COMPARATIVE STUDY ON MOLECULAR TECHNIQUES FOR LUMPY SKIN DISEASE VIRUS DIAGNOSIS IN SKIN NODULES OF INFECTED CATTLE AND CHORIOALLANTOIC MEMBRANES (CAMS) OF FERTILE EGGS

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ABSTRACT

A total of 22 skin nodules from clinical diseased cattle showed clinical signs suspected to be lumpy skin disease (LSD) were collected during summer 2010. Diseased cattle exhibited multiple skin nodules with or without fever. Polymerase chain reaction (PCR), dot blot hybridization (DBH) and histopathological examination were used for rapid diagnosis of the causative agent in the collected skin samples. The result revealed that viral DNA was detected in all field samples by PCR and in 18 samples by DBH. The intracytoplasmic inclusions detected in 8 samples. Trials for isolation of lumpy skin disease virus (LSDV) from collected samples was carried out via chorioallantoic membranes (CAMs) of ECEs aged 9 days. Four egg passages were carried out for each sample. The isolated virus was identified using the same previously mentioned techniques. After 4th passage in ECEs, both PCR and DBH detected the viral DNA in 16 samples and intracytoplasmic inclusions were detected in 14 samples. Real time PCR (RT-PCR) was used to confirm LSDV diagnosis using SYBR Green mix. The results indicate sensitivity of PCR over DBH in virus detection in field samples, while histopathological examination for detection of LSDV after serial passages in CAMS is preferable than direct detection on field samples. RT-PCR could be used for rapid and specific detection of LSDV nucleic acid.

Key Words: *Lumpy skin disease virus, polymerase chain reaction (PCR), dot blot hybridization (DBH), Real time-PCR, histopathology.*

INTRODUCTION

The genus Capripoxvirus within the subfamily Chordopoxvirinae, family Poxviridae comprises three closely related viruses, namely lumpy skin disease (LSD), sheeppox (SP) and goatpox (GP) viruses. These viruses are the etiological agents of economically important diseases which collectively constitute the most serious poxvirus diseases of production animals. The current criterium used for classifying capripoxviruses within the genus is based upon the animal species from which the virus was first isolated, LSDV from cattle, SPV from sheep and GPV from goat **(Babiuk et al., 2008)**. LSD is an acute, subacute or inapparent viral disease characterized by pyrexia, localized or generalized skin pox

lesions, and generalized lymphadenopathy **(Davies, 1991 and Mercer et al., 2007)**.

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 sever economic losses due to hide damage, loss of milk production, mastitis, infertility and death **(Weiss, 1968)**. LSD is endemic in Central and southern Africa **(Babiuk et al., 2008)**. In Egypt, the LSD was first appeared in 1988 after cattle importation from Somalia **(House et al., 1990)**. Lastly an outbreak was reported in 2006 having been introduced with foot and mouth disease by cattle imported from Ethiopia **(World Animal Health Information Database, OIE)**. Rapid and specific diagnosis of the disease is of vital importance. Following the diagnosis, rapid instigation of control measures such as slaughter, ring vaccination and movement restrictions are required to limit losses **(Carn, 1993)**.

Diagnosis of LSD is initially depending on clinical signs and laboratory diagnosis is based on virus isolation, histopathology and serological methods **(House et al., 1990, Tuppurainen, 2005 and Awad et al., 2009)**. These conventional diagnostic methods are time consuming, laborious and most of them of low specificity. Polymerase chain reaction (PCR) has been described for detection of LSDV and proved to be simple, rapid and specific diagnostic method **(Ireland and Binepal, 1998)**. Recently attention has been directed toward the application of Real time-PCR assays for rapid identification of capripoxviridae **(Bowden et al., 2008)**.

This study was performed to compare the results of PCR, DBH, and histopathology in diagnosis of LSDV in skin samples of infected cattle and in CAMs of embryonated chicken eggs. Also, using of Real time PCR (RT-PCR) to confirm LSDV diagnosis using SYBR Green mix.

MATERIAL AND METHODS

Collection of samples:

A total of 22 skin nodules from clinically diseased cows that were suspected to be infected with lumpy skin disease (LSD) were collected during summer 2010. Diseased cattle exhibited multiple skin nodules (figure 1) with or without fever. Part of each sample was taken in dry bottle containing phosphate buffer saline (PBS) for virus isolation, PCR, DBH and Real time-PCR. Another part was put in bottles containing neutral buffered formalin 10% for histopathological examination. Skin biopsies from three normal cows were included as negative controls.

Virus strain:

Tissue culture adapted LSDV/Ismailyia88 strain was kindly supplied from the Pox Vaccine Production and Research 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. This strain was used as a positive control.

Preparation of skin nodules for virus isolation:

Skin samples preparation was performed as described by **(OIE, 2004)** as follows: each sample was minced using sterile scissors and forceps and then homogenized in a mortar

containing sterile sand with a pestle. Phosphate buffered saline containing antibiotics (1000 U/ml penicillin, 1000µg/ml streptomycin and 500 µg/ml gentamycin) was added, making up a 20% (W/V) suspension. The suspension was frozen and thawed three times and centrifuged at 3000 r. p.m. for 10 min. The supernatant was collected and stored at -20°C till used.

Inoculation of prepared sample into embryonated chicken eggs (ECEs):

Inoculation of each prepared sample on chorioallantoic membranes (CAMs) of 9th days old ECEs was carried out according to **Van Rooyen et al., 1969**. The air space was created beneath the lateral walls of the egg by sucking the air through the blunt end of the egg with the help of teat. A volume of 0.1 ml of the inoculum was inoculated through the hole created over the air space. The holes were sealed with the help of the molten wax. The inoculated eggs were horizontally incubated for 6 days at 35°C. Finally, the CAMs were harvested aseptically and washed then examined for pock lesions. Four passages were carried out for each sample. After appearance of pock lesions, virus was identified using PCR, DBH and histopathology.

Polymerase chain reaction (PCR):

PCR was used to identify LSDV in field and egg passaged samples.

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 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 as described by **Viljoen et al., (2005)** using 0.5 mL of field samples and CAMs suspension digested with 20 µL Proteinase K (final concentration, 100µg/mL) at 56°C for 2 h. 100 µL Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and mixed by inversion then centrifuged at 13 000 r/min 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 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 r/min) for 15 minutes. The pellet was washed once with 70% ethanol and centrifuged at 12000 r/min for 10 min then air dried and resuspended in 50 µL TE buffer. Normal non-infected skin and CAM samples were included as a negative control sample.

Amplification of extracted DNA by PCR:

This was carried out as described to **Ireland and Binepal, (1998)**. Briefly, 10 µl sample of extracted DNA of each sample was placed in 50 µl of the final volume of 10 x re-

action mixture containing 50 mmol/L KCl, 10 mmol/L Tris–HCl (pH 8.3), 1.5 mmol/L MgCl2, 200 mmol/L of each dNTP, 100 pmol of each oligonucleotide primer and 2 U Taq-DNA polymerase. Then 40 ?L of mineral oil was added to prevent evaporation of components during thermocycling. The PCR had an initial cycle of 94°C for 5 min, followed by 34 cycles of 94°C for 1 min, 50°C for 30 s, 72°C for 1 min and a final elongation step of 72°C for 5 min.

Amplified product analysis

This was carried out according to **Viljoen et al., (2005)**. Briefly 10 µL of the amplified PCR product was mixed with 1 µL 10 x 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 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.

Dot blot hybridization (DBH):

DBH was applied to detect LSDV in field and egg passaged samples. Labelled DNA probe was prepared from the purified PCR product of the reference LSDV/Ismailyia 88 strain by ethanol precipitation according to **Sambrook et al., (1989)** and labeled with digoxigenin using nonradioactive labeling kit (Roche, Germany) for labeling of DNA as described in the instructions of the manufacturer's manual. Hybridization technique was applied according to **Khandijan, (1987)** by dotting 10 µl of DNA template on nitrocellulose membrane, following by denaturation step using denatured solution then fixation in UV crosslinker (Spectrolinker XL-1000, Spectronics cooperation, USA). Overnight incubation with the labeled probe at 55°C at hybridization incubator (LAB-line Instruments, USA) 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 nitro-blue tetrazolium and 5'-bromo-4 choloro-3-indobyl phosphate (X-phosphate) as substrate.

Histopathological examination:

Histopathological sections were carried out by fixing part skin nodules and CAMs in 10% neutral buffered formalin solution. The fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, cleaned in xylene, embedded in paraffin then sectioned (5 micron) and stained with hematoxyline and eosin according to **Bancroft et. al., (1998)**. The stained sections were examined microscopically.

Real time-PCR:

Three samples (two field samples and one CAM of ECE) were selected for confirmation of LSDV diagnosis by real time -PCR as described by Viljoen et al., (2005) as follows: It was performed in a final volume of 20 ul reaction mix containing 9 ul PCR grade water, 4 ul SYBR green master mix, 1 ul of 10 uM forward and reverse primers and 5 ul of extracted DNA. Cycling parameters were the following: 95°C for 10 min followed by 35 cycles of 95°C for 30 sec and 55°C for 30 sec and at 72°C for 1 min.

RESULTS & DISCUSSION

Viral DNA was detected in all 22 skin nodules collected from cattle using PCR and in 18 samples by DBH. The amplicon size of PCR product in positive samples had a molecular weight of 192 bp (Figure 2) and positive result of DBH was visualized as colour development. Concerning histopathological examination, intracytoplasmic inclusions detected in 8 crude skin samples (36.4%) (Figure 3). Inflammatory cells infiltration in dermis and hypodermis. The dermal fibrous connective tissue was hyalinized in some sections. In old lesions, necrosis involved all layers of the epidermis was observed.

Lumpy skin disease virus (LSDV) was isolated from 16 (out of 22) collected skin biopsies in percentage of (72.7%) and showed the characteristic pock lesions after four egg passages (figure 4). Identification of the isolated virus after the fourth passage revealed that both PCR and DBH detected the viral DNA in 16 samples. After 4^{th} passage in ECEs, intracytoplasmic inclusions detected in 14 samples (Figure 5). Table 1 summarizes results from the used diagnostic assays.

Concerning results of real time-PCR, three tested samples were positive, with threshold cycles (Ct): 23, 25 and 32 (Figure 6). No amplification was detected from negative non template control (NTC).

Capripoxviruses cause a severe and highly contagious disease in sheep, goats and cattle. LSD can be confused with skin lesions of pseudo-lumpy skin disease (caused by bovine herpes virus-2), insect bite, Demodex infection, onchocerciosis, besnoitiosis and dermatophilosis. So LSD needs a rapid and specific laboratory diagnosis after being suspected for rapid performing of control measures **(Esposito and Fenner, 2001 and Carn, 1993)**.

PCR, DBH and histopathological examination were used for detection of LSDV in 22 collected skin lesions serve as a rapid, effective and economic method for laboratory confirmation of disease. Viral DNA was detected in all skin biopsies collected from cattle by PCR and in 18 samples by DBH. This indicate sensitivity of PCR over DBH in virus detection in field samples and this results are in partial agreement with **Awad et al., 2009**, this may be due to low concentration of viral nucleic acid in some field samples and amplification of viral DNA in case of PCR but in DBH the viral DNA was blotted without amplification.

The result revealed that the characteristic pock lesions appeared in 16 out of 22 samples after inoculation on CAMs of ECEs after fourth passage as this passage is sufficient for appearance of pock lesions on CAMs as reported by **El-Kenawy and El-Tholoth, 2009**. These results were sustained by **Hassan et al., (1992), Ismael, (2000), Hamoda et al., (2002), Ahmed et al., (2005) and El-Kenawy and El-Tholoth et al., (2009)** who observed pock lesions on CAMs of inoculated ECEs and the lesion of the virus was maintained by serial passages. On the other hand Van **Rooyen et al., (1959)** and **Hassan, (1993)** failed to detect macroscopic lesions on CAMs of inoculated ECEs.

Identification of the isolated virus after the fourth passage revealed that both PCR and DBH detected the viral DNA in 16 samples

lower than numbers of samples detected directly in field samples. So detection of LSDV in skin samples is superior over detection after serial passages in ECEs. This may be due to high concentration of virus in skin lesions and poor replication of virus in CAMs **(Van Rooyen et al., 1959)**. This mean that PCR and DBH could be used in detection of LSDV in skin biobsy and isolated virus in CAMS. The finding is in concurrence with previous reports who recorded that PCR could be used in detection of capripoxvirus in biobsy samples, tissue culture and semen **(Tuppurainen, 2005)**, skin and blood samples **(Awad et al., 2009)** and in CAMs of ECEs **(El-Kenawy and El-Tholoth, 2010)**. The result, also, is in agreement with **Awad et al., 2009** who succeeded in identification of LSDV in skin samples by DBH.

Histopathological examination revealed intracytoplasmic inclusions in 8 skin samples (36.4%). While after 4th passage in ECEs, intracytoplasmic inclusions detected in 14 samples (63.6%), so histopathology for detection of the virus after the fourth passages in CAMS is superior to direct detection on field samples. The pathological changes in skin and CAMs sections were similar to those reported by **Van Rooyen et al., 1969, Bida, 1977, Ahmed et al., 2005 and Aly et al., 2006**.

Using of Real time-PCR for confirmation of LSD diagnosis is preferable as it do not need gel electrophoresis after PCR amplification and more rapid than conventional PCR (**Bowden et al ., 2008**).

Figures:

1- Skin nodules of LSD in infected calf.

2- PCR products of the attachment gene (192 bp) of LSDV DNA extracted from skin lesions in stained agarose gel electrophoresis, along with 100 bp DNA ladder (M). Lane 1: negative control sample. Lane 2, 3& 4 : The amplified DNA products prepared from skin nodules. Lane 5: positive control sample.

3- Skin section, showing intracytoplasmic inclusion bodies in the epidermal cells. H & E (original magnification X 520).

4- Characteristic pock lesions on CAM infected with isolated virus from skin lesion on the fourth passage.

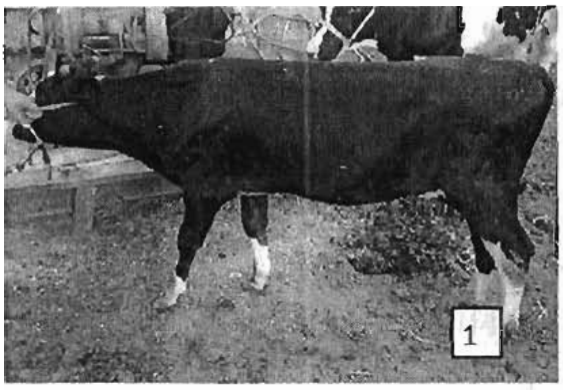
5- CAM section showing intracytoplasmic inclusion bodies. H & E (original magnification X 520).

6- Amplification plot of Real time-PCR in LSDV identification with Ct, 23.

Table 1: Comparative results of the virus identification in field samples and after 4th passage using PCR, DBH, and histopathology:

	Diagnostic tests		
	PCR	DBH	Histopathology (Presence of ICIB) *
Number positive (percent positive) in field sample. (n=22)	22 (100%)	18 (81.8 %)	8 (36.4 %)
Number positive (percent positive) after 4th passage in ECE. (n=22)	16 (72.7 %)	16 (72.7 %)	14 (63.6)

* ICIB = Intracytoplasmic inclusion bodies .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M
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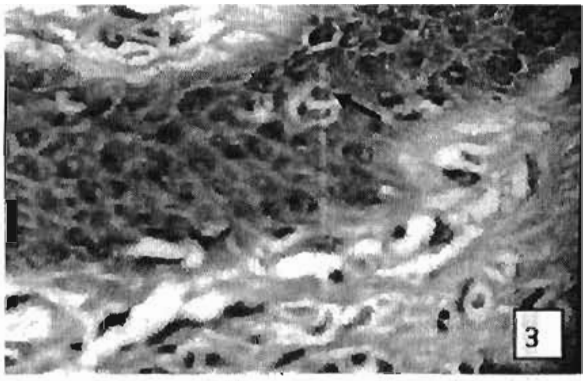
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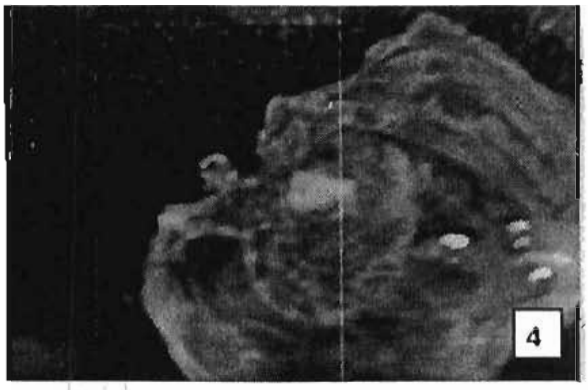
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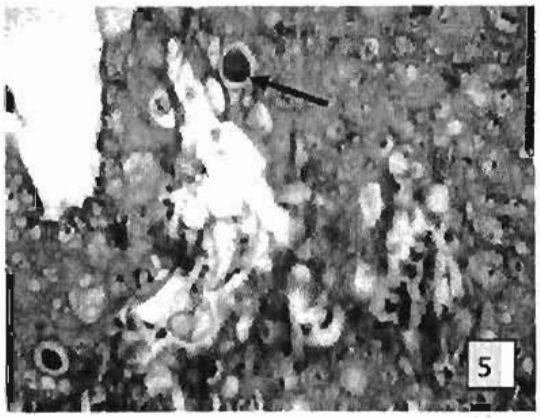
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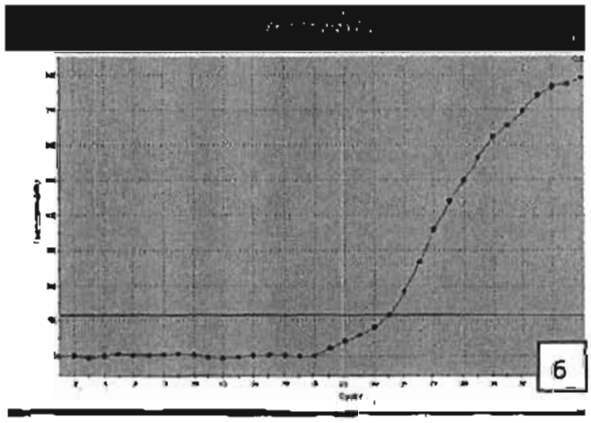
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الملخص العربى

دراسة مقارنة على استخدام التقنيات الجزيئية لتشخيص فيروس مرض الجلد العقدى بعقيدات جلد الابقار المصابة و الغشاء اللقانقى المشيمى للبيض المخصب

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استهدفت هذة الدراسة التشخيص الجزيئى لهذا الفيروس فى الابقار المصابة طبيعيا. حيث تم تجميع عدد 22 عينة من عقيدات الجلد من الابقار المصابة وتم استخدام تفاعل البلمرة المتسلسل والتهجين بعد التلطيخ التنقيطى والفحص الهستوباثولوجى للتشخيص السريع للمرض. تم عزل الفيروس على الغشاء اللقانقى المشيمى لبيض الدجاج المخصب و اجراء اربع تمريرات لكل عينة ثم تعريف الفيروس المعزول باستخدام الاختبارات السابق ذكرها. واخيرا استخدام تفاعل البلمرة المتسلسل (الترقيت الفعلى) لتأكيد تشخيص المرض.

و قد خلصت التجارب الى النتائج الاتية: تم الكشف عن و جود الحامض النووى لفيروس مرض الجلد العقدى فى جميع العينات الحقلية المجمعة بستخدام تفاعل البلمرة المتسلسل وفى 18 عينة باستخدام التهجين بعد التلطيخ التنقيطى و ظهور الاجسام الاحتوائية السيتوبلازمية فى 8 عينات. بينما بعد اجراء اربع تمريرات فى بيض الدجاج المخصب تم الكشف عن وجود الحامض النووى للفيروس فى 16 عينة بستخدام كلا من تفاعل البلمرة المتسلسل والتهجين بعد التلطيخ التنقيطى وظهور الاجسام الاحتوائية السيتوبلازمية فى 14 عينة. و هذة النتائج تدل ان تفاعل البلمرة المتسلسل اكثر حساسية من التهجين بعد التلطيخ التنقيطى والفحص الهستوباثولوجى للكشف عن الفيروس فى العينات الحقلية بينما يكون الفحص الهستوباثولوجى بعد اجراء التمريرات الاربعة فى بيض الدجاج المخصب افضل من اجراءة على العينات الحقلية. كما امكن تاكيد تشخيص الفيروس باستخدام تفاعل البلمرة المتسلسل (الترقيت الفعلى) فى عينات الجلد الحقلية وكذلك فى الغشاء اللقانقى المشيمى لبيض الدجاج المخصب.