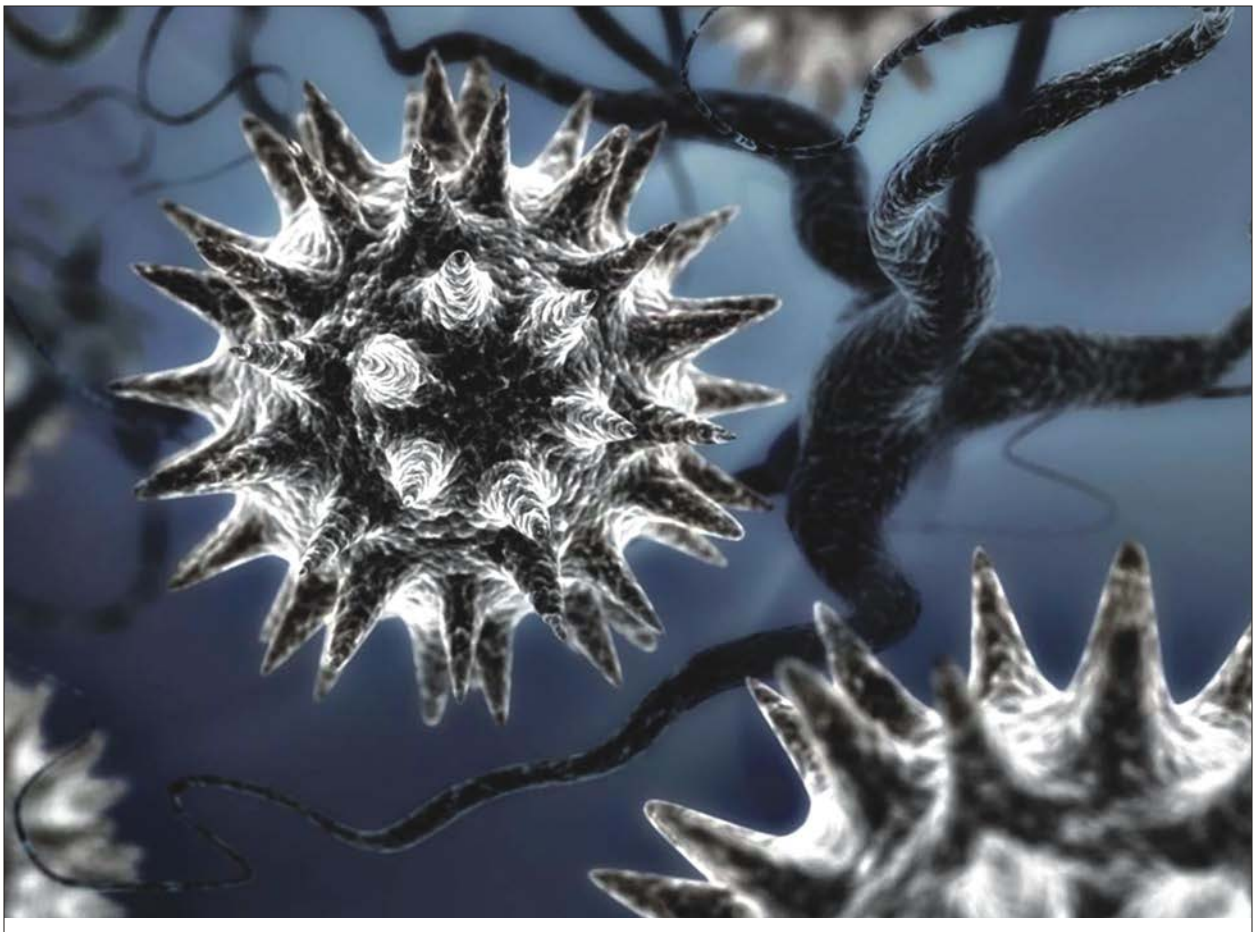

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Lumpy Skin Disease Virus Identification in Different Tissues of Naturally Infected Cattle and Chorioallantoic Membrane of Emberyonated Chicken Eggs Using Immunofluorescence, Immunoperoxidase Techniques and Polymerase Chain Reaction

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ABSTRACT

Lumpy Skin Disease Virus (LSDV) was detected in 23 samples collected from clinically diseased and slaughtered cattle showed clinical signs believed to be LSD. These samples include 7 skin lesions and 16 internal organs (lymph nodes (6), lung (4), kidney (3) and liver (3). Hyperimmune serum was prepared against reference LSDV (Ismailyia 88 strain). Immunofluorescence (IF), Immunoperoxidase (IP) techniques and Polymerase Chain Reaction (PCR) were used in our study. Chorioallantoic Membranes (CAMs) of Embryonated Chicken Eggs (ECEs) were inoculated with Known previously isolated and identified LSDV of $10^{4.8}$ EID_{50}/0.1 mL for virus follow up in these CAMs by using IF and IP techniques and PCR. The results indicate that IF and IP techniques are useful in the quick diagnosis of the disease in naturally infected cattle. PCR could be used for rapid and specific detection of LSDV nucleic acid in crude skin and internal organs samples. Also, LSDV could be detected in CAMs of ECEs using PCR at first day Post-inoculation (PI) and by IF and IP at second day post-inoculation before appearance of characteristic pock lesions on CAM.

Key words: Lumpy skin disease virus, chorioallantoic membrane, immunofluorescence, immunoperoxidase, polymerase chain reaction

INTRODUCTION

The genus Capripoxvirus is comprised of lumpy Skin Disease Virus (LSDV), Sheep Poxvirus (ShPV) and Goat Poxvirus (GPV), causing disease in cattle, sheep or goat, respectively (Esposito and Fenner, 2001). Lumpy skin disease is an acute, subacute or inapparent viral disease of cattle and occasionally buffaloes characterized by pyrexia, generalized skin lesions and generalized lymphadenopathy (Prozesky and Barnard, 1982; Davies, 1991; Hamoda *et al*., 2002). The disease is endemic in Central and South Africa. The first report of LSD outside Africa was from Kuwait in 1986-1988 (Anonymous, 1988), followed by Israel in 1989 (Shimshoney, 1990). In Egypt, the LSD was first appeared in Suez Governorate after cattle importation from Somalia followed by Ismailyia Governorate in 1988 (House *et al*., 1990) and two disease outbreaks were reported in 2005 and 2006 (Younis and Aboul Soud, 2005; OIE, 2006). The disease was considered a“ list A” disease by the Office International des Epizooties (OIE) due to its potential for rapid spread and ability to cause severs economic losses. The disease causes significant economic

loss due to hide damage, loss of milk production, mastitis, infertility and death (Weiss, 1968). Consequently, it is of uppermost importance to have rapid, sensitive and specific diagnostic methods.

Following diagnosis of the disease, rapid instigation of control measures such as slaughter, ring vaccination and movement restrictions are required to limit losses (Carn, 1993).

Diagnosis of the disease is depend initially on clinical signs and definitive diagnosis is provided by virus isolation or its demonstration by electron microscope and identification of antigen by fluorescent antibody, serum neutralization, agar gel precipitation, antigen capture ELISA, Dot ELISA and immunoperoxidase (Woods, 1988; El-Bagoury *et al*., 1995; Tuppurainen *et al*., 2005; Younis and Aboul Soud, 2005). Polymerase Chain Reaction (PCR) assay has been described for detection of LSDV (Ireland and Binepal, 1998; Heine *et al*., 1999; Tuppurainen *et al*., 2005; Ibrahim *et al*., 2006). The studies on using of Immunofluorescence (IF) and Immunoperoxidase (IP) antibody technique for detection of LSDV in naturally infected cows are insufficient so the present study aimed to use indirect immunofluorescence, indirect immunoperoxidase antibody techniques and PCR for direct detection of LSDV in skin and internal organs naturally infected cattle as rapid methods for the disease diagnosis and to improve understanding of LSD pathogenesis. We also follow up the virus in CAMs of ECEs using IF, IP and PCR.

MATERIALS AND METHODS

Collection of samples: A total of 23 samples were collected from clinically diseased and slaughtered cattle from Dakahlia Governorate, Egypt showed clinical signs believed to be LSD. These samples include 7 skin lesions and 16 internal organs (lymph nodes (6), lung (4), kidney (3) and liver (3). Samples from four normal cows were included as negative controls. Part of each samples was taken rapidly to the freezing chamber of a cryostat for IF testing. Another part was put in bottles containing neutral buffered formalin 10% for IP testing. Four samples from previously collected samples were selected to confirm LSDV diagnosis using Polymerase Chain Reaction (PCR).

Lumpy Skin Disease Virus (LSDV):

- Tissue culture adapted LSDV/Ismailyia 88 strain was kindly supplied from the Pox Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. It was prepared in Madian Darby Bovine Kidney (MDBK) and had a titre of $10^{4.5}$ TCID50/mL. It was used in Preparation of hyperimmune serum
- LSDV of $10^{4.8}$ $EID_{50/0.1}$ mL was previously isolated from naturally infected cattle on CAMs of ECEs and identified using Agar Gel precipitation Test (AGPT), Llatex Agglutination Test (LAT) and Reverse Passive Haem Agglutination (RPHA) (El-Kenawy and El-Tholoth, 2009)

Preparation of hyperimmune serum against standard reference LSD virus: It was prepared in rabbits according to Davies (1982).

Indirect immunofluorescent (IF) technique for LSDV antigen detection in samples collected from naturally infected cattle: IF test was used to detect lumpy skin disease virus according to Davies *et al*. (1971) and Mishra and Mallick (1997) as follow:

The collected tissues were taken rapidly to the freezing chamber of a cryostat and left for 15 min at -30°C. Tissues were sectioned at 3 microns thickness using a cryostat knife and then transferred

to slides. These sections were left to dry in air for 30 min, fixed with acetone for 10 min and then washed with PBS, pH 7.6. A few drops of 1:100 dilution of the prepared rabbit hyperimmune serum were added to the sections and the slides were kept in a humidified chamber for 1 h at 37°C. The slides were washed with PBS for 15 min 3 times then a few drops of 1:200 dilution of anti rabbit FITC conjugate (fluorescent conjugated goat IgG fraction to rabbit IgG (whole molecule), it was provided from ICN pharmaceuticals (lot no. 02671) was added. After 1 h of incubation at 37°C in the dark, the slides were thoroughly washed with PBS and counter stained with Evan's blue stain. They were then mounted with buffered glycerin, covered with a cover slip and examined under a fluorescent microscope.

Indirect immunoperoxidase (IP) technique for LSDV antigen detection: It was done according to Hamir and Moser (1994) and Mishra and Mallick (1997) as follow:

Formalin-fixed, paraffin embedded infected tissues were cut at 5 μm. Slides were heated at 55°C to melt the paraffin, deparaffinized in xylol, hydrated through graded ethanols and finally rinsed in phosphate buffered saline. Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide in methanol for 20 min and then slides were incubated with blocking solution (10% FCS) for 30 min. Sections were incubated with diluted primary antibody (1:100) for 60 min at room temperature in a humid chamber. After washing with PBS 3 times, 10 min for each cycle the sections were incubated with 1:200 dilution of anti-rabbit Horseraddish Peroxidase (HRP) conjugate (Peroxidase conjugated goat IgG fraction to rabbit IgG (whole molecule), it was provided from ICN pharmaceuticals (lot no.03782) and incubated for 60 min at room temperature in a humid chamber. The slides were then washed and drops of Diaminobenzidine (DAB) substrate solutions was added for 30 min. Sections were thoroughly washed under tap water and counter stained with hematoxylin. After this the reaction was detected under light microscope.

Confirmation of LSDV diagnosis using polymerase chain reaction (PCR): Four samples (skin lesions (2), lymph node (1) and lung (1) were selected to confirm LSDV diagnosis using Polymerase Chain Reaction (PCR) and LSDV Ismailia 88 strain used as positive control as follow:

Oligonucleotide primers: Oligonucleotide primers were designed according to Ireland and Binepal (1998) for amplification of the attachment gene of capripoxvirus. Oligonucleotide primers used in the PCR reactions were synthesized by Metabion International AG Company, Germany. The primers were received in lyophilized form and resuspended in Tris/EDTA (TE) buffer to reach a final concentration of 100 pmol μL^{-1} and were designed to amplify a specific segment of 192 bp. The primers sequences for PCR amplification were as follows: forward primer, 5′- TTTCCTGATTTTTCTTACTAT-3′ and reverse primer, 5′- AAATTATATACG TAAATAAC-3′.

DNA extraction: DNA extraction was done according to Viljoen *et al.* (2005). Using 0.5 mL of infected tissues suspension digested with 20 μL Proteinase K (final concentration, 100 μg mL^{-1}) at 56°C for 2 h. 100 μL Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and mixed by inversion then centrifuged at 13000 rpm min^{-1} for 5 min then the upper aqueous layer was transferred to a clean microcentrifuge tube and 2.5 volumes absolute ethanol and 1/10 volume of 5 mol L^{-1} sodium acetate (pH 5.3) were added and mixed thoroughly. The DNA was precipitated at -20°C overnight and pelleted by centrifugation at high speed (13 000 rpm min^{-1}) for 15 min. The

pellet was washed once with 70% ethanol and centrifuged at 12 000 rpm min^{-1} for 10 min then air dried and resuspended in 50 μL TE buffer. Normal non- infected skin samples were included as a negative control sample.

- **PCR amplification:** This was carried out as described previously by Ireland and Binepal (1998). Briefly, 10 μL sample of extracted genomic DNA was placed in 50 μL of the final volume of 10×reaction mixture containing 50 mmol L^{-1} KCl, 10 mmol L^{-1} Tris–HCl (pH 8.3), 1.5 mmol L^{-1} $MgCl_2$, 200 mmol L^{-1} of each dNTP, 100 pmol of each oligonucleotide primer and 2 U Taq-DNA polymerase. Then 40 μL of mineral oil was added to prevent evaporation of components during thermocycling. The PCR had an initial cycle of 94°C for 5 min, 50°C for 30 sec, 72°C for 1 min followed by 34 cycles of 94°C for 1 min, 50°C for 30 sec, 72°C for 1 min and a final elongation step of 72°C for 5 min

- **Amplified product analysis:** This was carried out as described previously by Ireland and Binepal (1998) and Viljoen *et al.* (2005). Briefly 10 μL of the PCR product was mixed with 1 μL 10×gel loading buffer and loaded to the individual wells of a 1.5% agarose gel. In addition, 2 μL of a 100 bp DNA molecular weight marker was loaded with 2 μL loading buffer in a single outside well to be used as DNA ladder. The amplified DNA products were detected in comparison with DNA ladder using the U.V. transilluminator. The gel was photographed

Follow up the LSDV on CAMs of ECEs: LSDV of $10^{4.8}$EID50/0.1 mL was inoculated on CAMs of 12 ECEs, 9 days old, by drop membrane route according to Van Rooyen *et al.* (1969) and CAMs of 2 eggs were collected daily for 6 days after the virus inoculation for detection of the isolated virus on CAM by indirect Immunofluoresent (IF), Indirect immunoperoxidase (IP) antibody techniques and PCR. Two eggs were used as negative controls.

RESULTS

Detection of the virus in skin and different internal organs of naturally infected cattle using Immunoflourescent (IF) and Immunoperoxidase (IP) techniques: The results obtained showed that LSD virus could be detected in all collected samples by both IF and IP (Fig. 1a-j). There was no significant different between the results obtained by the two tests. None of the control samples (normal skin) showed a positive reaction.

Confirmation of LSDV diagnosis using Polymerase Chain Reaction (PCR): Analysis of the PCR products obtained from the amplification reaction of extracted DNA of the four selected samples by agarose gel electrophoresis revealed the positive amplification of the attachment gene with correct size (192 bp) (Fig. 2).

Follow up of the LSDV virus in CAMs of inoculated ECEs using IF, IP techniques and PCR: The isolated virus was inoculated on CAMs of ECEs and the detection of the virus on these CAMs was done by using indirect Immunofluorescent (IF), indirect immunoperoxidase (IP) techniques and PCR. These CAMs were examined daily after inoculation as shown in Table 1.

The result obtained in Table 1 revealed that LSDV could be detected in CAMs of ECEs using PCR at first day Post- Inoculation (PI) and by IF and IP at second day post inoculation before appearance of characteristic pock lesions on CAMs.

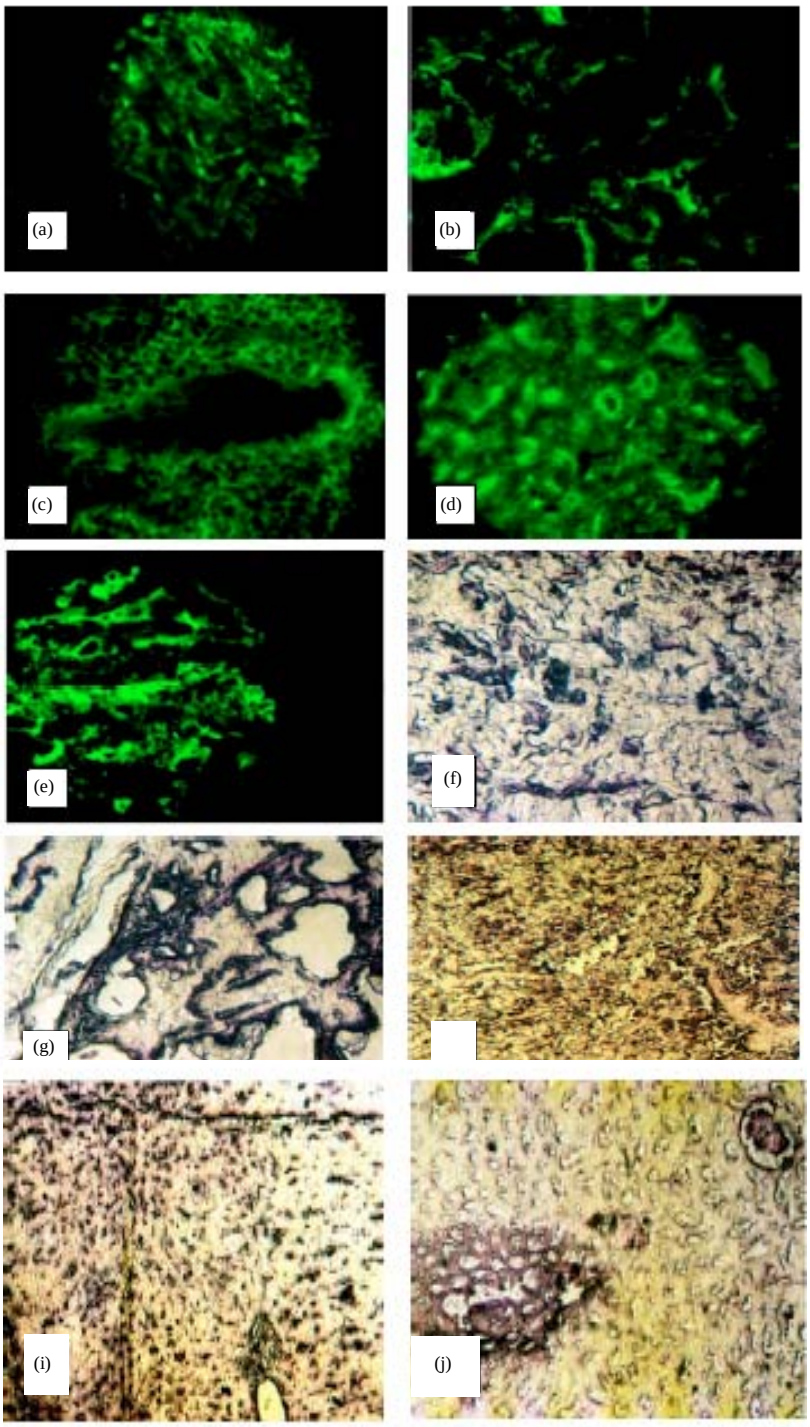

Fig. 1(a-j): (a-e) Immunoflourescent reaction in skin section, tissue of lung, lymph node and tissue of lung, respectively. (f-j) Deeply stained dark brown areas in skin, lung, lymph node, liver and kidney sections, respectively after indirect immunoperoxidase staining

Table 1: Detection of the isolated LSDV in CAMs of inoculated ECEs using IF, IP techniques and PCR

Days post inoculation (on CAM)	Gross appearance of CAM	Immunofluorescent technique	Immunoperoxidase technique	PCR
Day 1	Small hemorrhagic areas are seen at site of inoculation on CAM (non specific)	_	_	+
Day 2	Thickening of the membrane and become congested and hemorrhagic.	+	+	+
Day 3	White and opaque area around site of inoculation	++	+	+
Day 4	This white and opaque area increase in size.	++	++	+
Day 5	Opaque, pin point pock lesions arranged in streaks.	+++	++	+
Day 6	Opaque, pin point pock lesions arranged in streaks.	+++	++	+
Control	No gross lesions.	-	-	-

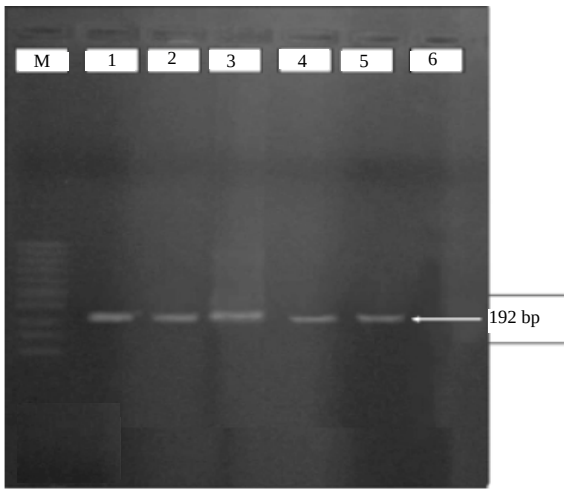

Fig. 2: PCR products of the attachment gene (192 bp) of LSDV DNA prepared from skin lesions (lane 1, 2) and internal organs samples (lane 3,4) in stained agarose gel electrophoresis, along with reference LSDV Ismailia88 strain (lane 5) and 50 bp DNA ladder (M). Lane 6: negative control sample

DISCUSSION

The results obtained showed that, the virus detected in the skin and different internal organs (lymph node, lung, liver and kidney) of naturally infected cattle in all collected samples by both IF

and IP. These results indicate that the immunofluorecence and immunoperoxidase techniques used for detection of LSDV in collected samples serve as a rapid, effective and economic method for laboratory confirmation of disease. The use of these techniques for direct detection of virus reduce the dependence on tissue culture and the time required to isolate the virus which may delay disease control.

Also these results indicate that the virus multiplication occur in these organs. It was suggested that dissemination of LSDV from the primary lesion to the draining lymph node and then to the systemic circulation, with localization to skin and other tissues including lung, liver, kidney and other lymph nodes. Our results were similar to that were obtained by Bowden *et al.* (2008).

No significant difference between the results obtained by using immunoflouroscent or immunoperoxidase for detection of LSDV in skin and different internal organs of naturally infected cows. Both tests are useful in the quick diagnosis of the disease. Sarma (2004) reported that immunoperoxidase is preferable than immunofluorescent test due to results can be read with a light microscope rather than a fluorescent microscope, sensitivity of the assay can enhanced by increasing the incubation period, endogenous enzyme activity can be blocked where as auto-fluorescence of cells interferes in some immunofluorescent assays, enzyme-antibody conjugates are more stable than fluorescent conjugates and fewer non specific reactions with enzyme antibody conjugates than with fluorescein-conjugated antibodies.

Isolated LSDV was inoculated on CAMs of ECEs and detection of the virus on these CAMs was carried out by immunofluorescent, immunoperoxidase techniques and PCR. The results obtained showed that LSDV could be detected in CAMs of ECEs using PCR at first day Post-Inoculation (PI) and by IF and IP at second day post inoculation before appearance of characteristic pock lesions in CAM. This result is in agreement with Davies *et al.* (1971), House *et al.* (1990), Ismael (2000), Tuppurainen *et al.* (2005) and Ahmed *et al.* (2005) who succeeded in detection of the virus in CAMs by immunofluorescent test.

The DNA product of the expected size (192 bp) was detected in 2 field skin samples, 2 internal organs samples and also LSDV Ismailia 88 strain used as positive control. This means that PCR could be used to detect LSDV genome in skin biopsy, internal organs and isolated virus in CAMS. This finding is in concurrence with Ireland and Binepal (1998), Irons *et al.* (2005), Tuppurainen *et al.* (2005) and Ibrahim *et al.* (2006) who recorded that PCR could be used to detect the capripoxvirus genome in biopsy, tissue culture, semen and blood samples. Using of PCR in LSDV genome detection is preferable as it do not require any specific reagents that can not be obtained commercially. Many firms sell custom primers for PCR and all the other reagents are common to all PCR reactions. It is also suitable for use in those countries in which the disease is not endemic and a live virus was not available (Ireland and Binepal, 1998; Heine *et al.*, 1999).

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