Rapid Virological and Molecular diagnosis of Lumpy skin disease virus

Hanaa A. Ahmed[1] and Eman M. AboHatab[2]

Genome Research Unit[1]. Virology Research Dept.[2]
Animal Health Research Institute, Dokki, Egypt

ABSTRACT

Lumpy skin disease is an infectious viral disease of cattle, which is an important serious skin disease of cattle in Africa. In present study, twenty one samples in the form of nodular lesion of skin were collected at different stages during the course of the disease from private farms in Ismailia and Fayoum governorates during April to June 2013. Different diagnostic tests used for Isolation of LSD virus from the nodular samples by inoculation on chorioallantoic membrane (CAM) of embryonated chicken eggs (ECE) SPF at 9-11 day and Madin Darby bovine kidney (MDBK) cell culture. Identification of virus by indirect immuno fluorescent antibody technique (IFA) which was considered a rapid and accurate method for LSD antigen detection. nodules were subjected directly to PCR and Real time PCR for rapid and specific detection of LSDV, sixteen out of the twenty one nodular samples were confirmed positive by molecular methods. Eleven out of twenty one nodular samples were positive by (IFAT) in CAM, which prove that PCR and Real Time PCR are much sensitive and rapid diagnostic tool of LSD reflecting their importance in controlling the rapid spread of disease in Egypt.

INTRODUCTION

Lumpy Skin Disease Virus (LSDV) was first originated in sub-Saharan Africa in 1929 from where it has spread north and south during the past 70 years (Woods, 1988). The endemic geographic range of LSDV is currently limited to the continent of Africa (including Madagascar), although outbreaks started in Egypt in 1988 (House et al., 1990) The outbreak occurred in Egypt in 2006 was introduced accompanying the foot and mouth disease in cattle imported from Ethiopia, and spread to Israel (World Animal Health Information Database, OIE) creating a real risk of LSDV establishing itself in the Middle East and spreading into Asia and Europe (Carn and Kitching 1995a). LSDV is a member of the family Poxviridae genus Capripoxviruses are double-stranded DNA viruses with genomes approximately 150 kbp in size which contains 156 putative genes (Tulman et al., 2001)., *Aedes aegypti* mosquitoes were capable of the mechanical transmission of LSDV over a period of 2–6 days post-infective feeding (Chihota *et al.,* 2001). The impacts of global climate change on insect vectors, established as a route of transmission for LSD suggesting that there were real risks of further spread of these diseases into other geographic regions (Hunter and Walla 2001).

The disease is of economic importance in endemic areas due to its rapid spread and severe economic losses such as hide damage, decrease in milk production and weight gain, mastitis, infertility in males and females, decreased semen quality, and death (OIE, 2010).

Strains of capripoxvirus that cause LSD have been adapted to grow on the chorioallantoic membrane of embryonated chicken eggs (ECE)

(Babiuk *et al.*, 2007). Isolation of virus occur by using CAM of 9-11day old fertile eggs and although the cytopathic effect CPE may be seen in Madin-Darby bovine kidney [MDBK] cells' culture within 7 days (OIE, 2010).

The utility of PCR was investigated for the detection of the DNA of LSDV in clinical specimens such as tissue specimens and milk samples where diagnosis of LSD outbreaks by PCR will facilitate rapid application of control measures (Sharawi and Abd El-Rahim, 2011). The strength of real-time PCR is its speed, its quantitative nature and the ability to include controls for detection of reaction inhibitors (Babiuk et al., 2008).

The aim of this study is to compare between some virological and molecular tools for diagnosis of LSDV.

MATERIALS AND METHODS

1- Samples: twenty one skin nodules were collected at different stages during the course of the disease from private farms in Ismailia and Fayoum governorates during April to June 2013 six months after vaccination. The samples were homogenized Lesion for virus isolation and antigen detection is minced using sterile scalpel blade and forceps and then ground with a pestle in a sterile mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (amphotericin, 2.5 µg/ml) in 10% suspension and used for the embryonated chicken egg (ECE) inoculation and PCR as mentioned by Burleson *et al.* (1997).

Reference Virus and Antiserum: Lumpy skin disease virus (LSDV) Local strain was obtained from Department of virology, Animal Health Research Institute, Dokki, Giza.

2. Virus isolation:

a. In Embryonated chicken (ECE):

0.2 ml from the prepared sample suspention were inoculated via chorio-allantoic membrane (CAM) of 9-11 day old SPF, according to (Van Rooyen *et al.* ,1969) and the harvested CAM washed 3 times in phosphate buffer saline and examined for pock lesion then the membranes kept at -20 °C for tissue culture inoculation.

b. Tissue culture:

Prepared nodular samples before were inoculated into confluent sheet of MDBK cell lines supplied from tissue culture unit of virology research department and observed daily for the presence of cytopathic effect according to (OIE, 2004).

3- Virus identification

1-Indirect Fluorescent Antibody Technique (IFAT): It was carried out on infected MDBK cells for detection of specific fluorescence of LSD virus according to **Majewska et al. (1984)**

2- Conventional PCR and Real Time PCR: DNA was extracted directly from nodules using Thermo Scientific GeneJET Genomic DNA Purification Kit (#K0721) following the manufacturer instruction .

Real Time PCR was done using Genetic PCR Solutions Kit (GPS) – Spain, with Premix Ex Taq (Probe qPCR), Takara in StepOne thermal block (Applied Biosystem, USA) specific for LSDV.

Conventional PCR was done following the method of Ireland and Binepal, (1998) recommended in OIE (2010) using th e following primers, 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'that amplify 192 bp from the viral attachment protein encoding gene. The mix was done using Taq Master 5X mix (Jena Bioscience) and the amplified product was electrophoresed in 1.5% agarose in TBE.

RESULTS

The harvested CAM revealed the presence of pock lesion 4 days post inoculation in 11samples out of 21 skin samples and increased by 2nd And 3rd passages as shown in figure (1).

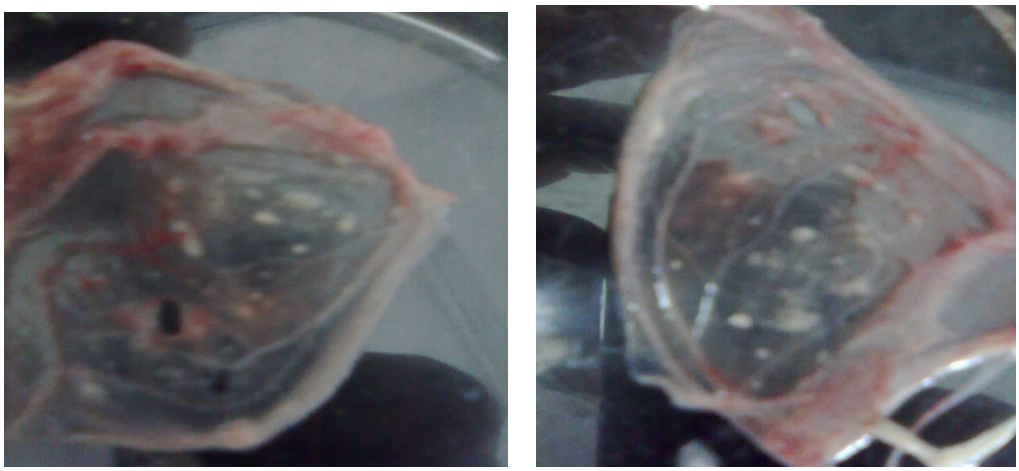


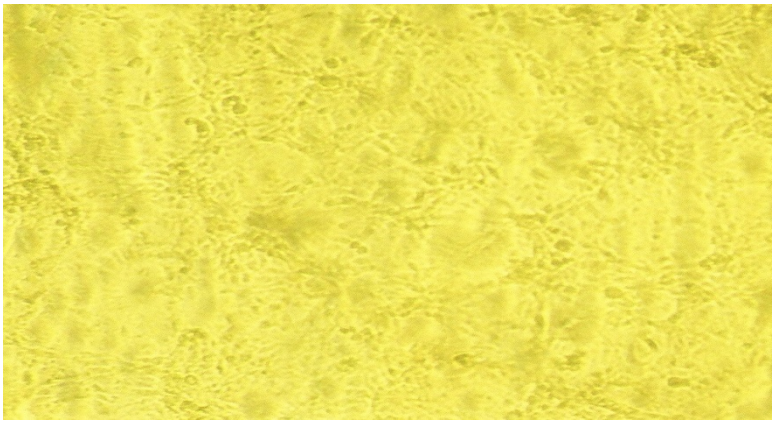
Fig. (1): lesion of LSDV on CAM varied from thickening of membrane In 1st passage to numerous white foci more pronounced by 2nd and 3rdpassag

The cytoplasmic pathogenicity CPE on MDBK cells developed 5-7 days post inoculation appeared in 11out of 21samples till distortion of the monolayer and cell detachment as figure 2 (a,b,and c)

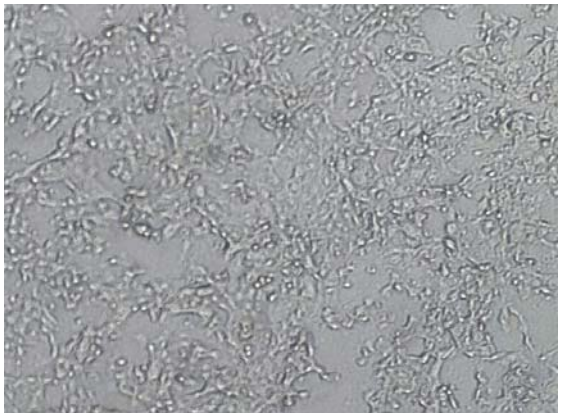
(a) Control cell complete monolayer sheet of MDBK

(b) The cell rounding, multinucleated cells , then

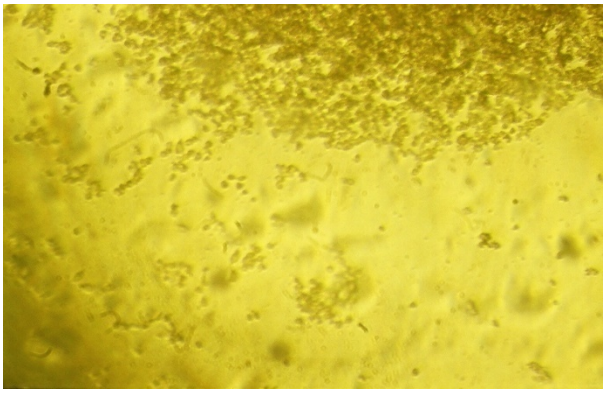
(c) Progressing of the CPE But the isolation of LSD virus in tissue culture needs long time until the clear appearance of CPE (5 days of inoculation and complete detachment after 7 days).



(a)Control MDBK Cell sheet (Magnified 400x)



(b) characteristic CPE of LSD isolates 5 days post inoculation MDBK



(c) complete cells detachment of sheet after 7 days post inoculation on MDBK (cells detachment)

Fig. (2): Typical CPE of LSD virus (b ,c) in comparison with normal control MDBK (a)

Identification Viral antigen by IFAT: the MDBK cells inoculated with the positive isolates and stained by fluorescence isothiocynate shown clear specific cytoplasmic diffusion yellowish green fluorescence granules emitted within 72 hrs post inoculation, negative one free from any fluorescence in Figure (3)

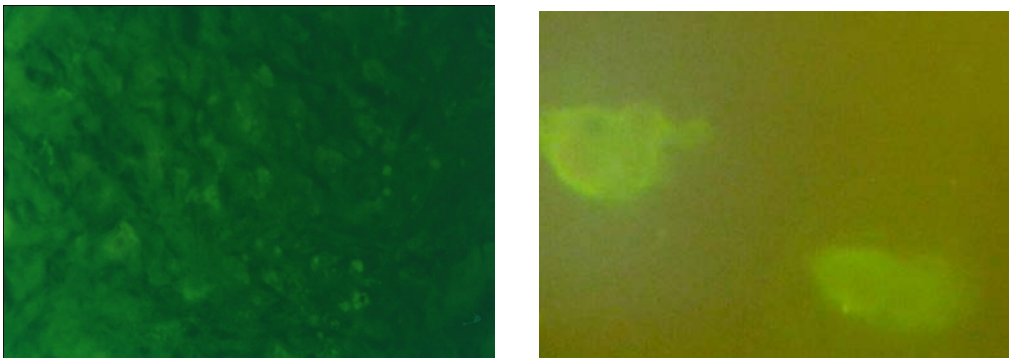


Fig. (3): MDBK cells infected by suspected local LSDV isolates and stained by fluorescence isothiocynate (Magnified 400x).
(Notice non specific intra-cytoplasmic yellowish green fluorescent granules)

Field samples were subjected directly to DNA extraction and both PCR and Real Time PCR was done simultaneously. By Real Time PCR, 16 out of 21 nodular samples were positive. In Fig (4) ten representative samples was used with the control positive, six were positive with different Ct and four were negative. The same samples tested with PCR with no results at the first time, by Re- PCR, 16 out of 21 gave positive for LSD virus with fragments of 192bp of the virus attachment gene.

Table (1) summarized the different techniques used in the study where Real time PCR was superior in sensitivity to conventional one followed by both virus isolation and IFAT.

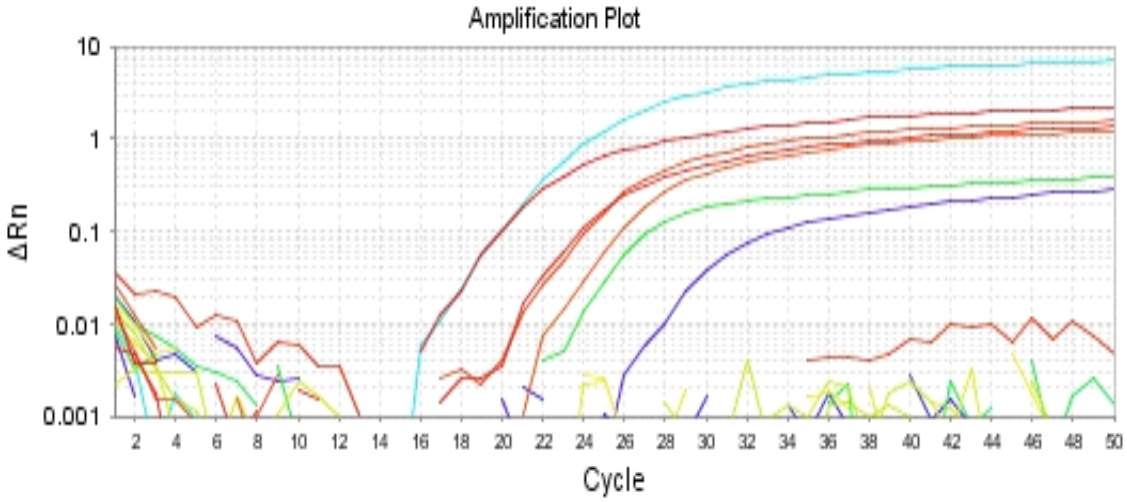


Fig. (4) : Amplification plot of six Representative LSDV samples from Nodules the control positive (CT was 24).

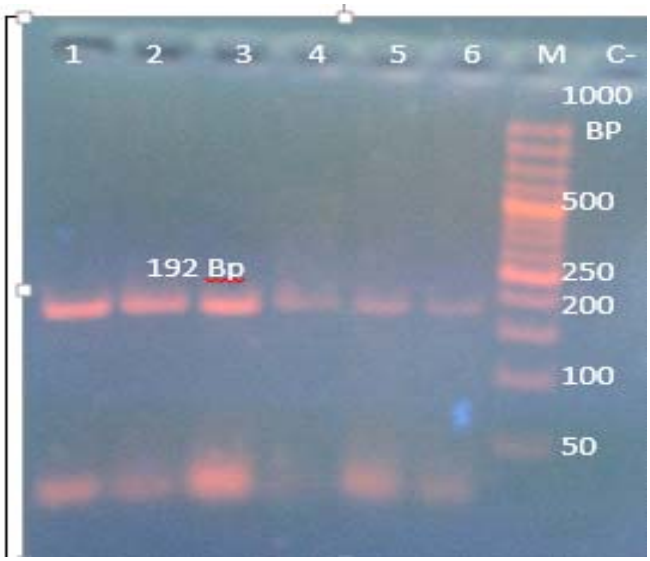


Fig. (5): showing the electrophoretic pattern of PCR products of six isolates of LSDV giving the specific 192 bp of the viral attachment gene. 1-6 field sample from nodule- M GeneRuler 50 bp DNA Ladder (Thermo # SM0241)- C- control negative

Table (1) : showing the comparison between different virological and molecular diagnostic techniques used for LSDV:

Technique	**Virus isolation**	**IFAT**	**Conventional PCR**	**Real Time PCR**
No/%	11/21 52.4%	11/21 52.4%	16/21 76.2%	16/21 76.2%

IFAT (Immuno fluorscent antibody technique)

DISCUSSION

LSD is an infectious disease 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 vaccination in Egypt depending on uses of sheep pox vaccine, it gives sufficient cross-protective immunity against LSD virus challenge (Saber *et al*., 1993).

In this study the harvested CAM revealed the presence of pock lesion 4 days post inoculation in 11samples out of 21 skin samples and increased by 2nd And 3rd passages as showen in figure (1) and this results agreed with Abd El-Rahim *et al*. (2002). The developed cytoplasmic pathogenicity CPE on MDBK cells 5-7 days post inoculation appeared in11out of 21samples the cell rounding, multinucleated cells , then progressing of the CPE till distortion of the monolayer and cell detachment as figure 2 (a,b,and c) But the isolation of LSD virus in tissue culture needs long time until the clear appearance of CPE (5 days of inoculation and complete detachment after 7 days) and in 3rd passage these findings were coincided with Prydie and Coackeley, 1959, Woods ,1988 and OIE ,2004).

Identification of the Cytopathic effect of Viral antigen by IFAT: the MDBK cells inoculated with the positive isolates and stained by fluorescence isothiocynate for differentiation between positive and negative one free from any fluorescence in Figure (3) according to (Majewska et al., 1984)

As showed the PCR to be more senstive in detecting LSD virus from skin samples. However, virus isolation is still required when the infectivity of the LSD virus is to be determined Tuppurainen et. al ., (2005). A similar results was recorded by El-Kholy et al. (2008) who confirmed the diagnosis of LSDV from Ethiopia imported cattle and found that PCR was 100% correlated with clinical signs and laboratory diagnosis of LSDV

Conclusion

Diagnosis based on clinical signs requires confirmation by rapid laboratory techniques. Real Time Polymerase Chain Reaction assay technique (qPCR) was the quick and more sensitive method for detection of LSDV from clinical samples in comparison with convensional virological virus isolation method which is time consuming. Also Real Time PCR proved to the same sensitive and time saving technique than conventional PCR

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