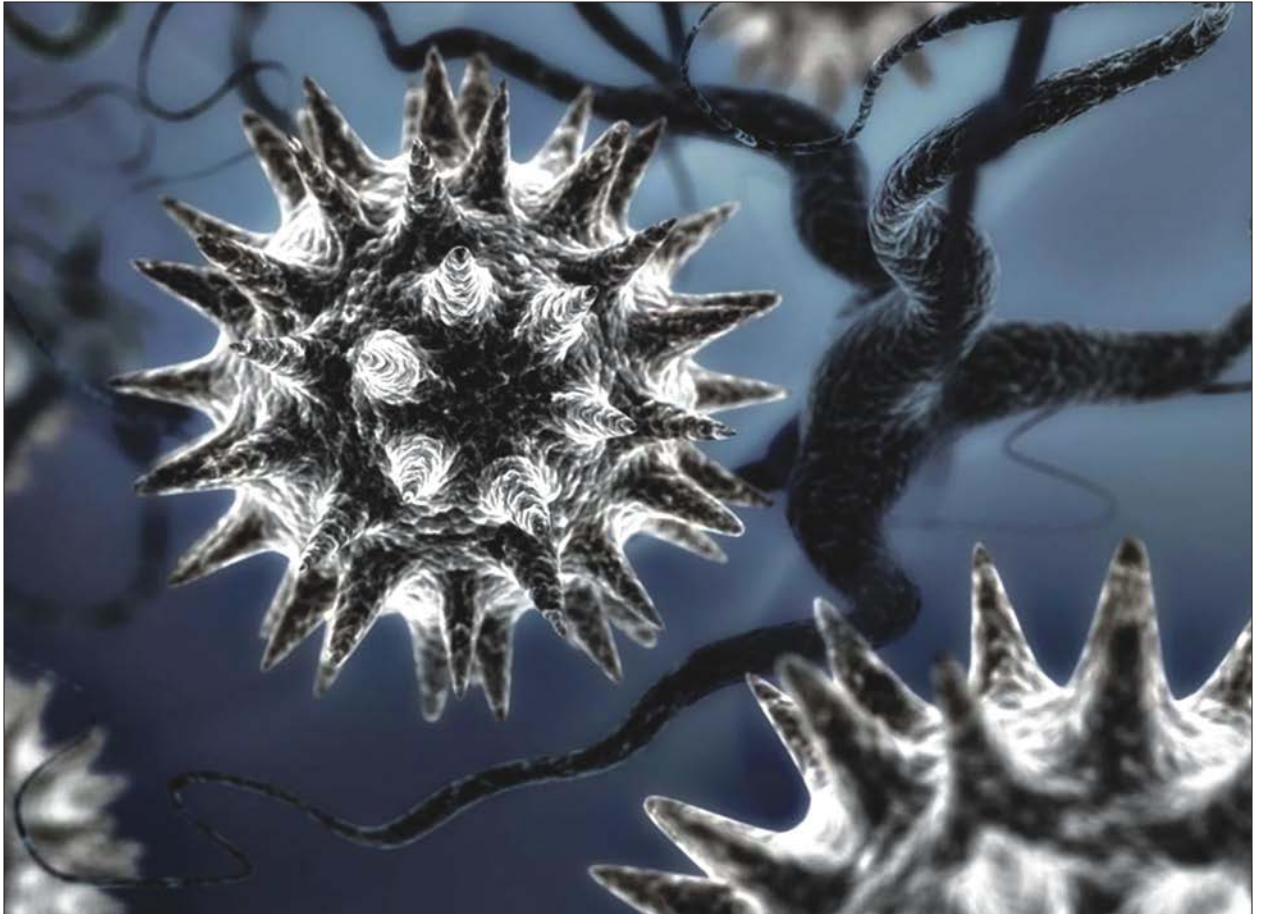




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Efficiency of PCR as Compared with Dot Blots Hybridization Techniques in Diagnosis of Lumpy Skin in Cattle in Egypt

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ABSTRACT

Lumpy Skin Disease (LSD) is one of the major threats of cattle stock industry in Egypt. There is many confusion in laboratory diagnosis of lumpy skin disease. Specific Lumpy Skin Disease Virus (LSDV) primers were used in Polymerase Chain Reaction assay (PCR) on field samples of skin biopsies and EDTA blood from clinically infected cattle located in Ismailia province, Egypt to assess their efficiency for LSD diagnosis. The specificity of the primers was confirmed by using other *Capripox viruses* (CaPVs) such as vaccinal strain of sheep poxvirus (SPPV) and local adapted tissue culture LSDV (Ismailia 88). Specific LSDV primers were successfully and specifically detected the LSDV in 100% of skin biopsies and 40% of EDTA blood samples and did not able to detect SPPV. Therefore, specific PCR is a valuable technique for accurate diagnosis of LSD without any confusion with other related viruses and differentiate LSDV from SPPV and must be used as routine diagnostic test for LSD. Detection of LSDV in field samples by using PCR and Dot Blot Hybridization (DBH) were compared. The results revealed that both PCR and DBH detected LSDV in 100% skin biopsies while detection rates in EDTA blood were 40 and 30% using PCR and DBH, respectively.

Key words: LSDV, SPPV, diagnosis, PCR, DBH

INTRODUCTION

Lumpy skin disease virus is a *Capripox virus* (CaPV) with a host range limited to ruminants (Aspden *et al.*, 2003), characterized by fever, nodular eruptions on the skin, mucous membranes and internal organs, in addition to emaciation, enlarged lymph nodes, oedema of the skin and sometimes death (OIE Manual, 2010) with morbidity varies from 3-85% and mortality never exceeds 3% (Barnard *et al.*, 1994).

Lumpy skin disease is currently endemic in most African countries and has recently spread out of Africa into the Middle East region and the risks of further spread into Turkey, Europe and Asia (Tuppurainen and Oura, 2012).

In Egypt, the first detection of the disease in Egypt among cattle was in May 1988 in Suez and Ismailia (House *et al.*, 1990). After that, LSD was recorded in restricted area during the period from April 1998 to June 1999 at Kaluobia government (Hamoda *et al.*, 2002). In early 2006, a massive LSD outbreak invaded cattle in different localities of Egypt exerting severe economic losses to livestock (El-Kholy *et al.*, 2008). During 2006 and 2007, two outbreaks of lumpy skin disease were recorded in Israel (Stram *et al.*, 2008).

Lumpy Skin Disease Virus (LSDV), Sheep Pox Virus (SPPV) and goat poxvirus (GTPV) are members of *Capripox viruses* (CaPV) genus in the family Poxviridae (Buller *et al.*, 2005). There are antigenic homology among all strains of CaPVs, thus recovery from infection with one strain provides immunity against all other strains. Because of this, there is potential to use a single vaccine strain to protect cattle, sheep and goats (Kitching, 2003). Serological techniques are not able to distinguish strains of CaPV as all viruses share a common major antigen (Lamien *et al.*, 2011a).

There is more than 95% homology amongst LSDV, SPPV and GTPV (El-Kholy *et al.*, 2008; Tulman *et al.*, 2002). Sequences and phylogenetic analysis have the ability to classify members of the CaPV into GTPV, SPPV and LSDV based on the P32 genomic sequence (Hosamani *et al.*, 2004).

Diagnosis based on clinical signs requires confirmation by rapid laboratory techniques. Polymerase Chain Reaction assay technique (PCR) was the quick and superior method for detection of LSDV from clinical samples in comparison with virus isolation which is time consuming (Awad *et al.*, 2010; Tuppurainen *et al.*, 2005). However, the PCR which based on primers for the viral attachment and fusion protein genes, does not allow differentiation between LSDV and SPPV (Ireland and Binopal, 1998). Therefore, a specific PCR system was developed which specifically amplify LSDV genome and not able to amplify other poxvirus genomes (Stram *et al.*, 2008). The efficacy of DBH used for diagnosis of LSD virus was similar to that of PCR assay (Awad *et al.*, 2010).

The objective of this study is the assessment PCR assay based on specific primers (lsd43U and lsd1262L) for LSD diagnosis under field conditions in Egypt. The Assessment of both PCR assay and dot blot hybridization techniques in detection of the LSDV.

MATERIALS AND METHODS

Clinical samples: Twenty-four dairy cattle located in a private farm belonging to Ismaillia governorate were examined for LSD. Nodular skin lesions (n = 14) were collected from cattle with lacrimation, salivation, nodular eruption all over the body, measured 2-5 cm in diameter, enlarged superficial lymph nodes and variable edema in one or more legs while EDTA blood samples (n = 10) were collected from infected cattle showing fever and few smaller skin nodules on the neck and chest wall. The nucleic acid (DNA) extracted from these samples were used for both PCR and DBH techniques.

Viruses: Pox department, Veterinary Serum and Vaccine Research Institute (VSVRI), Egypt kindly supplied local adapted tissue culture LSDV (Ismaillia 88) and SPPV (vaccine strain). These strains were used as positive controls.

Viral DNA extraction: DNA was extracted from whole blood samples by using whole blood DNA extraction kit spin-column (BioTeke Corporation, China) according to the manufacture's instructions. Briefly, blood samples were mixed with lysis buffer and 20 µL protease K (20 mg mL⁻¹) mix gently, incubate at 72°C in water bath for 10 min. Add 100 µL isopropanol and then overturn to mix thoroughly. The DNA samples (100 µL) were collected after loading on the spin-column of the kit. DNA from skin biopsies and viruses was extracted by using alkaline phenol-chloroform-isoamyl-alcohol (24:1) technique. The samples were stored at -20°C until used as templates for PCR and DBH.

Polymerase chain reaction assay: The primers used were viral attachment protein encoding gene (forward primer TTTCTGATTTTCTTACTAT and reverse primer AAATTATATACGTAAATA

AC, the amplicon size of PCR product is 192 bp (Ireland and Binepal, 1998). The LSD-specific primers used are (#lsd43UGTGGAAGCCAATTAAGTAGA and #lsd1262LTAAGAGGGACATTAG TTCT, the amplicon size of PCR product is 1237 bp (Stram *et al.*, 2008). The PCR was applied in a total volume of 25 μ L containing: 12.5 μ L of 2 $\times$ Taq PCR Master Mix (Biotek Corporation, China); 0.4 μ M (1.0 μ L) of each primer; 3 μ L of the DNA extract and PCR grade water up to 25 μ L. Then, the resulting mixture was carried out in a programmable thermocycler as follow: one cycle of 95°C for 1 min. This was followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 70 sec and a final extension step of 72°C for 5 min (Stram *et al.*, 2008). In each PCR run, a non-template control was included to detect possible external DNA contamination and control positive also used for confirmation.

Ten microliters of each amplified product were analyzed by agarose gel electrophoresis using a 100-bp DNA ladder (Biotek) on 1.5% agarose (Roko, Spain), containing 1 μ g mL⁻¹ ethidium bromide in TBE buffer and visualized and photographed under UV light.

Dot blot hybridization

DNA labeling: Purified PCR product of local adapted tissue culture LSDV (Ismaillia 88) labeled with digoxigenin using nonradioactive labeling kit (Roche, Germany).

Dot blot hybridization technique: DBH was applied according to Southern (1975) and by adding 5 μ L of DNA samples on nitrocellulose membrane, fixed by UV exposure in UV cross linker. Overnight incubation with the labeled probe at 55°C at hybridization incubator was done. DIG-labeled probes that hybridized to a target sequence were detected with an alkaline phosphatase-labeled anti-DIG antibody. Detection of positive samples with phosphatase activity was performed colorimetrically using the coloring agent nitroblue tetrazolium and 5'-bromo-4-chloro-3-indobyl phosphate (X-phosphate) as substrate.

RESULTS

Firstly viral attachment protein encoding gene primers are used for PCR analysis of extracted DNAs of clinical samples collected from clinically infected cattle, local adapted tissue culture LSDV (Ismaillia 88) and vaccinal strain of SPPV. The expected amplicon size of 192 base pairs (bp) is found in clinical samples, LSDV and SPPV. The results are confirmed by DBH (Fig. 1).



Fig 1: Results of DBH (black spots indicated positive amplification of LSDV in clinical samples and local reference strain and SPPV DNA

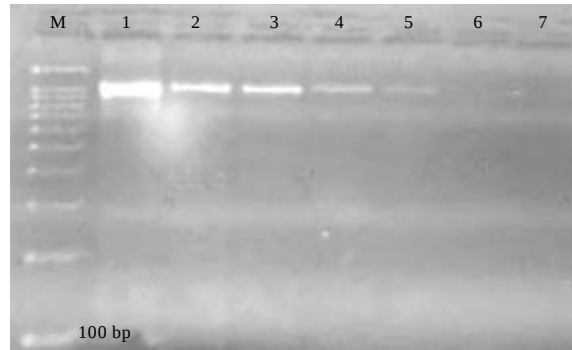


Fig. 2: Amplification of LSDV with LSD-specific primers (lsd43U and lsd1262L): M (DNA ladder 100 bp); Lane 1: Local strain of LSDV (Ismailia 88), Lane 2-5: Positive clinical samples, Lane 6: Negative blood sample and Lane 7: SPPV

Table 1: Detection of LSDV by PCR and DBH form based on viral attachment protein encoding gene primers

Sample/test	Skin biopsies (14)		Blood (10)	
	Positive	(%)	Positive	(%)
PCR	14	100	4	40
DBH	14	100	3	30

LSDV DNA is diagnosed in all skin biopsies (100%) by means of both PCR and DBH while detection rate in febrile blood samples is 40 and 30% by using PCR and DBH respectively (Table 1).

The second step in this study is to assess the specificity of PCR based on LSD-specific primers (lsd43U and lsd1262L) to detect LSDV specifically and not react with SPPV. To this clinical field samples from infected cattle with LSD, local strain of LSDV (Ismailia 88) and vaccine strain of SPPV are tested by LSD-specific primers by PCR. The expected amplicon size of 1237 base pairs (bp) specific for LSDV amplified from clinical samples and local isolate of LSDV (Ismailia 88) and do not support amplification of SPPV (Fig. 2).

DISCUSSION

In spite of the importance of LSD on animal industry its diagnosis was based on clinical signs but this can be confused with pseudo LSD especially in cases with few skin lesions and/or transient fever may be difficult for diagnosis (Barnard *et al.*, 1994). Also confusion with SPPV may occur, as it is rarely transmitted to cattle producing skin lesions (Kitching and Carn, 2008), also (Burdin and Prydie, 1959; Capstick, 1959) reported that an experimental infection of cattle with SPPV can produce similar lesions to LSD. Therefore, molecular techniques should be used for diagnosis of *Capripox viruses* instead of the traditional isolation and identification of the virus (Lamien *et al.*, 2011b).

In this study, two sets of PCR are used to overcome the confusion that occur for diagnosis of these viruses from clinically similar diseases. The first PCR assay set based on viral attachment protein gene and second based on specific LSD primers. The results of the first PCR assay set are

similar for local adapted tissue culture LSDV (Ismailia 88), SPPV and clinical samples at 192 bp. These results indicate the presence CaPV DNA but not able to differentiate between CaPVs and this also was confirmed by Ireland and Binepal (1998). Thus, the use of these primers for PCR assay is not the suitable method to differentiate between these viruses.

The second PCR assay set which based on specific LSD primers that were used for the first time in Egypt to assess their specificity to differentiate between LSDV and SPPV. Figure 2 show that the unique band of the expected size 1237 bp representing for DNA extracted from skin and blood samples of clinically diseased cattle and local adapted tissue culture LSDV (Ismailia 88) and does not detect SPPV DNA. Thus, these primers can be used to differentiate LSDV from SPPV, this PCR assay set was used to differentiate between LSDV from SPPV and confirmed by Stram *et al.* (2008).

Diagnosis of LSD virus is more specific and time saving by using the specific PCR assay set than diagnosis by traditional methods of viral isolation and identification (Awad *et al.*, 2010). Also the long serial passage needed for the virus isolation using cell culture may increase the contamination and failure rate of the cell line for diagnosis of the virus (Van Rooyen *et al.*, 1969).

In current study the sensitivity of PCR assay for detection of clinically infected cases revealed 100% for skin biopsies, this result agree with El-Kholy *et al.* (2008) and Awad *et al.* (2010). The high rate of detection of viral DNA in skin biopsies can be explained by viral tropism to skin tissues and its persistence in high concentration, this supported by Bowden *et al.* (2009), Carn and Kitching (1995), Davies (1991), Tuppurainen *et al.* (2005) and Weiss (1968) who added that the skin biopsies remained positive to LSDV for several months post infection.

The sensitivity of PCR in detection of LSDV DNA in EDTA blood are 40% in samples collected from febrile cases (Table 1), this may be due to presence of the virus in blood at low level of titer and for short period. (Carn and Kitching, 1995) support these results and Tuppurainen *et al.* (2005) who reported that PCR assay was negative for blood samples collected after 15 days post infection.

In the evaluation of DBH for detection of LSDV reveals 100% positive for skin biopsies while in blood samples are 30% positive. In comparison between DBH and PCR for detection of the virus in skin, the results were similar (100% positive). While in blood samples PCR assay result is 40% and in DBH 30%. These results agree with Awad *et al.* (2010). The lower percent of positive result in DBH than in PCR assay is attributed to the high requirement for many copies of DNA to be detected by DBH than that required by PCR (Awad *et al.*, 2010).

CONCLUSION

The use of LSD-specific PCR assay is valuable and more sensitive for accurate and rapid diagnosis of LSD virus; in addition, it can differentiate between LSDV and SPPV without the need for other molecular or traditional techniques, as these methods are less sensitive and time consuming. The best samples used for accurate diagnosis of LSD are Skin biopsies due to the higher concentration of the viral particles.

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