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# Original Paper

# Isolation and identification of Lumpy Skin Disease Virus from cattle in Menofeia Governorate during May 2019 to January 2020

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# ABSTRACT

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Lumpy skin disease virus (LSDV) is a significant and serious viral skin disease affecting cattle. In this study, isolation, and identification of LSDV from ten skin nodule samples collected from infected cattle at different localities of Menofeia Governorate, Egypt between May 2019 and January 2020. Seven out of ten nodular samples gave characteristic pock lesions by isolation of LSDV on chorioallantois membrane (CAM) of specific pathogen free embryonated chicken eggs (SPF-ECEs). On the other hand, four of ten nodular samples gave prominent CPE at the third passage on Madin Darby Bovine Kidney (MDBK) cell culture. Seven isolated samples tested positive by indirect fluorescent antibody test (IFAT). Using specific primers for the LSDV envelope protein (P32) gene, polymerase chain reaction (PCR) detected LSDV nucleic acid in seven of ten isolated samples and confirmed LSDV isolate with a specific amplified product of 192 bp. Accurate and fast diagnosis of LSD is critical for controlling the rapid spread of the disease. PCR is an effective, quick, and mDBK cells. Constant immunization against LSDV is required to protect animals and control the disease in Egypt.

### **1. INTRODUCTION**

Lumpy skin disease virus (LSDV) is a DNA epitheliotropic virus belonging to genus Capripox virus, one of eight genera in the subfamily chordopoxvirus of the Poxviridae family. Lumpy skin disease virus (LSDV), goat pox virus (GTPV) and sheep pox virus (SPPV), which causes disease in cattle, goats, and sheep, respectively, are all members of the genus Capripoxvirus. These viruses cause some of the most commercially significant domestic ruminants diseases in Africa and Asia (Mangana-Vougiouka et al., 2000; Acharya and Subedi., 2020).

The LSDV genome is about 151kbp in length, has 156 putative genes, and consists of a central coding region that is bounded by identical 2.4 k bp inverted terminal repeats (Tulman et al., 2001; Le Goff et al., 2009; OIE, 2020).

LSDV is typically associated with low mortality and high morbidity, which results in economic losses because of reduced milk production, poor growth, weight loss, permanent damage to hides, abortion, temporary or permanent infertility in cows and bulls, and sometimes death due to secondary bacterial infections (OIE, 2010; *Al-Salihi and Hassan*, 2015; *Sprygin et al.*, 2018).

Lumpy skin disease is the most highly risky disease affecting all ages and breeds of cattle, ranging from acute and severe to subclinical forms (Kara et al., 2003; Haftu, 2012). It is characterized by skin nodules, pyrexia, pox lesions which can sometimes impact internal organs, and lymphadenopathy (Younis and Aboul Soud, 2005). Significant LSD outbreaks have occurred in Egypt (El-Kenawy and El-Tholoth, 2011; OIE, 2017).

Lumpy skin disease virus is transmitted mechanically by Aedes aegypti female mosquitoes from infected cattle to susceptible cattle. The incubation period lasts between six to nine days (*Sprygin et al.*, 2018; *Sanz-Bernardo et al.*, 2021).

Clinical, subclinical, and mild forms of LSD require quick and reliable laboratory testing to confirm the diagnosis (OIE, 2010). Laboratory diagnosis comprises either isolation of the virus on fertile hen's egg and cell cultures, followed by identification using indirect fluorescent antibody test (IFAT) or using serological tests to detect its specific antibody (Tamam, 2006). Polymerase chain reaction (PCR) techniques has been developed, for the more precise and quick identification of LSDV in appropriate samples (Ahmed and Kawther, 2008; Stram et al., 2008; Manic et al., 2019). The main purpose of this work was isolation and identification of LSDV from clinically suspected skin nodules in cattle using serological and molecular techniques.

## 2. MATERIAL AND METHODS

#### Ethical approval

All of the experimental procedures were performed in the Virology Research Laboratory, Department of Virology, Faculty of Veterinary Medicine, Benha University, Egypt and were approved by Animal Ethical committees of the

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Benha University with ethical approval number (BUFVTM 23-10-22).

### 2.1. Tissue Specimens

Between May 2019 and January 2020, ten skin nodular samples were aseptically collected from infected cattle from different localities in Menofeia Governorate (Shebein El-Kom, Queesna and Alshuhada) with typical clinical signs of LSD. All samples were prepared for virological examination (Carn and Kitching, 1995) and stored at -80°C till used.

#### 2.2. Cultures for isolation of the virus:

The prepared samples were inoculated in SPF eleven -dayold embryonated chicken eggs (ECE) via the chorioallantois membrane (CAM) route (House et al., 1990) also, Madin Darby bovine kidney (MDBK) cell lines were used for virus isolation and propagated in Eagle's minimum essential medium (EMEM) with 10% fetal bovine serum (FBS) as a supplement (OIE, 2017).

2..2.1. Reference virus: The Neethling strain of LSDV was kindly provided by the Department of Virology, Animal Health Research Institute, Dokki, Giza.

2.2.2. Antiserum: Reference antiserum to LSDV was gotten from the Department of Virology, Animal Health Research Institute, Dokki, Giza and it was utilized for identification of virus by IFAT.

2.2.3. Conjugate: Anti-bovine IgG was conjugated with Fluorescent Isothiocyanate which was created in rabbits and obtained by Sigma (Organon teknika Corp. Westchester.PA. Cat #212-0081). It was utilized in IFAT.

2.2.4. Primers: The PCR primers (Table 1) used for amplification of partial LSDV envelope protein (P32) gene are specific for the LSDV) Cat. no.51304).

#### Table 1 Oligonucleotide primers sequences

Gene	Sequence	Amplified product	Reference
LSDV envelope protein (P32)	F: 5'-TTTCCTGATTTTTCTTACTAT-3') R: 5'-AAATTATATACGTAAATAAC-3')	192 bp	<i>Ireland</i> and <i>Binepal</i> (1998)

#### 2.3. Isolation of the virus:

Trials for isolation of LSDV were performed for three blind passages on the chorioallantois membrane of ECE (House et al., 1990) as well as in MDBK cells (OIE, 2017).

2.4. LSDV Isolates identification by Serological test: It was done by using IFAT (Mishra and Mallick, 1997).

#### 2.5. Molecular Identification of Virus Isolates:

Viral DNA Extraction: From infected CAM and MDBK cell isolates, viral DNA had been extracted and kept at -80 °C until used in PCR. (Sambrook et al., 2000).

### 2.6. Polymerase Chain Reaction (PCR):

It was carried out according to Ireland and Binepal procedures (1998). The primers of PCR were created from the LSDV envelope protein gene (P32). PCR reaction was done in a total volume of 50  $\mu$ l containing the following ingredients: 25  $\mu$ l 1 X PCR buffer (50 mM KCl and 20 mM Tris HCl pH 8.4); mixture of 0.2 mM deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP); 1.5 mM MgCl2; 1  $\mu$ l (20 pmol) of each primer; 2.5 units (U) of Thermus aquaticus Taq polymerase, 5  $\mu$ l of the viral DNA extracted and nuclease-free sterile double distilled water up to 50  $\mu$ l. In a programmable thermocycler, the following thermal profile was applied to the resulting mixture: One

cycle for 2 minutes at 94 ° C; 40 cycles for 50 seconds at 94°C, 50 seconds at 50° C and 1-minute at 72 °C; and one final cycle for 10 minutes at 72° C.

## 2.7. PCR amplification products analysis:

The resulting PCR product (amplicons) (10-15 µl) was analyzed in 1.5% agarose gel Electrophoresis (Sambrook et al., 2000).

### 3. RESULTS

### 3.1. Lumpy skin disease virus isolation

It revealed that seven samples from ten suspected nodular samples gave distinctive pock lesions on CAM of ECE (Fig. 1) and four of the ten nodular samples gave prominent CPE on MDBK cell line at the third passage starting on the third day after the inoculation and continuing until the cell sheet is completely detached after 5-7 days (Fig. 2). Serological identification of seven isolated samples (4 CAM samples and 3 MDBK samples) using IFAT revealed that seven isolates (4 CAM and 3 MDBK cell line) gave positive results, as shown in Table 2. IFAT positive results expressed by the appearance LSDV's distinctive yellowish green, fluorescent intracytoplasmic granules are shown in Figs (3&4).



Fig. 1 A- Characteristic pock lesions as numerous, scattered white foci on CAM of inoculated SPF-ECE after the third passage. B- Control normal non- infected CAM of SPF ECE.



Fig. 2 A: Inoculated MDBK cell line showing CPE at 3rd passage include cell rounding, cell aggregations and vacuoles formation followed by separation of cells from the sheet during isolation of suspected LSDV samples. B: Control a normal, non- infected, complete sheet of MDBK cells. Magnification power of microscope (10X)



Fig. 3 Stained infected cells of CAM suspension with FITC. Notice the green fluorescence emission as a positive result for local LSDV isolate. Table 2: Serological identification of LSDV isolates.

Test	Number of tested nodular samples	Number of positive samples	Number of isolates tested by IF	Number of positive isolates by IF
Isolation	10	*7	4/7	4
on CAM Isolation on DBK	10	**4	3/4	3

Positive results show presence of pock lesions on CAM of inoculated SPF-ECE. \*\* Positive results show presence of CPE on MDBK cells



Fig. 4 Specific intra-cytoplasmic yellowish-green, fluorescent granules in infected MDBK cells indicate presence of LSDV (400x magnification image taken with an Olympus fluorescent microscope camera).

#### 3.2. Molecular Identification:

The extracted genomic DNA products of three nodular samples (negative for isolation), three local isolates positive on CAM and (4) local isolates (positive for isolation on both CAM and MDBK cells) were subjected to conventional PCR for the amplification of the LSDV envelope protein (P32) gene using specific forward and reverse primers. The PCR amplicons were run using gel electrophoresis then analyzed and photographed using gel documenting system, which demonstrated the existence of a particular band at the correct predicted size of the LSDV envelope protein (P32) gene (192 bp). Only seven isolates were positive, and three samples were negative (Fig. 5).



Fig. 5 Ethidium bromide stained 1.5 % agarose gel electrophoresis of amplified PCR products of 3 skin nodules samples and 7 local isolates based on LSDV envelope protein (P32) gene. Lane M: High molecular weight nucleic acid marker (100bp); Lane P: Positive control (Reference virus). Lane N: negative control. Lanes1, 2 and 4: positive isolates from 3rd passage on CAM of SPF-ECE. Lanes 5, 7, 8 and 10: positive isolates from 3rd passage on MDBK cells. Lanes 3, 6, 9: negative nodular samples

### 4. DISCUSSION

Lumpy skin disease is endemic in the Middle East and Africa, including Egypt (Tuppurainen and Galon, 2016; OIE, 2017). The first natural outbreak and isolation of LSDV in Egypt was in1988 in Ismailia (House et al., 1990). Several outbreaks of LSD were recorded in different localities in Egypt (Beni-suef, Beheira, Ismailia and New Valley) in 2005 and 2006 (Younis and Aboul Soud, 2005; OIE, 2006; El-Kholy et al., 2008), followed by outbreaks in 2008 (Ahmed and Kawther, 2008), 2011 (El-Nahas et al., 2011; Sharawi and Abd El-Rahim, 2011), 2014 (Amin et al., 2015) and 2015 (Elhaig et al., 2017).

According to previous reports (Wainwright et al., 2013; OIE, 2020), the most recent LSD outbreaks were reported during warm, wet weather (from May to September) of the year while the incidence of LSD dropped throughout the cold season. In the present study, between May 2019 and January 2020, ten skin nodular samples were collected from clinically suspected cattle showed typical signs of LSD where skin nodules were scattered throughout the

body accompanied by edema in the limbs and enlargement of superficial lymph nodes. In this study LSD virus was isolated from samples via inoculation on the CAM of SPF-ECE. The obtained results showed the presence of pock lesions following the first passage, while they became clear and distinct after the third passage (Fig. 1). This result completely concurred with results of El Nahas et al. (2011), who detected pock lesions after the first passage, House et al. (1990) and Tamam (2006), who isolated LSDV on the ECE CAM successfully, and observed the distinctive pock lesions. In our study, MDBK cell culture demonstrated typical cytopathic effects (Fig. 2) within 72 hours of inoculation characterized by rounding cell, aggregation, and coalescence forming clusters which dispersed throughout the monolayer and grew gradually until 70-80% of the sheet. This was one of the hallmarks of CPE. These findings were consistent with those of Fahmy (2000), El Nahas et al. (2011) and Munyanduki et al. (2020). In this study, IFAT distinguished seven of the isolated LSDV (4 isolates from CAM and 3 from MDBK), revealing characteristic specific intracytoplasmic yellowish-green, fluorescent granules (Fig.3&4). This finding agreed with Davies (1991) and Ibrahim et al. (1999). Serological techniques are useful to identify LSD but consumed much time to be utilized as primary methods for diagnosis (Davies, 1991; Heine et al., 1999). As a result, PCR was chosen as the preferred technique for detecting and identifying the causal agent of the LSD outbreak (Kitching and Hammond, 1992, Manic et al., 2019). The PCR test was a precise technique to identify LSD virus in nodular samples, CAMs as well as tissue culture (El-Kholy et al. 2008, El-Kenawy and El-Tholoth, 2011). The molecular investigation in this study was used to confirm the presence of LSDV DNA using pair primers targeting the LSDV envelope protein gene and achieved success in amplifying the specific products (~192 bp) from the DNA products derived from nodular samples, infected CAM, and infected MDBK cells, as well as the LSDV Neethling reference strain, and the bands were clear and sharp.

#### **5. CONCLUSION**

The current study concluded that LSDV is spreading among cattle in the Menofeia Governorate, Egypt, between May 2019 and January 2020 as presented by isolation of the virus on CAM of SPF-ECE and MDBK cell line from skin nodules of infected animals with successful identification of the virus by IFAT in addition to PCR as a precise and rapid tool for diagnosis using primers specific for P32 gene demonstrating its significance in limiting the quick spread of the disease in Egypt. Constant immunization against LSDV is required to protect animals and control the disease in Egypt.

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