Original article

IDENTIFICATION AND CHARACTERISATION OF LUMPY SKIN DISEASE VIRUS RECENTLY ISOLATED FROM GIZA, EGYPT (2018)

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Summary

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Lumpy skin disease (LSD) is a viral disease, geographically distributed in Africa and now, vigorously spread in the Near East and also in Europe and Asia. It has a significant economic impact on cattle industry in Africa. The aim of this study was isolation and rapid identification of LSD virus circulating in Egypt from clinically suspected cattle based on clinical and molecular basis in a rapid and accurate way. Fifteen representative specimens (skin sitfasts) were collected in 2018 from clinically infected cattle in Giza governorate, Egypt. The virus was isolated on chorioallantoic membrane of specific pathogen free embryonated chicken eggs and Madin Darby Bovine Kidney tissue culture cells. The isolated virus was identified and confirmed by conventional polymerase chain reaction (PCR) and real-time PCR. Histopathological examination of the lesions showed a pathognomic intracytoplasmic inclusion body in dermal stroma section. The section of dermal layer revealed vasculitis with projection of its endothelial lining. It was concluded that LSD was enzootic in Egypt and still circulating among Egyptian cattle so that LSD virus could be isolated and identified by traditional and molecular diagnostic methodes. Real time PCR assay could be applied for rapid and accurate confirmation of the field isolate of LSD virus.

Key words: Egypt, histopathology, LSD, PCR, RT-PCR, virus isolation

INTRODUCTION

Lumpy skin disease (LSD) is a viral disease mainly affecting skin in cattle, also named pseudo-urticaria, bovine nodular exanthema, neethling and knopvelsiek. LSD inflicts high morbidity leading to huge economic losses as a result of decreased milk yield, weak body weight gain, abortion, infertility, decline in hide quality and deaths of young cattle in Africa, Asia, the Middle East, Russia, and Europe (Tageldin *et al.,* 2014; Abutarbush *et al.,* 2016; Tasioudi *et al.,* 2016; Sprygin

et al., 2018a,b) and now in China (Lu *et al.,* 2020).

LSD is an OIE list A disease, which shows its serious socio-economic status. LSD is caused by neethling pox virus – an enveloped *Capripoxvirus,* a genus from the *Poxviridae* family, and one of the largest viruses. Its genome comprises double-stranded DNA and is approximately 150,000 base pairs (bp) long (Tulman *et al.,* 2001).

LSD diagnosis is based on the characteristic clinical signs, virus isolation, electron microscopy, histopathological examination, serological and molecular techniques (OIE, 2018).

The characteristic clinical signs have been described in detail by several authors (Weiss, 1968; Prozesky & Barnard, 1982; Babiuk *et al.,* 2008; Salib & Osman, 2011). The incubation period in nature is estimated to be 1–4 weeks (Coetzer, 2004). Briefly, in the majority of cases, the initial evidence of infection is lacrimation and fever (40–41°C), but some cases are non-febrile. Subscapular and precrural lymph nodes become noticeably enlarged. Shortly after the onset of fever, skin nodules (1–5 cm in diameter) become apparent, in varying numbers, from few to multiple, covering the animal skin, with systemic effects including pyrexia, anorexia and pneumonia (Davies, 1991). In severe cases, ulcerative lesions in the mucous membranes of eyes, oral and nasal cavities are present, causing excessive lacrimation, salivation and nasal discharges. LSD virus lesions may also be present in the pharynx, larynx, trachea, lungs and throughout the alimentary tract. In post mortem examination of severe cases, LSD virus lesions may be found on the surface of almost all organs (Babiuk *et al.,* 2008). Some infected animals develop oedematous swelling of one or more legs associated with lameness (Salib & Osman, 2011). The clinical signs severity varies from subclinical to fatal depending on the virus strain virulence and the susceptibility of cattle breed (Lu *et al.,* 2020).

In LSD outbreaks, the morbidity rates vary widely depending on the immune status of hosts, the abundance of mechanical arthropod vectors and usually range from 3% to 85% (Prozesky & Barnard, 1982; Hamoda *et al.*, 2002; Buller *et al.,* 2005).

Mechanical transmission of LSD virus was recorded between all breeds and ages of cattle by haematophagous arthropod vectors such as mosquitoes and stable flies (Agianniotaki *et al.,* 2017a; Lu *et al.,* 2020). LSD is transmitted by direct contact between LSD infected cattle and other cattle or by direct contact between LSD infected cow and suckling calf, while sheep, goats, and water buffaloes in contact with LSD infected cattle remain clinically healthy. All pox viruses isolated from sheep are sheep pox virus (Rashid *et al.,* 2017*)*.

Diagnosis of LSD plays a crucial role in the control measures to save costs and continuity of the disease. LSD prevention should be adopted via routine vaccination. LSD can be easily diagnosed upon the characteristic clinical signs. However, inapparent and mild forms of LSD require available and rapid laboratory assays to endorse the diagnosis. Laboratory detection of LSD virus could be carried out by virus identification using several PCR tests, developed for more accurate and rapid detection of LSD virus in appropriate specimens (Khalefa *et al.*, 2015).

Real-time PCR assay is based on unique site in LSD044 used for the universal DNA detection from the field, vaccine, and recombinant strains of LSD virus (Alexander *et al.,* 2019).

The aim of this study was the rapid identification of LSD virus isolated from

cattle clinically suspected to be infected with LSD on clinical and molecular basis.

MATERIALS AND METHODS

Animals

The LSD infected cattle located in Giza governorate were investigated by measurement of body temperature, examination of the skin and superficial lymph nodes. Complications for all suspected animals were detected (Salib & Osman, 2011).

Ethical approval

Samples were collected as per standard procedure without any stress or harm to the animals. The work was conducted in line with Directive 2010/63/EU (Anonymous, 2010). All laboratory works were conducted at the Agriculture Research Center (ARC); Central Laboratory for Evaluation of Veterinary Biologics, Cairo, Egypt.

Samples

Fifteen sit-fast skin lesions were sporadically collected in 2018 from cattle that showed LSD characteristic clinical signs in Giza governorate. The samples were transferred to the laboratory in phosphate buffered solution supplemented with antibiotics and under chilled conditions and stored at −20 ℃ till used for LSD virus isolation and identification.

Sample preparation

For virus isolation, tissue samples were cut into fine pieces and minced using sterile scissors and forceps then ground with a pestle in a mortar containing sterile sand. Ten millilitres PBS containing gentamicin (0.1 mg/mL), ampicillin (0.05 mg/mL) and fungistatin were added. The suspension was kept overnight at 4 °C. The samples were partially clarified by centrifuga-

tion (2000 rpm, 3−5 min) to remove gross particles, supernatant was collected in sterile tubes and stored at −20 °C until used (OIE, 2018).

Embryonated chicken eggs

Specific pathogen free (SPF) eggs were obtained from the SPF Production Farm, Koum Osheim, El-Fayoum, Egypt. The eggs were kept in the incubator at 37 °C with humidity of 40-60% (House *et al*., 1990) and used for LSD virus isolation.

Lumpy skin disease virus (LSDV)

LSDV was supplied from Pox department, Veterinary Serum and Vaccine Research Institute (VSVRI) Abbasia Cairo, Egypt. Ismailia strain was isolated from Egypt during the outbreak of 1988 (House *et al*., 1990). The virus was adapted in Madin-Darby bovine kidney cells (MDBK) (Daoud *et al.,* 1998; Irons *et al.,* 2005).

Virus isolation

SPF embryonated chicken eggs, 9–11 days of age, were inoculated with the prepared samples via the chorioallantoic membrane (CAM) route (House *et al*., 1990; OIE, 2018). Two hundred μL from the supernatant fluid of each tissue homogenate were inoculated into the CAM of three embryonated chicken eggs, incubated at 37 °C with daily examination. The CAMs showing pox lesions were collected, minced and centrifuged at 3000 rpm for 10 min in a cooling centrifuge at 4 °C. The supernatant fluids were kept at −20°C for identification (Reed & Munch, 1938).

Madin Darby Bovine Kidney (MDBK) cell line from the Central Lab for Evaluation of Veterinary Biologic (CLEVB). Abbasia Cairo, was propagated with Eagle's minimum essential medium (EMEM), supplemented with foetal bo-

vine serum and used for virus isolation (Irons *et al.,* 2005; OIE, 2018). The supernatant (0.5 mL) was inoculated into monolayer of MDBK cell culture. Cultures were observed daily for 14 days for detection of the cytopathic effect (CPE).

Histopathological examination

The collected skin samples were fixed in 10% neutral buffered formalin solution, washed and dehydrated in ascending alcohol series, cleared in xylene, embedded in paraffin then sectioned (4–6 µm) and stained with haematoxylin and eosin. Semithin sections were done by fixation in 5% glutaraldehyde and sectioned by ultra microtome in thickness of one µm. Sections were stained by toluidine blue (Bancroft *et al.*, 1996; Suvarna, 2013).

Molecular identification of virus isolates

- Conventional PCR

Viral DNA extraction. Viral DNA was extracted from infected tissue culture samples (Sambrook *et al.,* 1989) and stored at −20 °C till used for PCR. The extraction method was carried out by using DNA extraction kits Easy Pure® Viral DNA/RNA Kit, lot # M 30904.

Oligonucleotide primers. The developed primers were from the viral attachment protein encoding gene. Expected amplicon size was 192 bp (Ireland & Binepal, 1998; OIE, 2018). The primers gene sequences were as followed: forward primer 5‟-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3‟ and reverse primer 5‟-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3‟.

The specific primers set amplified the DNA fragment of 192 bp equal to the expected amplification product size from LSD virus. The DNA Ladder 100 bp (2×Easy Taq@ PCR Super Mix-Trans. Lot# M31009) was used as a standard size

and the gel was visualised using an ultra-violet light transilluminator.

Amplification of DNA. The DNA amplification carried out in a final volume of 50 µL containing: 5 µL of 10× PCR buffer, 1.5 µL of MgCl$_2$ (50 mM), 1 µL of dNTP (10 mM), 1 µl of forward primer, 1 µL of reverse primer, 1 µL of DNA template (~10 ng), 0.5 µL of *Taq* DNA polymerase and 39 µL of nuclease-free water. As the volume of DNA template required may vary, nuclease-free water volume must be adjusted to the final volume of 50 µL (OIE, 2018 & Gamil *et al.*, 2019).

Thermocycler conditions. The samples in a thermal cycler were run as followed: first cycle − 2 min at 95 °C, second cycle − 45 s at 95 °C, 50 s at 50 °C and 1 min at 72 °C for 34 cycles; last cycle was 2 min at 72 °C and hold at 4 °C until analysis.

Ten µL of each sample were mixed with loading dye and loaded onto a 1.5% agarose gel in TAE (Tris/acetate containing EDTA) buffer. A parallel lane was loaded with 100 bp DNA-marker ladder. The products approximately were electrophoretically separated for 40–60 min (8–10 V/cm) and visualised.

- RT-PCR

It was carried out according to the method of Dejan *et al.* (2016).

DNA extraction. DNA extraction was done for RT-PCR according to the manufacturer's instructions by using Gene JET Genomic DNA Purification Kit (Thermo Fisher cat # K0721). In 180 µL of digestion buffer for 10 min at 37 °C, the sample (300 µL) was digested with 20 µL of proteinase K, then lysed with 200 µL of lysis buffer for additional 5 min. The mixture was centrifuged at 6000 rpm/2 min, 400 µL of 50% ethanol was added and the mixture transferred to the Gene JET column and centrifuged as described. Then

the column was washed twice with washing buffers and the DNA was eluted in 50 µL of elution buffer, and stored at − 20 °C till use.

Real Time PCR primers and probe. The primers flanked a conserved 151-bp region of LSD044 that is found among LSDV strains: zdf4ln (forward primer) CAA+AAA+CAA+TCG+TAAC+TAA TCC A, and zdr4ln (reverse primer) TG+GAG TTT TTA+TG+TCA TCG TC, and (Taqman® probe) zdpro4ln1 TC+ GT+CGT+CG+TT+TAA+AAC   TGA. The Taqman probe was labelled with 6-carboxy fluorescein (FAM), the reporter dye at the 5′-end and Black Hole Quencher-1 (BHQ-1) at the 3′-end. Selected primers were synthesised by Syntol (Moscow, Russia) and modified with locked nucleic acid (LNA) bases (Alexander *et al.,* 2019).

Real Time PCR. One positive LSD virus sample proved by conventional PCR (number 3 which was harvested from infected MDBK cell line) was analysed by RT-PCR to confirm it as LSD virus. It was done using Thermo Scientific AB solute Blue qPCR Low ROX kit cat # AB-4318/B according to the manufacturer's instruction. Briefly, 5 µL containing 30 µg of DNA was mixed with 12.5 µL of the 2× buffer, 100 pmol of each primer and 200 pmol of the probe. The reaction conditions were set as followed: 95 °C/ 15 min followed by 40 cycles of 95 °C/15 s, 60 °C/50 s. Fluorescence data were collected at the end of each extension using Agilent MX3005P thermal cycler.

RT-PCR assay

Threshold cycle (Ct) values were defined as the cycle number at which the amplification curve crossed the fluorescence threshold set at 0.1. An increase in fluorescence intensity above this level, cou-

pled to a cycle threshold of <40, was considered a positive result. If a sample did not register a Ct value, it was considered negative. The standard curve was done by using LSD Ismailia strain.

RESULTS

The suspected cattle showed clinical signs of LSD and its complications including pyrexia, salivation, ocular and nasal discharges, skin nodules, enlargement of lymph nodes, loss of body weight, pneumonia, corneal opacity. Most of the necrotic nodules were ulcerative and formed deep scabs called sitfast (Fig. 1).


**Fig. 1.** A calf affected with lumpy skin disease showing characteristic large skin nodules.

LSD isolation

On chorioallantoic membrane of SPF ECE, it was found that 12 out of 15 inoculated samples induced lesions on CAM from the 3rd passage forming typical pox lesion, death of embryo, thickening, oedema and haemorrhage of the membrane (Fig. 2). The isolated LSD virus was titrated in ECE with a virus titre of 10^7 EID_{50}/mL.

On MDBK cell line, negative cell cultures were blind-passaged after 14 days and the collected suspension inoculated onto
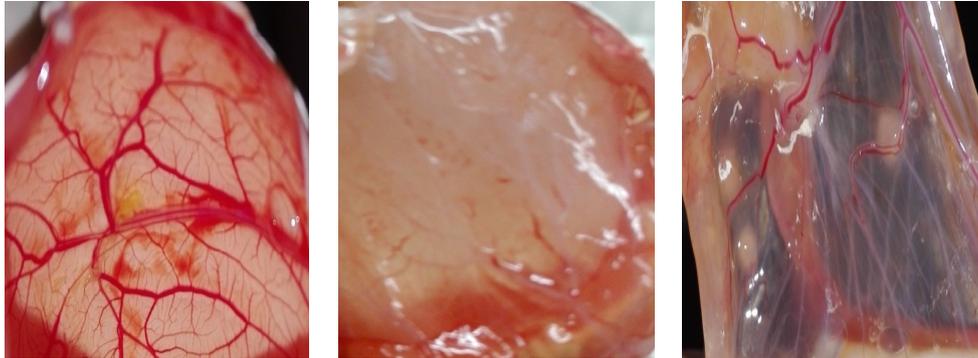
Fig. 2. LSDV lesion on CAM varying from thickening of membrane to numerous white foci of pock lesions with inflammatory signs.

A   **B**   **C**
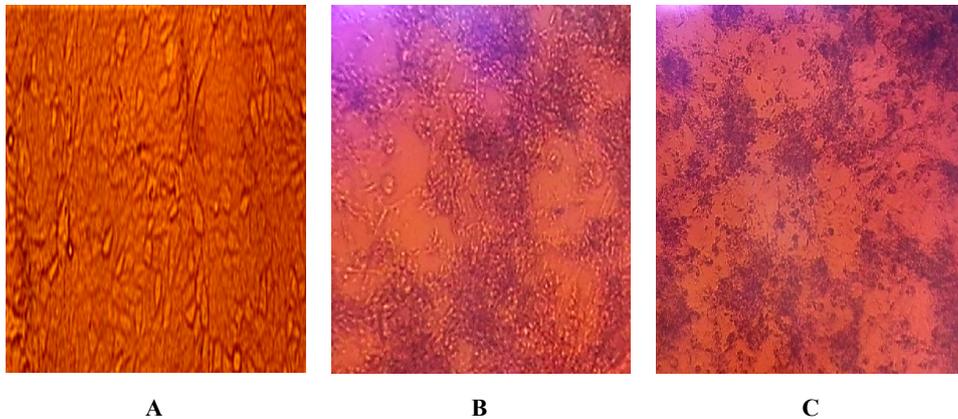
Fig. 3. A. Normal non infected MDBK cells (negative control cell monolayer without any virus inoculation); **B, C.** Characteristic CPE of LSDV by cell rounding, cell aggregation, coalesce together to form clusters within 5−6 days post inoculation.

fresh cell culture monolayer and observed for another 11−14 days 4 times until typical CPE changes occurred (Fig. 3).

The positive control was a cell monolayer inoculated with 0.5 mL LSD virus suspension at a titre of 4 $\log_{10}$ TCID$_{50}$.

Histopathological findings

Dermal layer histopathological examination revealed vasculitis with projection of its endothelial lining in the vessels lumen and focal aggregation of inflammatory cells surrounding the blood vessels (Fig. 4A). Specific intra-cytoplasmic inclusion

body of LSD infection appeared in the mixture of red blood cells, lymphocytes, neutrophils and macrophages which infiltrated in the semi-thin section of dermal stroma (Fig. 4B).

Molecular identification

Detection and identification of LSD virus from supernatant of the prepared sitfast sample, harvested from infected CAM and from infected MDBK cell line by using conventional PCR with specific primer of the specific gene (P32) viral attachment gene at 192 bp revealed that

the samples from suspected cattle were positive (Fig. 5).

In RT-PCR, positive Ct=19.97 was seen with the sample but not with the NTC (Fig. 6). The presence of specific Ct curves of DNA templates extracted from harvested MDBK infected cell were nearly similar to Ct of DNA template positive control of LSD Ismailia viral strain.

DISCUSSION

Lumpy skin disease is an economically important disease of cattle caused by lumpy skin disease virus (LSDV), one of

Capripoxvirus genus members. LSD is a viral disease of cattle that causes economic losses and is endemic to several countries of Africa and the Middle East, and recently recorded in Europe (Salib & Osman, 2011; Abutarbush *et al.,* 2015; Rashid *et al.,* 2017). LSD spread in China in 2020 and also in 23 countries in Asia (Roche *et al.,* 2020).

LSD was first recorded as an outbreak in Egypt during the summer of 1988 and within five to six months, it had spread to 22 out of 26 Egyptian governorates (House *et al.*, 1990). LSD has been making its way into Europe since 2014–2015
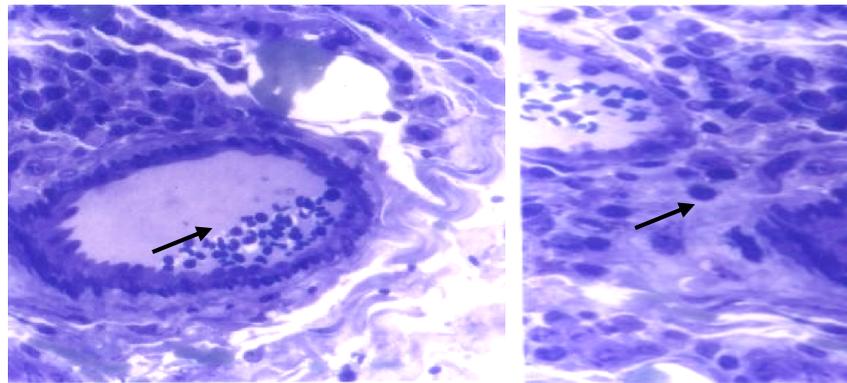
Fig. 4. A. Semithin section of dermal layer revealing vasculitis with projection of its endothelial lining. Toluidine blue, ×400; **B.** Semi-thin section of dermal stroma revealing intra-cytoplasmic inclusion body, Toluidine blue, ×1000.
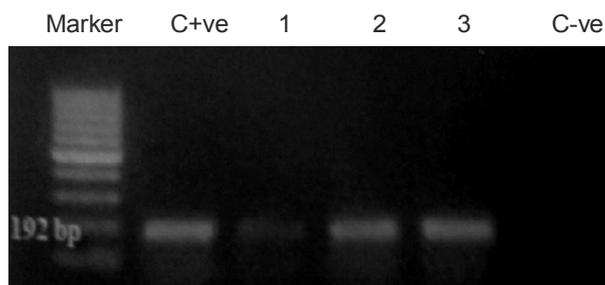
Fig. 5. PCR electrophoregram showing 3 positive LSD samples with molecular weight of 192 bp. Marker: 100-1000 bp; C+ve: positive control; lane 1: LSD virus from supernatant of the prepared sit-fast sample; lane 2: LSD virus harvested from infected CAM; lane 3: LSD virus harvested from infected MDBK cell line; C-ve: negative control.
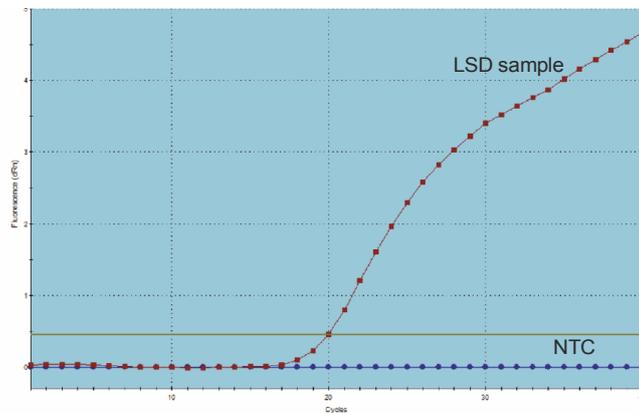
Fig. 6. Amplification plots. Positive Ct=19.97 was seen with the sample but not with the negative control (NTC). Threshold cycle (Ct) values were defined as the cycle number at which the amplification curve crosses the fluorescence threshold set at 0.1. An increase in fluorescence intensity above this level, coupled to a cycle threshold of <40, was considered a positive result. If a sample repeatedly tested inconclusive, it was considered positive. If a sample did not register a Ct value, it was considered negative (NTC).

(Beard, 2016).

The present study was a trial for isolation of LSD virus from skin nodule samples from 15 infected cows from Giza governorate in embryonated chicken eggs and tissue culture with molecular identification and characterisation of virus isolates using PCR and real time PCR.

The infected cattle showed characteristic clinical signs of LSD infection: fever, skin nodules all over the body and lymphadenopathy (Fig. 1), similar to recorded LSD lesions (Salib & Osman, 2011; Rashid *et al.,* 2017; Gamil *et al.,* 2019).

Serological assays are not useful tools in screening the prevalence of LSDV, they are too time-consuming to be used as primary diagnostic tools. Serum sample testing with LSDV antibodies may be difficult due to the cross-reactivity encountered with other poxviruses and the low antibody titres elicited in some animals following mild infection or vaccination (Lamien *et al.,* 2011*).*

Virus isolation is considered as the most important step for viruses diagnosis but it is time-consuming and needs some blind passages. Moreover, following these procedures, viral isolates have to be identified (Joshi *et al.,* 1996; Oguzoglu *et al.*, 2006).

LSD virus was isolated from sitfast of infected cattle on chorioallantoic membrane of 9 to 11 days old SPF embryonated chicken egg. The isolated virus was passed till the observation of characteristic pox lesions including inflammation, thickening, oedema and haemorrhage of the membrane. The pox lesions were observed after 2nd passage and became clear after 4th passage in only 12 from 15 isolated samples (Fig. 2). The absence of pock lesions in 3 samples could be interpreted by low titres in prepared samples. These findings agree with those of House *et al.* (1990), Tamam (2006), and with those of Kitching & Hammond (1992) and Gamil *et al.* (2019) who successfully cultivated LSDV on CAM of ECE and detected the characteristic pox lesions.

Also, MDBK cell culture showed characteristic cytopathic effect with all

fifteen inoculated samples (Fig. 3). CPE was characterised by cell rounding, cell aggregation and coalescence forming clusters 6–8 days post inoculation and gradually increased till 70–80% of sheet was completely detached. These findings were in line with previous reports (Davies, 1991; Ibrahim *et al.*, 1999; Fahmy, 2000) who isolated LSDV from skin biopsies on MDBK cell culture.

Histopathological changes revealed vasculitis with projection of its endothelial lining in the vascular lumens and focal aggregations of inflammatory cells surrounding the blood vessels. Specific intracytoplasmic inclusion bodies of LSD virus infection appeared in the mixture of red blood cells, lymphocytes, neutrophils and macrophages which infiltrated in the semi-thin section of dermal stroma as reported in other studies (Salib & Osman, 2011; Wainwright *et al.*, 2013; Tageldin *et al.,* 2014) who recorded skin nodules with congestion, haemorrhage, oedema, and vasculitis with consequent necrosis involving all dermis layers, subcutaneous tissue, and also adjacent musculature.

OIE (2018) affirmed the conventional gel-based PCR method is a simple, fast and sensitive method for the genome detection in different samples.Viral identification and characterisation of isolated LSD virus by conventional PCR recorded as clear strong running bands of 192 bp (Fig. 5) correlated with previous results (El-Bagoury *et al*., 2009; Sharawi & Abd El-Rahim, 2011; Khalefa *et al*., 2015; 2017; Fahmy & Gaafar, 2016; Gamil *et al.,* 2019) who considered PCR as the test of choice in the diagnosis of the causative agent of LSD.

Quantitative real-time PCR methods have been described as faster and more sensitive assay for detection of LSDV DNA in samples of low amounts of viral genomes and lowest Ct values, differentiating LSDV from other *Capripox* viruses (Balinsky *et al.*, 2008; Lamien *et al*., 2011; OIE, 2018; Gamil *et al.*, 2019).

Real-time PCR primers conserving the 151 bp region of LSD044 found among LSDV strains were used, whereas the probe was unique to LSD virus confirming the locally isolated virus as LSD virus in agreement with previous studies (Vidanovic *et al*., 2016; Agianniotaki *et al*., 2017b; Sprygin *et al*., 2018a,b; 2019a,b; Alexander *et al*., 2019; Gamil *et al.,* 2019) which all reported that TaqMan real-time PCR assay can detect LSDV DNA.

Clinical LSD signs appeared in cattle in different Egyptian governorates in 2018. The virus was isolated on CAM of SPF ECE and MDBK cell line and was identified using conventional and real time PCR to detect LSDV-DNA. All assays confirmed that cattle infection was caused by LSDV.

In conclusion, LSD is an important enzootic disease in Egypt that is still circulating among Egyptian cattle so that LSD virus could be isolated on CAM of ECE and on MDBK cell line and identified by histopathology and genetically by conventional and real time PCR assay. Real time PCR assay should be applied besides conventional techniques of LSD diagnosis as a rapid and accurate diagnostic approach.

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