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Isolation and Identification of Lumpy Skin Disease Virus from Naturally Infected Buffaloes at Kaluobia, Egypt

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Abstract: Lumpy skin disease virus (LSDV) was isolated from skin biopsies collected from clinically infected buffaloes at Kaluobia governorate. The virus was isolated on chorio-allantoic membrane (cam) of embryonated chicken eggs (eces) and identified by agar gel precipitation test (agpt) and indirect fluorescent antibody technique (ifat) using specific hyper immune serum against lsdv. Further investigations were carried out using electron microscopic (em) examination and polymerase chain reaction (PCR) for attachment protein gene confirmed LSDV isolate with specific amplified product 192bp.

Key words: Lumpy Skin Disease • Buffaloes • Isolation • Egypt

INTRODUCTION

Lumpy skin disease virus (LSDV) is a member of genus capripoxvirus, together with sheep pox and goat pox viruses, within the chordo-poxvirinae subfamily of Poxviridae. The genomic sequence of LSDV, about 151-kbp in length, consists of a central coding region bounded by identical 2.4 kbp-inverted terminal repeats and contains 156 putative genes. Genomic comparisons revealed that LSDV is closely related to other members of the Chordopoxvirinae, it however contains a unique complement of genes responsible for viral host range and virulence [1]. LSDV is the etiologic agent of an economically important disease of cattle in the Middle east and Africa [2]. In affected cattle, LSDV exists in skin nodules, crusts of skin lesions, blood, saliva, nasal discharge, semen and milk [3].

In Egypt, LSDV was first isolated and identified from cattle during two outbreaks in Suez and Ismailia governorates during 1989 [4,5] Diagnosis of LSD is often based on characteristic clinical signs. However, mild and subclinical forms require rapid and reliable laboratory testing to confirm diagnosis. Laboratory diagnosis of LSD comprised either identification of the virus using: electron microscopy, egg inoculation, isolation in cell cultures, fluorescent antibody test; or detection of its specific antibody using serological tests. Several polymerase chain reaction (PCR) assays have been developed recently for more accurate and rapid detection of LSDV in

suitable specimens [6,7] the key objective of this work was to detect LSDV from clinically suspected skin nodules from buffaloes based on serological and molecular basis.

MATERIALS AND METHODS

Tissue Specimens: Skin nodules were aseptically collected from infected buffaloes with atypical clinical signs of lumpy skin disease. Each sample was prepared for virological examination [8] and stored at -70°C till used.

Cultures for Virus Isolation: SPF Eight-day-old embryonated chicken eggs (ECE) were inoculated with the prepared samples via the chorioallantoic membrane (CAM) route. Madin Darby bovine kidney (MDBK) cell line propagated with Eagle's minimum essential medium (EMEM) supplemented with 10%fetal bovine serum (FBS) were used for virus isolation.

Reference Virus: Lumpy skin disease virus (LSDV) Neethling strain was kindly obtained from the Department of virology, Animal health Research Institute, Dokki, Giza.

Antiserum: Reference antiserum to LSDV was supplied by the Department of virology, Animal health Research Institute, Dokki and Giza. It was used for viral identification by AGPT and IFAT.

Conjugate: Anti-bovine IgG conjugated with fluorescent Isothiocyanate developed in rabbits and supplied by Sigma. It was used in IFAT.

Primers: The PCR primers were developed from the gene for viral attachment protein with the following sequences: forward primer 5'-TTTCCTGATTTTTCTTACTAT-3' and reverse primer 5'-AAATTATATACGTAAATAAC-3' the amplicon size of the PCR product is 192 bp. It was manufactured in the laboratories of the Midland Certified Reagent company Inc. of Midland, Texas.

Virus Isolation: Trials for LSDV isolation was carried out on CAM of ECE [4].and also on MDBK cells [9] for three blind passages.

Serological Identification of LSDV Isolates: It was carried out using IFAT [5] and AGPT [10].

Electron Microscopy: Inoculated CAM was prepared and examined under the transmission electron microscope [11], at transmission electron microscope unit, National Research Center

Molecular Identification of Virus Isolates

Extraction of Viral DNA: Viral DNA was extracted from skin biopsy [12] and from infected CAM and MDBK cells [13], stored at -20°C until used in PCR.

Polymerase Chain Reaction (PCR) Assay: It was performed according to the procedures of Ireland and Binopal [14]. The PCR primers were developed from the gene for viral attachment protein was used. PCR reaction was applied in a total volume of 50 µl containing: 1 X PCR buffer (20 mM Tris HCl pH 8.4 and 50 mM KCl); 1.5 mM MgCl₂; 0.2 mM deoxynucleosides triphosphates mixture (dATP, dCTP, dGTP and dTTP); 20 pmol of each primer; 2.5 units (U) *Thermus aquaticus* Taq polymerase 0.1µg of extracted viral DNA and nuclease-free sterile double distilled water up to 50.0 µl. Then, the resulting mixture was subjected to precise thermal profile in a programmable thermocycler as follows: One cycle of: 94 ° C for 2 min; 40 cycles of: 94°C for 50 sec, 50° C for 50 sec and 72° C for 1 min; followed by one final cycle of 72° C for 10 min.

Analysis of PCR Amplification Products (Amplicons): The resulting PCR amplicons (10-15 µl) were analyzed by 2% agarose gel Electrophoresis [15].

RESULTS

Isolation of LSDV revealed the characteristic pock lesion on CAM of ECE (Fig. 1) and prominent CPE on MDBK cells started from third day post inoculation until complete destruction of cell sheet (Fig. 2).

Serological identification indicated that 3 isolates from 2 skin nodules show line of white precipitation with AGPT and also positive results for IFAT with specific antisera for LSDV (Table 1). The specific intracytoplasmic yellowish green fluorescent granules characteristic for LSDV using IFAT demonstrated by Fig. (3).

Examination of isolated LSDV by electron microscopy revealed the virion as brick shaped Fig. (3).



Fig. 1: Pock lesion of LSDV on CAM numerous, small, scattered white foci.

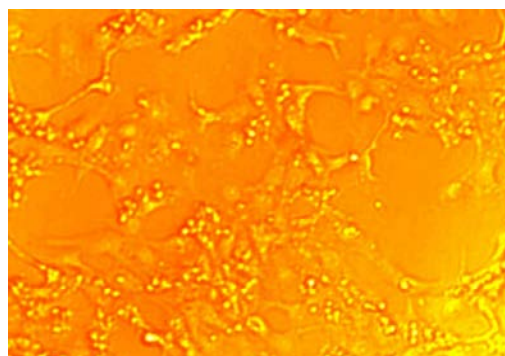


Fig. 2: Characteristic CPE of LSD in the form of clusters of cell rounding, cell aggregations and vacuoles then cell beginning of detachment

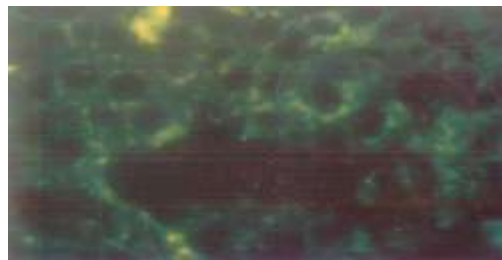


Fig. 3: The specific intracytoplasmic yellowish green fluorescent granules.

Table 1: Serological identification of lsdv isolates in ece

Number of skin nodules	Number of positive samples on ece	Number of positive samples on MDBK	Number of positive samples by serological identification	
			agpt*	fat**
3	2	2	2	2

*clear precipitation lines **yellowish green fluorescent in cell cytoplasm

Molecular Identification: The specific primers set amplified a DNA fragment of 192 bp equivalent to the expected amplification product (amplicon) size from LSDV. The LSDV reference strain and the local isolate from skin nodules, infected CAM and MDBK cells had the same size of attachment protein gene fragment 192 bp, without significant differences between the strains (Fig. 2). Subsequently, it was certain that these specimens contained DNA of LSDV.

DISCUSSION

The present study concerned with serosurvey and trials for isolation of LSD V from skin nodule samples from infected buffaloes on fertile chicken eggs and tissue culture with further identification by means of conventional serological tests as AGPT and IFAT in addition to electron microscopic examination of LSDV as well as advanced molecular characterization of virus isolate using PCR.

LSDV was isolated from samples collected from naturally infected buffaloes by inoculation on CAM of SPF-ECE. Characteristic pock lesions were observed after 1st passage and become clear after 3rd passage (Fig. 2). These findings come in complete agreement with those of House *et al.* [4] and Tamam [16] who successfully cultivated LSDV on CAM of ECE and detected the characteristic pock lesions. Also MDBK cell culture showed characteristic cytopathic effect as shown in fig (3). CPE was characterized by cell rounding, cell aggregation, coalesce together to form clusters that scattered all over the monolayer within 72 hr post inoculation and gradually increased till 70-80 % of sheet was completely detached. These findings agree with those of Ibrahim [17] and Fahmy [18].

Isolated LSDV was identified by serological tests (Table 1). Clear precipitation lines were appeared in AGPT by using reference LSDV antisera and Characteristic specific Intracytoplasmic yellowish green fluorescent

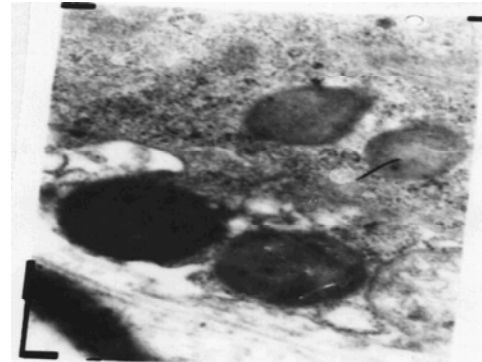


Fig. 4: Electron micrograph showing the virion appeared as ovoid in shape, with rounded ends and characteristic ball of wool appearance.

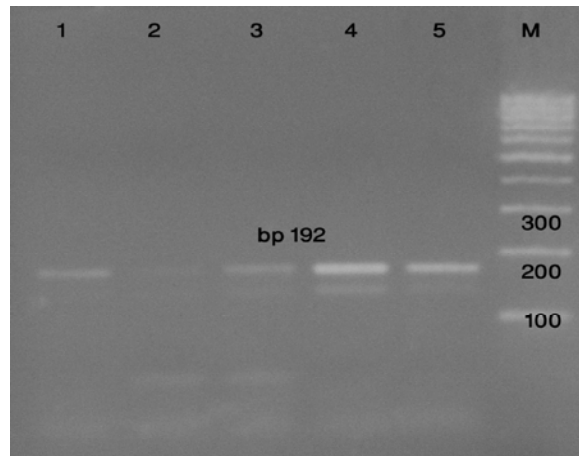


Photo 5: Electrophoretic pattern of PCR amplification where Lane M: High molecular weight nucleic acid marker, Lane 1: Original Nodule sample Lane 2: negative control sample, Lane 3: (T.C) 3rd Passage sample, Lane 4: (CAM) 3rd passage sample Lane 5: Positive control (Reference virus)

granules were appeared in IFAT (Fig. 4) as observed by Davies [5]. Electron microscope examination showed the characteristic ovoid shaped virion with "ball of wool" appearance (Fig 5), the same results were recorded by Woods [19] and Ahmed *et al.*, [20].

Serological methods are useful for confirming retrospectively LSD but are too time consuming to be used as primary diagnostic methods [5,6]. Serological assessment of antibodies to a capripoxvirus may sometimes be difficult due to the cross-reactivity encountered with other poxviruses as well as to the low antibody titres elicited in some animals following mild infection or vaccination [21]. Therefore, PCR was the test of choice for rapid detection and identification of the LSD outbreak causative agent. The PCR assay used in this work showed high specificity as a unique band of the expected size (~ 192 bp) was obtained for DNA samples derived from skin biopsies; infected CAM, infected MDBK cells and Neethling reference strain of LSDV.

In conclusion, LSDV antibodies were prevalent throughout the Egyptian governorates and PCR assay should be applied besides conventional techniques for any cases with skin lesions as early as possible to diagnosis and apply adequate control measures.

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