Polymerase chain reaction and Dot blots hybridization techniques for diagnosis of lumpy skin disease in cattle in Egypt

Mahmoud M. ElHaig[1], Abdelfatah Selim[2] and Abdelwahab Shahira[3]

[1&3]Faculty of Veterinary Medicine, Suez Canal University, P.O. Box 41522, Ismailia, Egypt. [2]Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh, Egypt Correspondence author Email: mahmoud_mohyeldeen@vet.suez.edu.eg

----

Abstract

Lumpy skin disease (LSD) is one of the major threats of cattle stock industry in Egypt. There is much 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 Ismaillia, 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 (Ismaillia 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.

Keywords: LSDV, SPPV, diagnosis, PCR, DBH

Introduction

Lumpy skin disease virus is a capripoxvirus (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, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death (OIE, 2010) with morbidity varies from 3 to 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 disease was first described among cattle in May 1988 in Suez and Ismaillia (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 was 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 occurred in Israel (Stram et al., 2008).

LSDV, SPPV and goat poxvirus (GTPV) are members of 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 from cattle, sheep or goats 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. PCR was quick and superior in detecting 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 Binepal, 1998). Therefore, a specific PCR system was developed which specifically amplify LSDV 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. Comparison between PCR assay and dot blot hybridization 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 & 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 20µl protease K (20mg/ml) mix gently, incubate at $72^{o}C$ in water bath for 10min. Add 100µl 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 (Sambrock et al., 1989). The samples were stored at -20 ºC until used as templates for PCR and DBH.

Polymerase chain reaction assay

The primers(5'-3') used were viral attachment protein encoding gene (forward primer TTTCCTGATTTTTCTTACTAT, reverse primer AAATTATATACGTAAATAAC, the amplicon size of PCR product is 192 bp (Ireland & Binepal, 1998) and LSD-specific primers (lsd43UGTGGAAGCCAATTAAGTAGA, lsd1262LTAAGAGGGACATTAGTTCT, the amplicon size of PCR product is 1237 bp,

(Stram et al., 2008). PCR was applied in a total volume of 25μl containing: 12.5μl of 2× Taq PCR Master Mix (Bioteke Corporation , China); 0.4μM (1.0μl) of each primer; 3μl of the DNA extract and PCR grade water up to 25μl. 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 30s, 58°C for 30s and 72°C for 70s and a final extension step of 72°C for 5min (Stram et al., 2008). In each PCR run, a non-template control was included to detect possible external DNA contamination and control positive were used for confirmation.

Ten microliters of each amplified product were analyzed by agarose gel electrophoresis using a 100-bp DNA ladder (Bioteke) on 1.5% agarose (Roko, Spain), containing 1μg/ml 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 Khandijan (1987) by adding 5μl of DNA samples on nitrocellulose membrane, fixed by UV exposure in UV cross linker. Overnight incubation with the labeled probe at 550C 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 choloro-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 (figure 1)

LSDV DNA is diagnosed in all skin biopsies (100%) by means of both PCR and DBH, while detection rate in fevered 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 (Ismaillia 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 (Ismaillia 88) and do not support amplification of SPPV. (Figure 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 Capripoxviruses 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 (Ismaillia 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 1237bp representing for DNA extracted from skin and blood samples of clinically diseased cattle and local adapted tissue culture LSDV (Ismaillia 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 (Prydie and Coackly, 1959 and 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, 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 after15 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).

Conclusions

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.

Acknowledgements

References

Aspden, K., J. A. Passmore, F. Tiedt and A. L. Williamson. 2003. Evaluation of lumpy skin disease virus, a capripoxvirus, as a replication-deficient vaccine vector. The Journal of general virology 84(Pt 8):1985-1996.

Awad, W. S., A. L. Ibrahim, K. Mahran, K. M. Fararh and M. I. Abdel Moniem. 2010. Evaluation of different diagnostic methods for diagnosis of Lumpy skin disease in cows. Tropical animal health and production 42(4):777-783.

Barnard, B. J., E. Munz, K. Dumbell and L. Proesky. 1994. Lumpy skin disease. Vol. 1. Infectious diseases of livestock with Special Reference to Southern Africa. Oxford University Press, Cape Town, South Africa, 1:604–612.

Bowden, T. R., B. E. Coupar, S. L. Babiuk, J. R. White, V. Boyd, C. J. Duch, B. J. Shiell, N. Ueda, G. R. Parkyn, J. S. Copps and D. B. Boyle. 2009. Detection of antibodies specific for sheeppox and goatpox viruses using recombinant capripoxvirus antigens in an indirect enzyme-linked immunosorbent assay. Journal of virological methods 161(1):19-29.

Buller, R. M., B. M. Arif, D. N. Black, K. R. Dumbell, J. J. Esposito, E. J. Lefkowitz, G. McFadden, B. Moss, A. A. Mercer, R. W. Moyer, M. A. Skinner and D. N. Tripathy. 2005. Virus Taxonomy: Classification and Nomenclature of Viruses. Pages 117–133 in The International Committee on Taxonomy of Viruses. Vol. Eighth Report. Elsevier Academic Press, San Diego.

Burdin, M. L. and J. Prydie. 1959. Lumpy skin disease of cattle in Kenya. Nature 183(4666):949-950.

Capstick, P. B. 1959. Lumpy skin disease-experimental infection. Bull. Epizoot. Dis. Afr. 7(51-62).

Carn, V. M. and R. P. Kitching. 1995.The clinical response of cattle experimentally infected with lumpy skin disease (Neethling) virus. Archives of virology 140(3):503-513

Davies, F. G .1991. Lumpy skin disease, an African capripox virus disease of cattle. The British veterinary journal 147(6):489-503.

El-Kholy, A. A., H. M. T. Soliman and K. A. Abdelrahman. 2008. Polymerase chain reaction for rapid diagnosis of a recent lumpy skin disease virus incursion to Egypt. Arab J. Biotechnol. 11:293–302.

Hamoda, F. K., E. A. Aboul Soud, M. S. Magda, M. A. Shahein, A. Michael and A. M. Daoud. 2002. Field and laboratory studies on lumpy skin disease. J. Egypt Vet. Med. Ass. 62(5):183-199.

Hosamani, M., B. Mondal, P. A. Tembhurne, S. K. Bandyopadhyay, R. K. Singh and T. J. Rasool.2004. Differentiation of sheep pox and goat poxviruses by sequence analysis and PCR-RFLP of P32 gene. Virus genes 29(1):73-80.

House J A, T. M. Wilson, S. El Nakashly, I. A. Karim, I. Ismail, N. El Danaf, A. M. Moussa and N. N. Ayoub. 1990. The isolation of lumpy skin disease virus and bovine herpesvirus-4 from cattle in Egypt. Journal of veterinary diagnostic investigation: official

publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 2(2):111-115.

Ireland, D. C. and Y. S. Binepal. 1998. Improved detection of capripoxvirus in biopsy samples by PCR. Journal of virological methods 74(1):1-7.

Kitching, R. P. 2003. Vaccines for lumpy skin disease, sheep pox and goat pox. Developments in biologicals 114:161-167.

Kitching, R. P. and V. M. Carn. 2008. Sheep pox and goat pox. Office international des Epizooties of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). OIE Paris:1058-1068.

Lamien, C. E., C. Le Goff, R. Silber, D. B. Wallace, V. Gulyaz, E. Tuppurainen, H. Madani, H. Caufour, T. Adam, M. El Harrak, A. G. Luckins, E. Albina and A. Diallo. 2011. Use of the Capripoxvirus homologue of Vaccinia virus 30 kDa RNA polymerase subunit (RPO30) gene as a novel diagnostic and genotyping target: development of a classical PCR method to differentiate Goat poxvirus from Sheep poxvirus. Veterinary microbiology 149(1-2):30-39.

Lamien, C. E.,M. Lelenta,W. Goger,R. Silber, E. Tuppurainen, M. Matijevic and G. A. Luckins, A. Diallo. 2011. Real time PCR method for simultaneous detection, quantitation and differentiation of capripoxviruses. Journal of virological methods 171(1):134-140

OIE, T. M. 2010. lumpy skin disease. Office International des Epizooties chapter 2.4.14:1-13.

Stram, Y., L. Kuznetzova, O. Friedgut , B. Gelman,H. Yadin and M. Rubinstein-Guini. 2008. The use of lumpy skin disease virus genome termini for detection and phylogenetic analysis. Journal of virological methods 151(2):225-229.

Tulman, E. R., C. L. Afonso, Z. Lu, L.Zsak, J. H. Sur, N. T. Sandybaev, U. Z. Kerembekova,V. L. Zaitsev, G. F. Kutish and D. L. Rock. 2002. The genomes of sheeppox and goatpox viruses. Journal of virology 76(12):6054-6061.

Tuppurainen, E. S. and C. A. Oura. 2012. Review: lumpy skin disease: an emerging threat to Europe, the Middle East and Asia. Transboundary and emerging diseases 59(1):40-48.

Tuppurainen, E. S., E. H. Venter, and J. A. Coetzer. 2005. The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. The Onderstepoort journal of veterinary research 72(2):153-164.

Van Rooyen, P. J., E. K. Munz and K. E. Weiss. 1969. The optimal conditions for the multiplication of Neethling-type lumpy skin disease virus in embryonated eggs. The Onderstepoort journal of veterinary research 36(2):165-174.

Weiss, K. E. 1968. lumpy skin disease virus. Virology Monographs 3:111-131.

Test	PCR		DBH	
	Positive	%	Positive	%
Blood (10)	**4**	**40**	**3**	**30**
Skin biopsies (14)	**14**	**100**	**14**	**100**

Table1. Detection of LSDV by PCR and DBH based on viral attachment protein encoding gene primers

Figure captions

Figure1. Results of DBH (black spots indicated positive amplification of LSDV in clinical samples and local reference strain and SPPV DNA
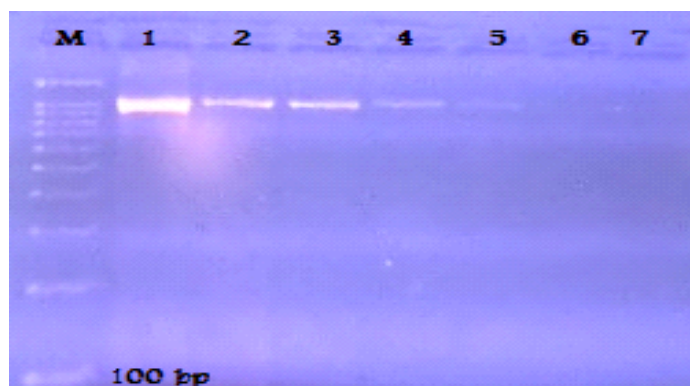
Figure2. Amplification of LSDV with LSD-specific primers (lsd43U and lsd1262L) at (1237 bp)**: M (DNA ladder 100bp) ; Lane 1, local strain of LSDV (Ismaillia 88); Lanes 2-5, positive clinical samples; Lane 6, negative blood sample; Lane 7, SPPV.**