol syntheselabor Gmb H Berlin, Germany were used in application of PCR assays.(**Irelnd and Binepal, 1989)**. The PCR procedures were performed according Ireland and Binepal **(Sambrooket al.;2000)**. The primers were developed from the gene for viral attachment protein was used. PCR was applied in volume of 50 µl containing 1 X PCR buffer (20 mM Tris HCl pH 8.4 and 50 mM KCl); 1.5 mM MgCl2; 0.2 mM deoxynucleosides triphosphates mixture (dATP, dCTP, dGTP and dTTP); 20 pmol of each primer; 2.5 units (U) Thermus aquaticus Taq polymerase 0.1µg of extracted viral DNA and nuclease-free sterile double distilled water up to 50.0 µl. After that, the mixture was subjected to 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.

The Analysis of PCR of Amplicons:

The PCR amplicons (10-15 µl) were analyzed by 2% agarose gel Electrophoresis. The specific primers set amplified a DNA fragment of 192 bp equal to the expected amplification product size from LSDV. The local isolates from skin nodules, infected CAM and MDBK cells and the Lumpy Skin Disease Virus reference strain had the same size of attachment protein gene fragment 192 bp, without significant differences between the strains. Therefore it was certain that, these specimens contained DNA of LSDV.

Primer set	nucleotide sequence	Tm	Amplicon size (bp)
Capripox (Ks.1.5/Ks.1.6)	F 5`- TTTCCTGATTTTTC-TTACTAR 3` P_2 5` - AAATTATATACG-TAAATAAC 3`	60 48	192

RESULTS

Isolation of Lumpy Skin Disease Virus revealed the characteristic pock lesion on Choroallantoic membrane of ECE **(Fig. 1)** and prominent Cytopathic effect on MDBK cells started from third day post inoculation until complete destruction of cell sheet **(Fig. 3).** Serological identification of isolated samples indicated that 2 isolates. From (23) skin nodules were also positive results for IFAT with specific antisera for LSD. The specific intracytoplasmic yellowish green fluorescent granules characteristic for Lumpy Skin Disease Virus using IFAT were demonstrated by **Fig. (5).**

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. 6)**. Therefore, it was certain that these specimens contained DNA of LSDV. Antibodies against LSDV were detected by serum neutralization test. **Table (1)** revealed that neutralizing antibodies against LSDV were 55% from total serum samples.

Fig. (1): lesion of LSDV on CAM varied from thickening of membraneIn 1st passage to numerous white foci more pronounced by 2nd and 3rdpassag

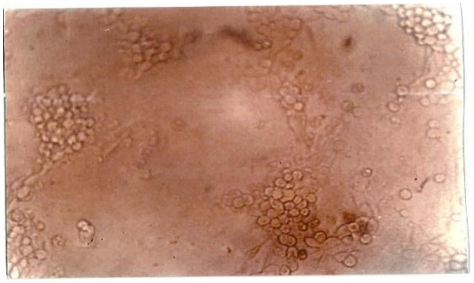

Fig. (2): characteristic CPE of LSDV by cell round in, cell aggregation, coalesce together to form clusters within 72 hr. post inoculation Meg 400x

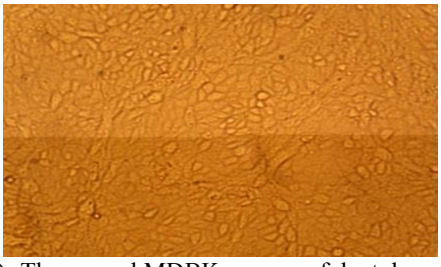

Fig. (3): The normal MDBK monoconfulant layer Meg 400x

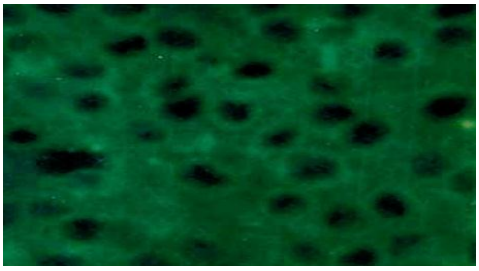

Fig. (4): Negative IFAT

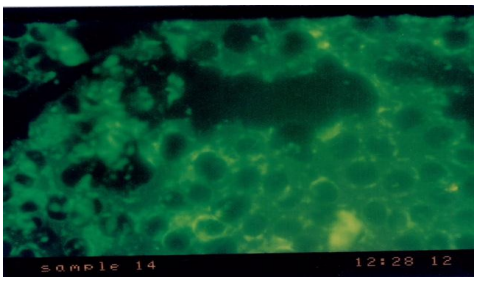

Fig. (5): Specific yellowish green fluorescent granules emitted from the normal inoculated cell culture indicating presence of LSDV

Fig. (6): Detection of DNA of LSDV by PCR (agarose gel electrophoresis of the polymerase chain reaction products) Lanes: (M) 100 bp DNA ladder (Fermentas); (1) Reference of LSDV "Neethling" strian; (2) Old Egyptian LSDV strain; (3, 4, 5, 6, 7) +ve samples; (8) -ve control (no primers); (9) Negative control DNA of MDBK cells; (10) -ve control (only primers without DNA template).

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					
			4		8		16	
100	55	55%	No.	%	No.	%	No,	%
			8	14.5	22	40	25	45.5

DISCUSSION

LSD is an infectious disease of cattle characterized by rapid spread and sudden appearance of lumps in skin after fever. The control of the disease to decrease the economic loss is depending on rapid and accurate diagnosis **(Cran, 1993).** The importance of this disease increase gradually as the way of eradication and control is very difficult.The present study concerned with isolation of LSDV from skin nodule of infected cattle on fertile chicken eggs and MDBK tissue culture with further identification by IFAT in addition to, molecular characterization of virus isolate using PCR. LSDV was isolated from samples collected from naturally infected cattle by inoculation on MDBK and CAM. LSDV can be propagated on the chorioallantoic membrane (CAM) of embryonated chicken eggs. After the first passage, the characteristic pock lesions were observed and become clear after the third passage (**photo** 1). Maximum yield of LSDV was obtained in the CAM of 7- to 9-day-old embryos. Prominent CPE on MDBK cells started from third day post inoculation until complete destruction of cell sheet

(photo 3).CPE 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 increased till 70-80 % of sheet was completely detached .Our results agreed with **(House *et al.;* 1990 and El-Nahas et al.; 2011)** who isolated LSDV from skin biopsies on CAM and MDBK cell culture. 2/23 of Isolated LSDV was identified by IFAT which showed Characteristic specific intracytoplasmic yellowish green fluorescent granules (Photo 4).Our results came in agreement with those of **(Davies 1991 and Ibrahim et al.; 1999**). Serum samples were tested neutralizing antibodies by serum neutralization test. 55% of tested serum samples gave protective antibody titer. SNT as serological test is useful for confirming retrospectively LSD but are too time consuming to be used as primary diagnostic methods (**Davies, 1991).** 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)**. Therefore, PCR was the test of choice for rapid detection and identification of the LSD outbreak causative agent.Isolated skin nodules samples were tested by conventional PCR for the presence of LSDV.5 samples were positive for LSD 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. This result correlate with **Sharawi and Abd El- Rahim, (2011).** This may be attributed to the viral tropism to skin tissues and its persistence in high concentration in Kalubeya governorate.

CONCLUSION

It could be concluded that:

Lumpy Skin Disease is an important enzootic disease in Egypt. Lumpy Skin Disease virus (LSDV) is circulating among cattle in Egypt. 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.

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