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ORIGINAL ARTICLE

Isolation of Lumpy Skin Disease Virus Isolated from SPPV Vaccinated Cattle

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Abstract
Massive outbreaks of suspected LSDV in cattle population were observed during 2017 and 2018 at Beni-Suef governorate. Samples from diseased cattle, previously SPPV vaccinated with SPPV, were inoculated in embryonated chicken eggs (ECE) using chorio-allantoic membrane (CAM) route. Histopathological examination of the inoculated CAMs showed large eosinophilic intracytoplasmic inclusion bodies characteristic for LSDV. The inoculated CAMs were hemorrhagic with congestion blood vessels appeared by the 1st passage then become more pronounced after the second - fourth passages. Characteristic pock lesions were observed after the 1st passage and become clear after the third passage and become clearly observed 4 days post inoculation. For molecular identification, DNA was extracted from a pool of the infected CAMs. Two pairs of primers specific for LSDV including one flanking a 554 bp product of the G gene and second flanking 172 of the RP030 gene were used. In conclusion, LSDV infections have been detected and the virus has been isolated and identified by PCR from cattle previously vaccinated with SPPV vaccine during the summer of 2018.

Keywords
Egypt; LSDV; PCR; Sheep pox vaccine

1. Introduction
Lumpy skin disease (LSD) is a devastating viral transboundary high-impact cattle disease causing significant loss in production, **Ochwo et al. (2018).** It is characterized by fever, skin and visceral nodules as well as generalized lymphadenopathy and skin edema of affected animals, **Coetzer (2004)** as well as pneumonia as a common sequel with lesions in the respiratory tract, **OIE (2010).**

The disease is caused by a protype lumpy skin disease virus (LSDV) Neethling virus, as a member of genus *Capripoxvirus* in family *Poxviridae*, and subfamily *Chordopoxvirinae* **Buller et al. (2005)**. LSDV has double stranded DNA genome, encodes 30 homologues structural or nonstructural, viral proteins. It showed close antigenic relation to sheep pox virus (SPPV) and goat pox virus (GTPV) with nucleotide sequence identities of 96% among that species **(Tulman et al. 2001; Tulman et al. 2002; Bhanuprakasha et al. 2006).**

The LSD inducing severe economic losses due to decreased milk production, weight loss, poor growth, abortion, infertility and skin damage pneumonia, mastitis, infertility as well as mortalities especially in free areas **(El-Bagoury et al. 1995; Irons et al. 2005; Salib and Osman 2011; Coetzer 2004; Babiuk et al.**

2008). In addition, the cost of treatment and control measures such as vaccination campaigns as well as indirect costs of the compulsory limitations in animal movement that causes significant financial losses on a national level **(Tuppurainen and Oura 2012)**. The viral replication in cells such as the pericytes and endothelial cells in lymphatics and blood vessels walls; gives rise to vasculitis and lymphangitis. In some severe cases thrombosis and infarction may be the end result that cause edema in the affected areas **(Vorster and Mapham 2008).**
Laboratory diagnosis of LSDV can be performed by using serological techniques, PCR and by virus isolation in ECE and cell cultures, beside transmission electron microscopy, **(OIE 2010)**.

Routine tests for LSDV diagnostics include molecular group methods for the detection of a CaPV**, (Bowden et al. 2008; Stubbs et al. 2012; Haegeman et al 2013; Tuppurainen 2005; Balinsky et al. 2008).**

Despite the implemented control measures, the disease is currently spreading widely in different governorates in Egypt in animals vaccinated with live attenuated sheep pox vaccine. Therefore, it was of importance to direct the aim of the present study to investigate the current status of LSDV in Beni-Suef Governorate, Egypt through isolation and molecular characterization of LSDV circulating in vaccinated cattle during 2017-2018.

2. Materials and Methods

2.1. Cattle samples

Twenty clinically suspected cattle showing typical signs for lumpy skin disease were used for collection of samples. Skin nodules biopsies were collected in sterile containers containing sterile phosphate buffer saline (pH 7.4) and also on 50% glycerol saline.

2.2. Virus isolation

An amount of 0.2 ml of the prepared tissue homogenate was inoculated on chorioallantoic membrane (CAM) of ECE (3 eggs for each sample). The inoculated ECEs were incubated for 6 days at 35°C and examined for presence of pock lesions. The CAMs from the first passage were kept at -20 ^{0}C for next passage. Three serial passages were carried out by the same manner as in the second passages **(Van Rooyen et al. 1969).**

2.3. Histopathological examination

Sections from CAMs of the third passage were routinely examined. The tissue biopsies (preserved in 50% glycerol) were fixed in 10% buffered neutral formalin, paraffin embedded then stained with prior to their examination using light microscopy **(Bancroft and Gamble 2002)**.

2.4. Polymerase Chain Reaction (PCR)

LSDV genome was extracted from the suspected material (tissue homogenate) and positive virus control using GF-1 tissue DNA extraction kit (Vivantis Technologies Malaysia) as described in the manufacture's instruction. Two virus positive controls were used including LSDV/Ismailia 88 strain and Sheep poxvirus vaccine were kindly supplied from a Pox Vaccine Production and Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo, Egypt. Amplification of target genes LSDV was carried out in a final volume of 25µl. Two PCR reactions were conducted for each samples using two sets of pair specific primers flanking CRG and RPO30 genes **(Table 1)** according to **Viljoen et al. (2005).** The amplified DNA products were detected using agarose gel electrophoresis and DNA molecular weight marker.

Table 1. Oligonucleotide sequences used for the PCR amplification of LSDV.

Name	Gene-specific sequence primers	Amplicon size
CRG*	LSD F 5' AGT ACA GTT AGT AGC GCA ACC-3' LSD R 5' GGG TGA ACT ACA GCT AGG TAT C- 3'	554 bp
RPO30	LSD F5'TCTATGTCTTGATATGTGGTGGTAG3' LSD R5'AGTGATTAGGtGGTGTATTATTTTCC-3'	172bp

***CRG:** Coupled chemokine receptor gene.

3. Results

3.1. Clinical symptoms

Recurrent outbreaks of suspected LSD in cattle population were observed during 2017-2018 at Beni-Suef Governorate. Suspected cattle demonstrated skin nodules and scabs scattered all over the body. All the examined animals were vaccinated with a local modified live sheep pox vaccine before the occurrence of the disease with variable periods of 4-6 months

3.2. Virus Isolation

It was found that the inoculated samples induced lesions on CAMs by the 1st-4th passages **(Table 2).** The collected CAMs showed hemorrhages, congested BVs by the 1st passage. The pock lesions were detected in the form of white opaque large pock lesions and sometimes hemorrhagic which became more prominent at the 2nd - 4th passages **(Fig. 1a).**

3.3. Histopathological examination

Histopathological examination of inoculated CAM with suspected LSD viral samples showed large eosinophilic intracytoplasmic inclusion bodies characteristic for Poxviridae **(Fig. 1).**

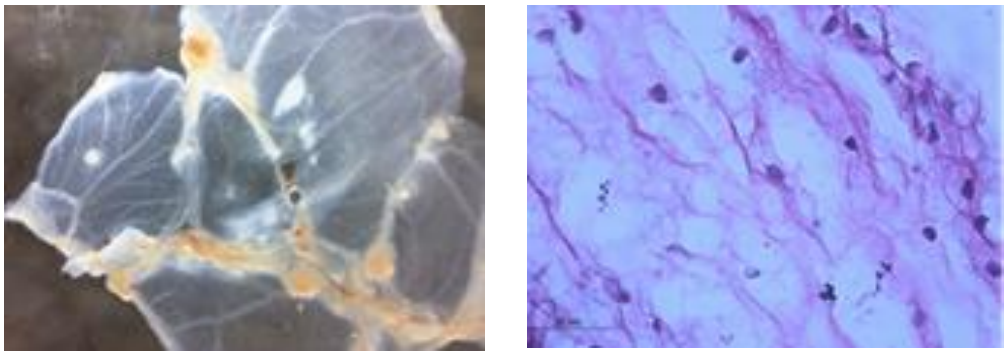
3.4. Polymerase Chain Reaction (PCR)

An extracted viral DNA from pooled CAMs of ECE was used for detection of LSDV using PCR. Primers specific for GPCR gene amplified a 554 bp product from LSDV genome confirming positivity of the sample for presence of LSD viral genome **(Fig. 2)** while primers set targeting RP030 gene amplified a 172 bp product from LSDV genome confirming positivity of the sample for presence of LSD viral genome. Tissue culture adapted SPPV vaccinal strain amplified a (152 bp) which was easily distinguishable relative to the LSDV amplicons **(Fig. 2).**

Table 2. Positivity and intensity of pock lesions on CAM in different embryo passages.

Sample number	Intensity of pock lesions in different embryo passages			
	1st	2nd	3rd	4th
1	-	+	+	++
2	-	+	+	++
3	+	+	++	++
4	+	+	++	++

- No growth + white opaque pock lesion, + + Large hemorrhagic pock lesion



(a) (b)

Fig. 1. Gross and histopathology of CAM inoculated with skin homogenate from diseased cattle with skin lesion **(a)** Characteristic pock lesions of infected CAM **(b)** Stained inoculated CAM showing eosinophilic intracytoplasmic inclusions (H and E 100X, oil emersion lens).

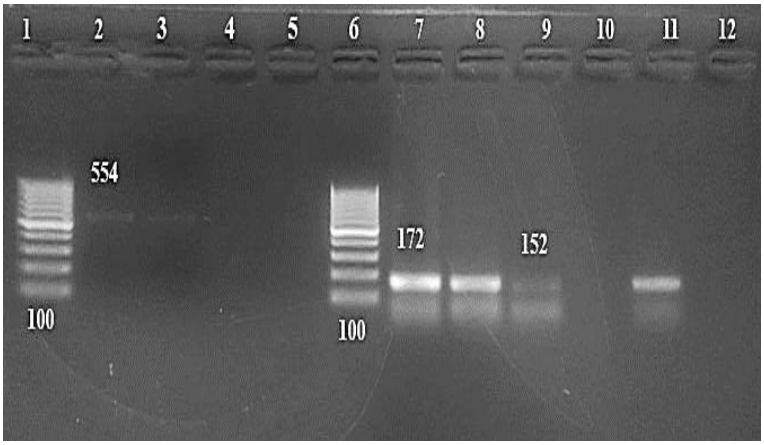


Fig. 2. Gel electrophoresis of PCR products using two LSDV specific primers, Lane 1 and 6: 100 bp DNA ladder, lane 2 and 3: LSDV suspected samples (554 bp), lane 4 and 5: sample pools showed negative results. Lane 7, 8 and 11: samples showed positive result (172 bp), lane 9: sheep pox vaccine (152), lane 10: control negative sample, Lane 11: control positive sample.

4. Discussion

Massive outbreaks of LSDV in cattle population were observed during 2017 and 2018 at Beni-Suef governorate. Animals were vaccinated before the occurrence of the disease with variable periods of 4-6 months. These outbreaks were observed in previously vaccinated cattle with the Romanian sheep poxvirus (SPPV) vaccine. The appearance of these outbreaks in vaccinated cattle become a questionable and raises the concern of the efficacy of the SPPV vaccine. A study conducted by (**Elkady 2016**) showed that animals suffered from severe signs differed from that reported in the previous LSD outbreaks, in terms of severity and deaths at different governorates including Beni-Suef governorate.

LSD suspected cattle showed multiple localized or generalized skin nodules. Skin lumps varied in size but were mostly 1-3 cm in diameter and were quite hard in consistency and affected the full thickness anywhere on the animal body. Similar lesions appeared in the mucosa of the mouth, nose, vagina and conjunctiva. A circular dark line of necrosis appeared around the surface of the lesions and produced the 'sit-fast' that is so typical of the disease. Central necrotic plug eventually sloughed and left a raw granulating ulcer or pock. Such findings were similar to the clinical manifestation of LSD previously described by **(Awad et al. 2009; Abdelwahab 2016; Elkady 2016).** Skin lesions were preceded by fever. Edema in the legs and brisket was observed in many cases. The observed edema may be explained on the basis of the basic pathogenic mechanism by which the LSDV seems to cause lesions is viral replication in cells such as the pericytes and endothelial cells in lymphatics and blood vessels walls; giving rise to vasculitis as reported by **Vorster and Mapham (2008).**

Abdallah et al. (2018a) reported sporadic cases of LSD in cattle previously vaccinated with Romanian SPPV vaccine in Sharkia Governorate. The present study confirmed the isolation of LSDV from skin lesions of cattle on the CAM of -ECE and the virus nucleic acid was identified in the conventional PCR using two different pairs of primers.

For clarifying the reasons of reappearance of LSDV among cattle previously vaccinated with the Romanian live attenuated sheep pox virus vaccine, skin nodules from clinically diseased cattle were collected as aseptically as possible and used for isolation of the virus. Skin lesions have been reported to be the most useful samples for virus isolation **(Bowden et al. 2008)** because the virus can be isolated for up to 35 days after the first appearance of the skin nodules **(Tuppurainen 2005; Wiess 1968).** In the current study, the lesions observed of CAMs after inoculation of the LSDV agree with **(Hamoda et al. 2002; Ahmed et al. 2005; Tamam 2006; El-Kenawy and El-Tholoth 2009)** who observed pock lesions on CAMs of inoculated ECEs and the pock lesions of the virus became clearer after serial passages. The development of lesions on CAM was firstly detected by **(Alexander et al. 1957; Van**

Rooyen et al. 1969) and varied from thickening and congestion **(El-Kenawy and El-Tholoth 2011)** to clearly visible pock lesion **(House et al. 1990)** that looked like numerous, small, scattered white foci **(El-Nahas et al. 2011).**

Histopathological examination of infected CAMs **showed** characteristic eosinophilic intracytoplasmic inclusion bodies characteristic for poxviridae appeared in ectodermal cell and mesodermal cell layers of CAM infected with suspected LSD viral sample and stained hematoxylin and eosin. These inclusions were suggestive for the presence of LSDV as a member of capripoxviruses replicating in the cytoplasm. Such results agree with those reported by **(Tamam 2006; Sohier et al. 2008; Abdelwahab 2016; Elkady 2016)** who observed formation of intracytoplasmic inclusion bodies which indicate extensive replication of LSDV.

For molecular identification, an extracted viral DNA from pooled CAMs of ECE was used for detection of LSDV with PCR. Two types of Oligonucleotide primer pairs were used for detection of the local LSDV strain in infected CAM. The first primer was specific for GPCR gene which is expected to amplify the specific product 554 bp. The second primer targeted the LSDV RP030 gene which is expected to amplify the specific product 172 bp from the extracted DNA products using PCR.

Primers specific for GPCR gene amplified a 554 bp product from LSDV genome confirming positivity of the samples for presence of LSD viral genome (**photo 3)** while primers set targeting RP030 gene amplified a 172 bp product from LSDV genome confirming positivity of the samples for presence of LSD viral genome. Tissue culture adapted SPPV vaccinal strain amplified a (152 bp) which was easily distinguishable relative to the LSDV amplicons, **photo (3).** The PCR proved to be a specific assay for specific detection of LSD virus in skin lesion **(Stram et al. 2008; El-Nahas et al. 2011),** CAMs and cell culture **(El-Kholy et al. 2008; El-Kenawy and El-Tholoth 2011),** semen **(Bagla et al. 2006)** and blood and skin samples **(Tuppurainen et al. 2005).** PCR assay in this study provides a very cooperative technique and could easily distinguish between these viruses going to a clear cut between them even at low concentration of the virus in field sample as reported by **Stram et al. (2008).**

In Egypt, the vaccination policy adopts Romanian sheep pox vaccine strain and a Kenyan sheep pox tissue culture vaccine strain produced in Vero cell line **((Davies 1991; Michael et al. 1994).** Meanwhile, the live modified lumpy skin disease virus vaccine (Ismailia strain) was produced and proved to be safe, potent and able to protect cattle against challenge with virulent LSDV **(Daoud et al. 1998; Abdelwahab et al. 2016).** On the other hand, inactivated LSDV vaccines were prepared and evaluated **(Saber et al. 2000; El-Desawy 2001).** LSDV vaccine adjuvanted with *Nigella sativa* oil was safe and elicited high antibody response against LSD either when used alone or in combination with sheep pox vaccine (**Madbouly et al. 2002)**. In contrast, the Romanian sheep poxvirus (SPPV) vaccine is still used in spite of the appearance of the disease in cattle vaccinated with this vaccine that showed low protection and leading to sever economic losses.

5. Conclusion

In conclusion, LSDV infections have been detected and the virus has been isolated and identified by PCR from cattle previously vaccinated with SPPV during the summer of 2018 in Beni-Suef. Evaluation of the vaccine efficacy under field conditions is necessary to select the most effective and protective vaccine.

6. Conflict of interest

The authors declare no conflict of interest.

7. References

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