Virological and pathological studies on lumpy skin disease in naturally infected cattle

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SUMMARY
During 2006 outbreak, a total of 39 skin nodules and 115 blood samples were collected from different breeding farms at El-Kaluobia and Alexandria governorates. Isolation of LSD virus by inoculation of the grounding skin lesions fluid suspension onto Embryonated chicken eggs was successfully done. Detection of isolates was applied by agar gel precipitation test (AGPT) and serum neutralization test. Further identification by appearance of bright granular fluorescence on CAM around the blood vessels in the dermal layer. More confirmation by transmission electron microscopy of skin nodules which revealed virions in different stages of development in the cytoplasm of infected macrophage, fibroblasts and endothelial cells. Also, a typical pock lesions were observed on inoculated CAM. The virion were remarkably in size and structural appearance to those of vaccinia virus.

INTRODUCTION
Lumpy skin disease (LSD) is an infectious eruptive viral disease affecting cattle at all ages and sexes (Ayre-Smith, 1960). It caused by lumpy skin virus which antigenically related to sheep pox in the genes Capripoxvirus (Fenner, 1976). The disease is characterized by fever, generalized cutaneous eruption of round, firm nodules, varying from 0.5 cm to 5.0 cm in diameter. Similar lesions may be present in the skeletal muscle and the mucosa of the digestive and respiratory tracts. A subcutaneous edema of the limbs and ventral parts of the body and generalized lymphadenopathy are also characteristic of the disease. Economic losses occur from debilitation, loss of milk and meat production, damage to hides and reproductive wastage due to abortions and temporary sterility in bulls (Henning, 1956 and Thomson, 1988 and Youssef et al., 1990a).

LSD was reported in Kenya 1957 (Burdin, 1959), Niger 1973 and Nigeria 1974 (Bida, 1976). The disease spread rapidly to the north and west of Africa (Ali and Obeid, 1977), and reported in Kuwait in 1986-1988 (House et al.,
In Egypt, the disease was introduced for the first time through cattle imported from Somalia in May 1988 at governmental farm near the quarantine station in Suez area (Agag et al., 1989). A second outbreak was reported in October, 1988 in Tul El-Kabir, Ismailia governorate, where LSDV was isolated (Youssef et al., 1990). At 1998, several cattle in Smallot–Minia governorate showed skin nodules of LSD (Abd El-Rahim et al., 2002).

The aim of this study was to monitor the clinical signs of the infected cattle and to ascertain virus isolation. Further confirmation was applied by electron microscopy, immunohistopathological, virological and histopathological approaches.

MATERIALS AND METHODS

Samples:

Thirty-nine skin nodules were collected from infected cattle in breeding farms at EL Kanater - Kaluobia (17 cases) and El-Noubaria- Alexandria governorate (22 cases). Apart of these lesions were grounded and made in a suspension containing 10% antibiotics, then centrifuged 2000 r.p.m. for 15 minutes. The supernatant was collected and used for inoculation of Chorio-allantoid membrane (CAM). Other parts were send for pathological and ultra structural studies.

1. Virological Studies:
   a. Blood samples:-
      One hundred and fifteen blood samples (29 samples from El-Kanater and 86 from El-Noubaria) were collected from infected cattle and used for the determination of the presence of antibodies against lumpy skin disease virus by Serum neutralization test (SNT) (Soleha, 1991) using standard known antigen obtained from pox Department, Serum and vaccine Research Institute, Abbassia, Cairo.

   b. Virus isolation by Embryonated chicken egg :-
      The supernatant fluid from the ground skin lesions of infected cattle was inoculated into the CAM of 9-11 days old fertile eggs (Alexander et al., 1957 and House et al., 1990). It was propagated twice on CAM and the virus was submitted to identified.

   c. Identification of LSDV by Agar gel precipitation test (AGPT):-
      It was carried out according to Albana (1978). Using reference LSDV positive serum.

2. Histopathological studies:

   Samples from infected skin nodules were fixed in 10% neutral buffered formalin, then processed by the standard paraffin method, sectioned at 5 µ thickness and stained with haematoxylin and eosin (Bancroft et al., 1990).
3. Ultrastructure studies:

1 mm skin nodules and CAM suspension were fixed in 4% glutaraldehyde in phosphate buffer then osmium tetraoxide 1%, dehydrated in alcohol and embedded in epoxy resin. Semi thin sections stained with toulidin blue. Then ultra-thin sections were stained with uranyl acetate and lead citrate prior for examination with Jeol tooex electron microscope according to Bozzala and Russell (1992).

4. Immunohistopathological examination:

Paraffin sections from skin (cleared with zylene and hydrated with grades of ethyl alchol) and CAM suspension were subjected to fluorescent antibody technique and examined with ultraviolet light (Goldman, 1968 and Davies et al., 1971).

Virological examination:

Table (1): Virus isolates from different samples adapted on CAM & Identified Of isolates by AGPT and IFAT.

<table>
<thead>
<tr>
<th>Governorates</th>
<th>No. of nodules</th>
<th>Isolates on CAM</th>
<th>Identified isolation by AGPT</th>
<th>Identified isolates by IFAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Kaluobia</td>
<td>17</td>
<td>9</td>
<td>52.3</td>
<td>4</td>
</tr>
<tr>
<td>Alexandria</td>
<td>22</td>
<td>14</td>
<td>63.6</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>23</td>
<td>58.9</td>
<td>11</td>
</tr>
</tbody>
</table>

RESULTS

The clinical findings:

The clinical observations among different cases of cattle revealed salivation, nasal and lacrimal discharges, fever (40-41 °C), depression and inappetance. Subcutaneous edema of different parts of the body accompanied by swelling of the superficial lymph nodes. Most of the affected animals revealed obvious cutaneous nodules all over the body. Such nodules were firm, circumscribed, flat-tapped measuring 1-3 cm in diameter (Fig., 1). The nodules involve all layers of the skin, and may coalesce forming large elevation measuring 10 cm in diameter or more. On cut section showing clear serous or purulent exudates.
The prepared skin nodules inoculated on CAM of embryonated chicken egg of age 9-11 days. 23 positive samples out of 39 collected samples with 58.97% gave thickening and specific pock lesion of LSD on CAM (Fig. 2). These positive samples were identified by using reference Antisera against LSDV through AGPT showing results as in table (1).

IFAT was successfully carried out on the previous positive samples on CAM. sixteen identified positive samples out of 23 showing bright greenish yellow fluorescence in CAM (Fig. 3). Two highly positive samples for LSD on CAM was used for EM studies (Fig. 4). The serological investigations of the collected sera by using SNT showing high titer ranging between 1/16-1/64.

**Histopathological examination:**

The histopathological examination of skin nodules of different cases (39) revealed hyperkeratosis, parakeratosis and acanthosis within the epidermis. The prickle cell layer were swollen and vacuolated. Coagulative necrosis and ulceration within epidermal layer was seen associated with presence of numerous homogenous eosinophilic intracytoplasmic inclusion bodies (Fig. 5) that were confirmed by phloxin tartrazin stain (Fig. 6). The dermis showed areas of graulomatous reaction composed mainly of centrally injured blood vessel surrounded by lymphocytes, macrophages, plasma cells and polymorphnuclear cells (Fig. 7). Vasculitis and thrombosis were also seen within different vasculature (Fig. 8). Also coagulative necrosis, calcification and degenerated polymorphnuclear leucocytes were seen in the surrounding tissue (Fig. 9). In severely affected cases, the dermis was greatly infiltrated by numerous numbers of mononuclear cells and neutrophils (Fig. 10 & 11). In some cases, the epithelial cells lining the hair follicles showing vacuolar degeneration, necrosed, destructed and the surrounding areas were infiltrated by neutrophils and mononuclear cells (Fig. 12). Concerning the muscle layer, zinker’s necrosis was seen associated with mononuclear cells infiltrated between the muscle, around blood vessels and nerve ending (Figs. 13 & 14). Moreover cystic dilatation of some sweat glands with periductal edema were also seen (Fig. 15). Intracytoplasmic inclusion bodies were seen within the histocytes and giant cells in the dermal layer (Figs. 16-17).

**Ultrastructural studies:**

Transmission electron microscopic (TEM) of skin nodules revealed virions in different stages of development seen in the cytoplasm of infected macrophages, fibroblasts and endothelial cells. Some virions were enclosed in a membrane (membrane enclosed developing...
virions MEDV) while others were empty and in some cases developed to nearly electron dense complete viral particles. Mature virions appeared oval or rectangular particles with rounded corners. There was central chromatocytosis of the nucleus of infected cells with margination of chromatin at prephary of the nucleus (Fig. 18 a & b). Dilatation of the endoplasmic reticulum (ER) were seen (Fig. 19). In some cases the virions were seen adhered to the dilated endoplasmic reticulum (Fig. 20) while in others invagination of the dilated endoplasmic reticulum resulted in the formation of multi-layered structure within which the developing virions appeared. Mitochondrial alterations included swelling with loss of its cristae (Fig. 21).

**Indirect fluorescent antibody technique (IFAT):**

Indirect fluorescent antibody technique (IFAT) revealed specific immunofluorescent staining reactions within the round cells infiltrating the epidermal layer and around the blood vessels in the dermal layer (Fig. 22 a & b).
Fig. (1): Animal infected with LSD showing cutaneous nodules all over the body and enlargement of the superficial lymph node.

Fig. (2): Thickening and specific pock lesion of LSD on CAM.
Fig. (3): Bright greenish fluorescence in the cytoplasm of infected CAM (x 400).

Fig. (4): Electron micrograph of LSD in CAM infected cell. (x 78000).
Fig. (5 & 6): Skin of cattle showing ulceration, vacuolation and homogenous eosinophilic intracytoplasmic inclusion bodies in the prickle cell of the epidermis. (5: H & E stain x 100, 6: phloxintartrazin stain x 1000).

Fig. (7): Skin of cattle showing vasculitis, perivascular collections of round cells and necrosis of the blood vessel wall in the dermal layer. (H & E stain x 200).
Fig. (8): Skin of cattle showing vasculitis and thrombus the blood vessel (H & E stain x 400).

Fig. (9): Skin of cattle showing necrosis of the dermis surrounded by fragmented neutrophils and calcification. (H & E stain x 200).
Fig. (10): Skin of cattle showing severe collections of mononuclear cells, fragmented neutrophils with focal area of necrosis and calcification in the dermal layer. (H & E stain x 200).

Fig. (11): High powered of the previous figure. (H & E stain x 400).
Skin of cattle showing (Fig., 12) vacuolar degeneration with destruction of the epithelium lining of hair follicles. (H & E stain x 200). Fig. (13) Zenker’s necrosis of the muscle (H & E stain x 200). Fig. (14) perivascular collection of round cells and around nerve ending (H & E stain x 400).
Fig. (15): Skin of cattle showing dilatation of the sweat glands associated with periductual edema. (H & E stain x 400).

Fig. (16 & 17): Skin of cattle showing (16): inclusion bodies within the histocytes (Arrow). (H & E stain x 400). (17) inclusion bodies in the dermis. (Phloxin tartrazin stain x 1000).
Fig. (18): Ultrastructure of skin showing central chromatolysis with oval MEDV within the cytoplasm of (a) the endothelial cells and (b) fibroblast. TEM. a) x 21000       b) x 10000.

Fig. (19): Ultrastructure of skin showing severe dilatation of ER in the fibroblast. (TEM x 14000).
Fig. (20): Ultrastructure of skin showing virions adhered to dilated ER. (TEM x 10000).

Fig. (21): Ultrastructure of skin showing multilaminated structure (arrow) and swollen mitochondria with loss of its cristea (m) (TEM x 16500).
Fig. (22 a & b): Skin of cattle showing specific immunoflourescent reaction infiltrating the dermis and around blood vessel. (IFAT x 200).

DISCUSSION

Lumpy skin disease (LSD) is an infectious viral disease of cattle caused by capripox virus and characterized by eruption of skin nodules and many systemic effects as pyrexia, anorexia and pneumonia (Davies, 1991). On the basis of virological, serological and experimental infection, LSD was firstly diagnosed in Egypt by Agag et al. (1989). Sporadic cases of the disease with classical signs were observed in Minia governorate 1998. In spite of, cattle protective programme applied in Egypt against LSD by vaccination with Kenyan sheep pox (SP) tissue culture vaccine, skin eruptive outbreak was observed during 2006 in Kaluobia and Alexandria governorate. Most of the affected animals revealed obvious cutaneous nodules all over the body. The associated clinical findings and gross lesions in this study were completely agreed with Ayre-Smith (1960); Ali et al. (1990); Nagi et al. (1990); Anthony and Werner (1992); Manal (2003) and Aly et al. (2006). The observed outbreak might be due to improper vaccination or to those cattle which didn’t receive vaccine especially in young cattle. In the current work, LSD virus was isolated by propagation of skin lesion suspension on CAM for three successive passage (Alexander et al., 1957). Multiple pin-head pock lesions were observed on CAM in the second passage. The lesions were demonstrated as stricks or strips and sometimes as
thickening and congestion of the membrane. These results were parallel with those observed by El-Allawy et al. (1992) who inoculated twenty local isolates of LSD virus in Assuit into 9-11 days old eggs.

The result of serological detection of LSD virus by AGPT were similar to those obtained by Alba-na (1978). Serum neutralization test revealed variable titre of antibodies ranging from 1/16 to 1/64 in the sera of infected cows. These results were highly augmented by several workers (House et al., 1990 and Ismael, 2000).

The detecting isolates were identified and confirmed by IFAT. Bright greenish yellow fluorescence were observed on CAM (House et al., 1990 and Abo Ul-Soud, 1995).

Further identification of the virus by EM on the 2nd passage of inoculated CAM showing clear macroscopic pock lesions. The results of EM investigation, have provided an additional evidence in support that the virus as a member of the pox group. There are two distinct forms of virus particles, the first is distinguished by irregular threads on its surface, the other by a capsule consisting of three distinct components surrounding an inner body. These features according to Muller and Peters (1963) are characteristic of pox virus.

It is known that lesions produced by different viruses vary significantly according to their tropism to certain tissues. The vascular endothelium is infected in many viral disease (Alexander et al., 1957 and Plowright and Ferris 1959). The lesions in the vascular endothelium in such viral disease were attributed to inclusion bodies or viral antigens (Alexander et al., 1957 and Hook et al., 1962).

In the present study, LSD virus showed direct cytoplasmic effect on the vascular endothelium of the skin. Vasculitis, thrombosis and necrosis of the lining endothelium were observed. Hence, it could be concluded that vasculitis and thrombosis have an evident role in the pathogenesis of LSD (Prozesky & Barnard, 1982). These findings were also observed by Jubb et al. (1985); Nagi et al. (1990); Anthony & Werner (1992); Manal (2003) and Aly et al. (2006). Prozesky and Barnard (1982) correlated such endothelial damage either to the virus itself or the virions.

The epidermal changes including edema, necrosis and ulceration with intracytoplasmic inclusion bodies were observed in the current study. Similar findings were also described by Nawath et al. (1982); Jubb et al. (1985); Prozesky and
Barnard (1982); Ali et al. (1990); Nagi et al. (1990); Manal (2003) and Aly et al. (2006). The vascular changes were associated with necrosis (Prozesky and Barnard, 1982). Joklik (1966) suggested that the virus can also have a proliferative effect on few necrotic endothelial cells which devoid of virus.

The current work revealed that the light and ultrastructural studies were identical. Intracytoplasmic inclusion bodies and MEDV which observed was considered to be diagnostic (Prozesky and Barnard, 1982). Ultrastructurally, some virions granular were observed as fibrillar matrix in various stages of development and agreed with those observed by Prozesky and Barnard (1982). Other virions like granular material with numerous membrane enclosed sub virions as those observed by Conroy and Meyer (1971) in swine pox. Typical brick-shaped pox like virus were also observed by Nawath et al. (1982). Virion appeared adhered to dilated ER and multilaminated structure of the dilated ER were observed in the present work. These multilaminated may be myelin figures which are lipid membrane residues originate from necrotic organelles or they may be formed by intracisternal sequestration which denotes laminar profiles of ER lying within the dilated ER. Marked swelling of mitochon-

dria associated with disruption of the internal structure were observed as a common sequel of viral replication. These observation and discussion were similar to those mentioned by Prozesky and Barnard (1982). Also crystalloid deposits in mitochondria of pig epidermal infected cells in swine pox (Conroy & Meyer, 1971).

Central chromatolysis of the nucleus as well as migration of the chromatin and formation of a central nuclear vacuole were observed. These changes may be attributed to metabolic dysfunction and to the presence of myeline figures in nuclei of infected cells. Similar findings were observed by Nagi et al. (1990); Prozesky and Barnard (1982); Conroy and Myar (1971) and Anthony and Werner (1992).

Specific bright granular fluorescence was observed by using IFAT in the cytoplasm of epidermal cells and around the blood vessels in the dermal layer. These results were parallel to those reported by Davies et al. (1971) and Anthony and Werner (1992).

From the previously discussed results, we can concluded that, lumpy skin disease is one of the major cattle diseases in Egypt. So that the animal should be quarantined and preventive measures must be done. Controlling of insect vector will reduce the incidence, reapp-
earance and spreading of the disease among the susceptible animals. The further studies by using IFAT and EM with demonstration of intracytoplasmic inclusion in the tissue were valuable in diagnosis of the disease.

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