DETECTION OF LUMPY SKIN DISEASE VIRUS ANTIGEN AND DNA IN FORMALIN FIXED PARAFFIN EMBEDDED TISSUES FROM A NATURAL OUTBREAK IN EGYPT

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ABSTRACT

An epidemic of Lumpy skin disease (LSD) affecting 16 provinces in Egypt was reported during 2006. Formalin fixed tissues including biopsies and postmortem tissue samples were collected from calves showed typical clinical signs of LSD from a private dairy farm in Damietta province, Egypt a period from March to May, 2006. Formalin fixed paraffin embedded tissue samples (FFPET) were assessed using histopathology as the skin lesions were classified into either acute or subacute-chronic. Both LSD viral DNA detected by polymerase chain reaction (PCR) of extracted DNA and LSD antigen detected by immunohistochemistry (IHC) using a capripoxvirus specific monoclonal antibody were observed in the acute skin lesions and in some subacute-chronic skin lesions.

Key words: *Lumpy skin disease virus; formalin fixed paraffin embedded tissues; Egypt*

INTRODUCTION

Lumpy skin disease (LSD) is an economically important infectious and occasionally fatal disease of cattle **(Coetzer et al., 1994; Davies, 1991; Fenner, 1996)**. LSD viruses (LSDV) as well as sheeppox and goatpox viruses (SGPV) belong to the genus Capripoxvirus within the subfamily Chordopoxvirinae of the family Poxviridae **(Buller et al., 2005)**. Collectively, these viruses caused the most serious poxvirus diseases in production animals. The disease was characterized by rapid eruption of multiple circumscribed skin nodules, and generalized lymphadenitis and fever and may result in mastitis and orchitis **(Coetzer, 2004)**. Other lesions, visible at postmortem examination, include necrotic plaques in the membranes, chiefly of the upper respiratory tract, the oral cavity and lungs. LSD was first described in northern Rhodesia in 1929 **(MacDonald, 1931)**. The first outbreak in Egypt occurred in 1988 in and around Suez and Ismailia **(Ali et al., 1990)**. The disease reappeared during the summer of 1989 in 22 out of 26 Egyptian governorates causing 2% morbidity of the whole

cattle population **(Davies, 1991)**. A severe LSD outbreak again struck foreign and local cattle populations in 16 provinces including Damietta, Egypt during 2006 (http// www.oie.int./eng/norms/mcode/A 00036. htm). Here we describe the detection of LSD viral DNA and antigen in some FFPET samples by PCR and by using monoclonal antibody as a primary antibody in imported (Holstein) and native breeds of calves following the natural outbreak in Egypt.

MATERIALS AND METHODS

In a private dairy farm (outdoor system), Damietta province located near to quarantine (Sanad's farm), a disease clinically resembling LSD was affecting calves from native and imported breeds during March to May 2006. Skin biopsies comprising epidermis and dermis of the nodular skin lesions were collected from six -three- month old female calves (numbers=1-6) clinically ill for 2 weeks. The two necropsied calves were 3-4 month old (numbers=7-8) (1 female and 1 male). Samples from skin nodules, lungs, superficial lymph nodes, heart, liver and spleen were fixed in 10% neutral formalin for one week and submitted for examination. Table (2) displays a summary of calf number, age, sex, breed and the obtained samples.

After receiving the samples, they were processed at once then embedded in paraffin wax until they were assessed for diagnostic evaluation. All blocks were sectioned and stained with hematoxylin and eosin (HE) for microscopic examination.

At the time of IHC application, samples of all tissues were sectioned and picked up on charged slides. The monoclonal antibody F80G5 was generated using E.coli expressed capripoxvirus ORF 057, a viral core protein, was used for detection of LSD antigen. The monoclonal antibody was titrated at 1: 500, 1:1000 and 1:1500 dilutions to determine the appropriate dilution. 3% hydrogen peroxide was used as a blocker. The antigen retrieval method, dilution, incubation temperature & duration, secondary antibody and chromogen were summarized in Table (1). Sections were counterstained with Mayer's hematoxylin then dehydrated, cleared and mounted. Slides from bovine cutaneous papilloma were used as a negative control and were stained with each set of test slides. The laboratory methods were carried out at Department of Veterinary Pathology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan.

DNA was extracted from 12 FFPET blocks, each contained one of the following samples skin, subcutis, lymph node and lungs using DNA tissue kits (TAKARA Bio Inc Shiga, Japan) according to the manufacturer's instructions. 1-3 sections were freshly cut from each paraffin block at 5?m thick then placed directly into 1.5 ml micro-centrifuge tubes into which 0.5 ml (10-12 drops) of Takara DEXPAT lysis buffer was added. The micro-centrifuge tubes were incubated at 100°C for 10 minutes after piercing the cover lid with sterile new 23 gauge needles and being covered with aluminum foils. Centrifugation was done at 12,000 g for 10 minutes at 4°C then lysate was aspirated from layers just under the paraffin cap and transferred to new fresh tubes and immediately stored at -20°C until time of PCR assay. A denaturation step just prior to PCR amplification was performed by

incubating 10 µl of extract at 95°C for 5 minutes in a thermal cycler followed by immediate chilling and addition to the PCR solution.

The PCR primers were specific for the viral attachment protein encoding gene (32) and had the following sequences: forward primer 5'-d TTTCCTGATTTTTCTTACTAT3' and reverse primer 5'-d AAATTATATACGTAAATAAC 3' (Ireland and Binepal, 1998). The size of the amplicon was 192 bp (Ireland and Binepal, 1998). A DNA amplification was carried out in a final volume of 50 µl containing: 5 µl of 10x PCR buffer, 4 µl of four dNTP (10nM), 0.4µl of forward primer, 0.4 µl of reverse primer, 10 µl of DNA template after being incubated at 95°C for 5 minutes, 0.2 µl of Taq DNA polymerase and 30 µl of nuclease free water. The samples were placed in a thermal cycler (BIORAD icycler ™): first cycle 95°C for 2 min initial denaturation step, second cycle: 95°C for 45seconds, 50 °C for 50 seconds and 1 minute at 72°C. The second cycle was repeated 45 times. Last cycle: 72°C for 2 minutes (a final elongation step to complete the extension of the primers) (OIE Terrestrial Manual, 2008). Amplified products were analyzed using a 100 bp DNA ladder (Whitehead Scientific Ltd) as a molecular marker on 1.5% agarose gels in TBE buffer. Amplicons were visualized using an UV trans-illuminator at a wavelength of 590 nm and positive reactions were confirmed according to size.

RESULTS

90/400 of calves (22.5%) were affected. Clinical examination showed fever, decreased body weight, increased salivation, oval to circular skin nodules either singular or multiple covering the body surface and varying in size from several millimeters to 2-4 cm in diameter Fig. (1&2). On section the nodules were white with multiple hemorrhages. Central areas of some skin nodules were indurated in 1 case forming sitfasts. There was edema in lower limbs with enlarged peripheral lymph nodes (LN) 3-5 times more than normal size in all cases with the presence of pin-point hemorrhages in their cut section in some cases Fig. (3). Subcutaneous brisket edema was observed in one calf. Nasal epistaxis was observed in one calf due to the presence of few ulcerated pox lesions in nasal mucosa and in some cases respiratory signs were seen. A high mortality rate of 39% 35/90 was observed in affected animals. In 2 necropsied animals, following removal of the skin, congestion, hemorrhage and necrosis were observed in the subcutaneous tissues and thoracic wall after removal of skin adjoining areas of skin nodules. Lungs in one calf showed the presence of circular to oval grey nodules Fig. (4), with hydrothorax and areas of fibrinous adhesions detected in the thoracic cavity while in the other animal, chronic interstitial pneumonia was found. No gross lesions were found in the other organs.

Histological lesions of skin nodules were classified into acute, subacute and chronic. The acute stage was seen in 1 case (number 7) which showed minimal hydropic degeneration and round to oval eosinophilic intracytoplasmic inclusion bodies (EICIB) in keratinocytes and epithelium of hair follicles Fig. (5&6). Macrophages with EICIB infiltrating the dermis and necrotic subcutaneous tissues were observed Fig. (7&8). There was prominent superficial and deep dermatitis characterized by necrosis, mononuclear cell infiltra-

tion Fig. (9), extensive edema and neutrophilic peri-vascular reaction with some hemorrhage, vasculitis and thrombosis. Severe and diffuse infiltration with inflammatory cells mainly neutrophils, were observed in the underlying muscular tissue. Subacute and chronic stages showed mild to moderate inflammatory reaction in dermis of 6 skin nodules (numbers 1, 3, 4, 5, 6 & 8) with more severe fibroplasia and more eosinophils and mast cells. The inflammatory cells aggregated in small pockets inside the fibrotic areas Fig. (10) while significant changes in epidermis and EICIB were not seen. The center of the indurated nodules or sitfast was seen in 1 case (number 2) and was characterized by diffuse caseation necrosis. Lymph nodes collectively showed lymphoid hyperplasia, sinus catarrah, histiocytosis, perivascular fibrosis and edema. Lungs showed necrotizing pneumonia in 1 animal (number 7) in which basophilic bacterial colonies were observed. In the other animal (number 8) non suppurative chronic active interstitial pneumonia was detected. Non specific reactions were seen in spleen, heart and liver in 2 necropsied animals. The spleen displayed lymphoid hyperplasia, congestion and hemorrhage. In the heart perivascular fibrosis, edema and degeneration in cardiac muscle fibers, with mononuclear cell infiltration besides, the occasional presence of sarcocysts inside cardiac muscle fibers were observed. The liver displayed degenerative lesions and mononuclear cells aggregation.

The optimum dilution of monoclonal antibody was 1:500. LSD antigen stained most clearly with DAB as brown color. In skin nodules, positive IHC reaction was detected in epidermis and dermis of 3 samples; 1 sample from skin nodule with acute stage and 2 samples from skin nodule with subacute – chronic stages Fig. (11). In acute and subacute to chronic cases, the distribution of IHC staining in skin nodules is similar as the epidermal cells and macrophages infiltrating the dermis were the only kind of cells showed the positive reaction meanwhile, the intensity of IHC staining is slightly deeper in skin nodule with acute stage than those with subacute-chronic stages. In subcutis, positive reaction was also recorded mainly inside macrophages Fig. (12), while a weak positive reaction was seen inside macrophages infiltrating parenchyma of lymph nodes (0-3 cells per field) Fig. (13). No staining was observed in lungs, liver, spleen and heart. Table (2) summarizes the IHC results. Using the autoclaving method for antigen retrieval in a high pH (9) retrieval solution at 121ºC for 10 minutes was the most effective in demonstrating LSD antigen particularly in skin samples.

DNA was extracted from 12 specimens and was subsequently used in a PCR assay to detect LSD genome. The specific primers set amplified a DNA fragment of 192 bp equivalent to the expected amplification product (amplicon) size from LSD. Six samples were positive for this PCR assay including 3 blocks from skin nodules, 1 block from subcutaneous tissue and 2 blocks from superficial lymph nodes. None of the negative controls produced any amplicon Fig. (14). The PCR results are summarized in Table (2).

DISCUSSION

Several reports have previously described diagnostic methods for detecting LSD following either experimental **(Babiuk et al., 2008)**

or natural infection (**Al-kholy et al., 2008**). PCR has been previously demonstrated for LSD genome detection in skin lesions and blood. However detection of LSD by PCR following extraction from FFPET has not been previously described. Immunohistochemistry has been developed for detecting LSD antigen in skin nodules from experimentally infected Holstein calves using a monoclonal antibody generated to a capripoxvirus viral core protein (ORF 057) expressed in Escherichia coli (**Babiuk et al., 2008**).

This work represents the first description of the possibility of detection of LSD viral antigen and DNA in FFPET samples. Effective control of LSD requires rapid and accurate laboratory diagnosis supported by clinical findings **(Tuppurainen et al., 2005).** In our findings, gross and microscopic pathology were similar to those previously described by **(Barnard et al., 1994; House et al., 1990)**. Clinical signs and gross pathology of LSD were found to range from mild with only a few secondary skin nodules to generalized infection of varying severity. Unknown genetic factors were thought to influence the disease severity **(Babiuk et al., 2008)**. Until the last few years, the exact pathogenesis of lesions development associated with LSD has not been as well understood as the pathogenesis of sheeppoxvirus prototype member of the capripoxvirus genus **(Fenner et al., 1987)**. Recently, **Babiuk et al., (2008)** mentioned that the pathogenesis and tissue tropism of LSD showed some similarities to capripoxvirus infections in sheep and goats caused by SGPV **(Bowden et al., 2008)**. The similarity included a marked lymphadenopathy in absence of high titers in lymphoid tissue. Moreover, the immunological-pathological process responsible for lymphadenopathy is likely similar in LSD as well as sheeppox and goatpox. Indeed, the lesions in superficial lymph nodes were characteristically similar in our findings. Multiple organ involvement has been observed in naturally infected cattle **(Woods, 1988).** However, lung lesions were not seen in calves experimentally infected with LSD **(Kitching and Taylor 1985; Bowden et al., 2008)**. This observation may be explained by the presence of additional stresses on cattle in the field may exacerbate the disease **(Babiuk et al., 2008)**. In this destination, lungs of one necropsied animal showed non suppurative chronic active interstitial pneumonia as been described by **(Annandale et al., 2010)**, while lungs of the other animal showed the presence of necrotizing lesions corresponding to gross grey nodules. Grey pink nodules with caseous necrotic cores were previously reported in muscles, subcutis, lungs and other organs of animals infected with LSD **(Geering et al., 1995).** LSD could be differentiated clinically from pseudo-lumpy skin disease (bovine herpes mammilitis; herpesvirus 2) by the fact that the lesions of the second involve only the epidermis and leave a scab after sloughing and systemic signs do not develop (**Geering et al., 1995**). LSD might be differentiated histologically from urticaria, insect and tick bites, or insect stings by the absence of eosinophils and the presence of a deep vasculitis and from herpesvirus, cutaneous lymphosarcoma, streptothrichosis and tuberculosis by the presence EICIB in keratinocytes and germinal cells of affected hair follicles and sebaceous glands in early lesions **(House et al., 1990).** However, it is impossible to diagnose LSD histologically in cases of absence of severe

inflammatory and necrotic changes and EICIB as in the subacute and chronic stages of the disease. **Brenner, et al., (2006)** reported that the presence of intra-cytoplasmic inclusion bodies in LSD was occasional and they were found in many cell types only in early lesions **(Prozesky and Barnard, 1982, Yager and Scott 1985)**. In addition, however, vasculitis and thrombosis, leading to edema and necrosis were central to the pathogenesis of the lesions in LSD (**Prozesky and Barnard 1982),** these findings were observed only in 1 skin nodule sample. The variation in histological findings of LSD among infected cattle has been also described by **(Tuppurainen et al., 2005)**.

Immunohistochemistry using monoclonal antibody to a virion core protein with antigen retrieval in a high pH (9) retrieval solution at 121ºC has been previously reported by (**Babiuk et al., 2008)** and it appeared effective in demonstrating LSD antigen particularly in skin samples. LSD antigen was detected in some samples (from foreign breeds) inside cytoplasm of epidermal cells, interstitial macrophages infiltrating dermis, subcutaneous tissue and parenchyma of lymph nodes.

PCR is the test of choice for rapid detection and identification of LSD as the causative agent in a LSD outbreak **(Al-kholy et al., 2008)**. PCR successfully detected LSD in 6/12 FFPET samples (from foreign breeds) as been showed in (Table 2) after being subjected to a denaturation step just prior to PCR amplification. **Bonin et al., (2003)** mentioned that it was possible to amplify longer sequences ranging up to 300 bases from postmortem tissues, with no modification to the usual DNA extraction procedures through a pre-PCR restoration treatment by filling single strand breaks, followed by a vigorous denaturation step. However, our samples were mostly biopsies or obtained shortly after necropsy no amplification occurred without the addition of a denaturation step. This may be due to the use of un-buffered formalin or from the long time preservation of samples in paraffin blocks until time of PCR application (4 years, from 2006 until 2010). Although many sources for virus detection were reported such as blood, semen and milk, skin biopsies are likely the best choice to sample since they contain greater numbers of viral particles facilitating easier detection by PCR **(Tuppurainen et al., 2005; Babiuk et al., 2008).** The PCR used in this work showed high specificity as a unique band of the expected size (?192 bp) was obtained for 3/8 DNA samples derived from skin nodules, in 2/2 lymph nodes and in 1 subcutaneous tissue sample. In this study, it is not fully understood why LSD antigen and DNA were expressed in some samples but not in all by IHC and PCR respectively. The possible explanations for this might be different breeds or different stages at which skin nodules were collected. **Tuppurainen et al., (2005)** found a variable persistence of the PCR positive result in skin biopsies from experimentally infected bulls as LSD viral DNA was demonstrated in skin biopsies of four bulls until days 92 85, 25 and 18 post infection (p.i). To the best of our knowledge, there were no previous reports describing detection of LSD virion by PCR after being extracted from FFPET.

Finally, we could conclude the following:

* LSD in this destination varied from mild

with few secondary skin nodules to generalized infection of varying severity, and was characterized by morbidity with mortality mainly among calves.

* PCR and IHC can be used for diagnosis of LSD in FFPET samples on condition that there are several samples from acute, subcute to chronic stages of the disease.
* The results obtained by IHC were compatible with those of PCR.
* It is recommended to add a denaturation step before PCR application especially if the used formalin is not or improperly buffered, also to use autoclaving antigen retrieval method (121ºC /10 min) for IHC.
* The applied monoclonal antibody found to be able to detect LSDV antigen in some skin nodules from animals with acute and subacute to chronic forms of the disease.
* LSD caused the most serious lesions in foreign breeds as it is depicted to become endemic in Egyptian native breed.
* The difference in breed and immune status of calves may have a role on the variation of histological lesions, expression of LSD antigen and DNA by IHC and PCR respectively among samples. **Amills et al., (1998)** thought that the genetic resistance as determined by major histocompatibility complexes found on cell surfaces of individuals is responsible for different animal responses to infection with LSDV. The genetic variation in LSD viruses is not been thought to have a role on the fore-mentioned since the virus has a very limited genetic variation **(Kara et al., 2003)**.
* The detection of viral antigen and DNA in samples from lymph nodes indicated that lymph nodes could be taken as samples with skin nodules for diagnosis of LSD during systemic infection.

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Table 1. Antigen retrieval method, dilution, incubation temperature & duration, secondary antibody and chromogen.

Antigen retrieval	Dilution	Incubation Temperature & duration	Secondary antibody	Chromogen
Actinase E	0.1%	37°c/10 min	Envision+anti-mouse HRP*	ImmPACT™ DAB
Pronase	Ready to use	Room temp/5min	(LSAB+System-AP+ Fuchsin chromogen)†	
Target retrieval solution (pH 9)®	1:10	121°c /10 min	Envision+anti-mouse HRP	ImmPACT™ DAB

Min =minutes; temp = temperature Actinase E was from FUNAKOSHI corp. Pronase was from ImmunoBioScience Corp.

• was from DAKO (Carpinteria,CA). DAB was from Burlingame, CA USA. †was from DAKO (Carpinteria,CA) ® was from DAKO Denmark A/S

Table 2: Comparison between the results of PCR and IHC

Case No.	Sex	Age	Breed	Assessment of histological lesions	Samples obtained	PCR	IHC
1	♀	3 m	Holstein	Subacute-chronic	Skin biopsies	-	-
2	♀	3 m	Holstein	Sitfast	Skin biopsies	ND	-
3	♀	3 m	Native	Subacute-chronic	Skin biopsies	-	-
4	♀	3 m	Native	Subacute-chronic	Skin biopsies	-	-
5	♀	3 m	Holstein	Subacute-chronic	Skin nodules	+	+
6	♀	3 m	Native	Subacute-chronic	Skin nodules	-	-
7	♀	3 m	Holstein	Acute	Skin nodules Subcutis Lungs Lymph nodes Heart Liver	+ + - + ND ND	+ + - + - -
8	♂	4 m	Holstein	Subacute-chronic	Skin nodules Lungs Lymph nodes Heart Spleen Liver	+ - + ND ND ND	+ - + - - -

♀female, ♂male, m month, + positive - negative ND not done

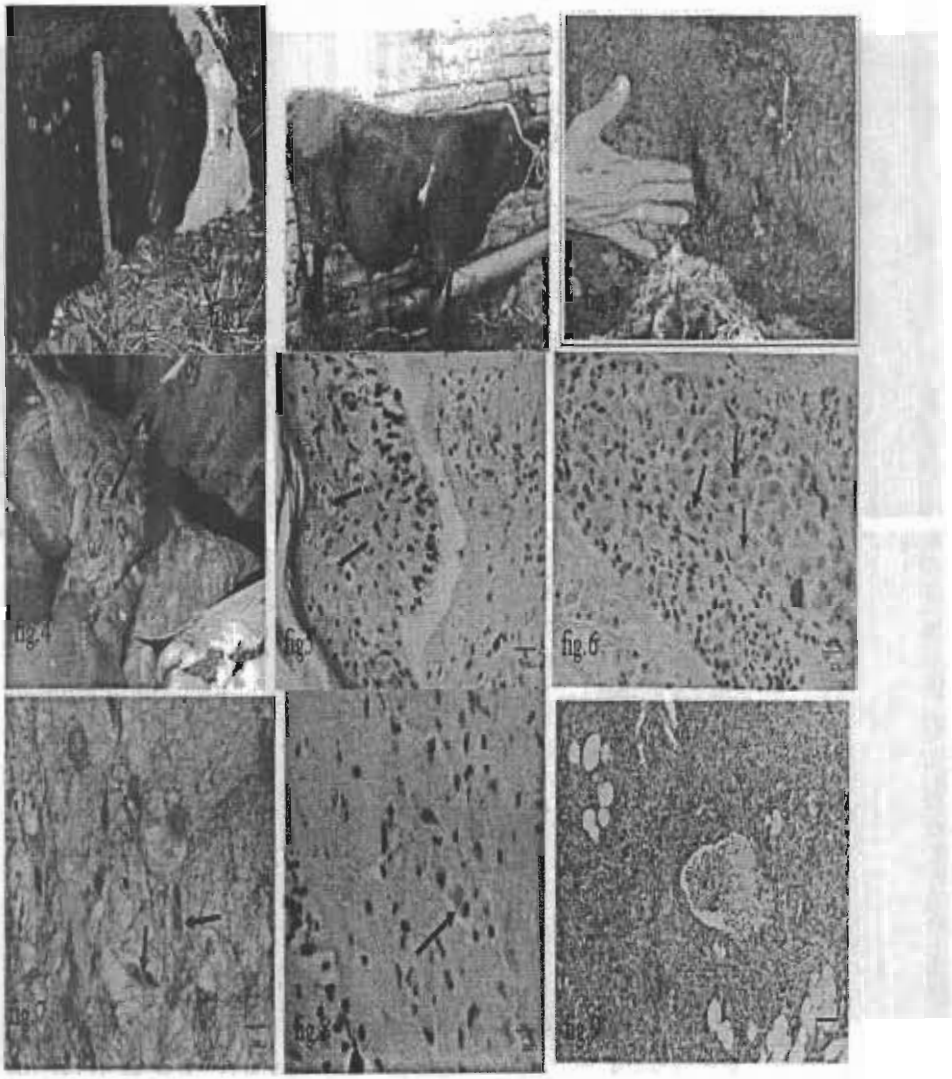

Fig. (1): Semintal calf showing the characteristic skin lesions of LSD spreading in head and neck.

Fig. (2): Native breed calf displaying characteristic skin lesions of LSD on the skin covering the head, neck and hind limbs.

Fig. (3): Calf showing swollen prefemoral lymph node.

Fig. (4): Gross pathology of calf lung showing the presence of nearly circular grey-red areas on pleural surface of the lung associated with skin nodules.

Fig. (5): H&E of calf skin nodule from acute stage with the presence of eosinophilic intracytoplasmic inclusion bodies in epidermal cells with mild hydropic degeneration (white arrows).

Fig. (6): H&E of calf skin nodule from acute stage with the presence of eosinophilic intracytoplasmic inclusion bodies in epithelium of hair follicle (black arrows).

Fig. (7): H&E of calf subcutaneous tissue showing the presence of eosinophilic intracytoplasmic inclusion bodies inside macrophages infiltrating subcutis (arrows).

Fig (8): H&E of calf skin nodule from acute stage with the presence of eosinophilic intracytoplasmic inclusion bodies in macrophages infiltrating dermis (arrow).

Fig. (9): H&E of calf skin nodule from acute stage with severe inflammatory and necrotic changes with edema in dermis.

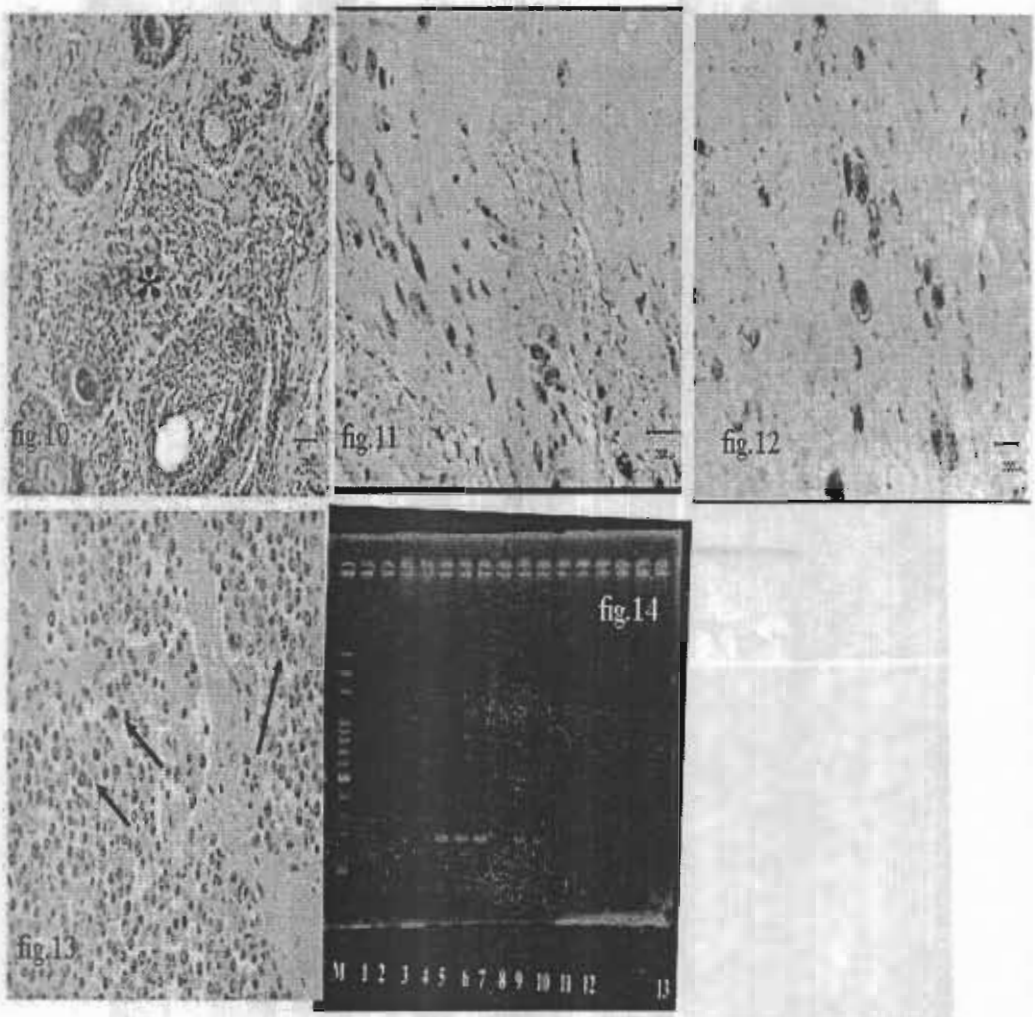

Fig. (10): H&E of calf skin nodule from subacute to chronic stage with the inflammatory cells aggregated in small pockets inside the fibrotic areas (asterisk).

Fig. (11): IHC counterstained with Mayer's hematoxylin of calf skin nodule with subacute-chronic stage showing a positive reaction in epidermal cells and inside macrophages infiltrating the dermis.

Fig. (12): IHC counterstained with Mayer's hematoxylin of calf subcutaneous tissue displaying a strong positive reaction inside macrophages infiltrating the subcutis.

Fig (13): IHC counterstained with Mayer's hematoxylin of calf lymph node displaying a weak positive reaction inside macrophages in the parenchyma of lymph node (arrows).

Fig (14): Agarose gel electrophoresis of PCR amplified gene encoding P32 of Lumpy skin disease virus. Bands at 192 bp showing that the virus is LSDV. Lane M DNA marker, lane 1 was subcutis, 2-8 were skin sample, 9-10 were lymph node samples, 11-12 were lung samples and Lane 13 was negative control.

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الملخص العربى

تحديد وجود انتيچينات ڤيروس إلتهاب الجلد العقدى فى بعض أنسجة العجول المحفوظة فى الفورمالين

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هذا العمل كان نتيجة لاستقصاء بحثى تم فى مصر من العام ٢٠٠٦ إلى ٢٠٠٧، وقد أنجز الإمينوهستوكميسترى وتفاعل البلمرة المتسلسل فى معمل الباثولوچيا بجامعة أوبيهيرو للزراعة والطب البيطرى باليابان، عرضت الدراسة إمكانية تشخيص مرض الجلد العقدى فى الأبقار والذى أصاب مزرعة أبقار حلاب بمحافظة دمياط حيث إعتمد التشخيص على تاريخ حدوث المرض وعلى الأعراض والدراسات الباثولوچية (العينية والمجهرية) ثم الإمينو هستوكميسترى وتفاعل البلمرة المتسلسل، صاحب هذا المرض إصابات عديدة (٢٢,٥٪) ونفوق فى العجول (٩٪)، وتدرجت أعراض المرض من شكل خفيف تميز بعد قليل من العقد الجلدية إلى مرض عام مختلف الضراوة وتراوح متوسط قطر العقد من ٢-٤سم، كما قمنا بذبح عجلين مصابين لفحص التغيرات العينية بهما، فتبين من فحص القطاع العرضى للعقد الجلدية أن لونها من الداخل أبيض ويوجد بعض النقط نزفية، كما لوحظ زيادة فى حجم الغدد الليمفاوية الظاهرية بمعدل ٣-٥ أضعاف حجمها الطبيعى مع وجود نقط نزفية فى قطاعها العرضى وأظهر فحص الرئتين وجود مناطق دائرية رمادية اللون على سطحها الخارجى مصاحبة لوجد ارتشاحات فى القفص الصدرى والتصاقات فى عجل منهما، بينما أظهرت الرئتين فى العجل الآخر إلتهاباً بينياً مزمناً، أظهر الفحص الهيستوباثولوچى اختلاف الصورة المجهرية للعقد الجلدية، حيث قسمت حسب شدة ودرجة الالتهاب ونوع الخلايا المرتشحة إلى حاد ومابعد الحاد والمزمن، وتميز النوع الحاد عن غيره من الأنواع بوجود أجسام احتوائية حمراء فى سيتوبلازم خلايا قشرة الجلد والبلاعم مع وجود أعداد كبيرة من الخلايا المتعادلة، تورم، جلطات عديدة والتهاب فى جدر الأوعية الدموية والليمفاوية فى أدمة الجلد فى حالة واحدة فقط، وظهر الأمينو هستوكميسترى وتفاعل البلمرة المتسلسل قادران على تشخيص الڤيروس المسبب للمرض فى بعض عينات الأنسجة المحفوظة فى الفورمالين وكانت نتائج الإمينوهستوكميسترى مطابقة لنتائج تفاعل البلمرة المتسلسل سواء فى النوع الحاد أو مابعد الحاد أو المزمن.

ووجد أن اختلاف السلالة والحالة المناعية للحيوان قد يلعبا دوراً فى اختلاف الصورة المجهرية ونتيجة كلاً من الإمينوهستوكميسترى وتفاعل البلمرة المتسلسل، بينما لايظهر لاختلاف التركيب الوراثى للفيروسات المسببة للمرض أى دور فى ذلك.

يوصى بإضافة خطوة التحلل للحامض النووى قبل إجراء تفاعل البلمرة المتسلسل خاصة إذا كان وسط الفورمالين المستعمل فى حفظ العينات غير متعادل، وباستخدام محلول عالى القلوية لإعادة تصحيح الانتيجات عند درجة حرارة عالية ١٢١ه س لمدة ١٠ دقائق وذلك فى أوتوكلاف لإجراء الإمينوهستوكميسترى خاصة على عينات الجلد المصابة.