Polymerase chain reaction for rapid diagnosis of a recent lumpy skin disease virus incursion to Egypt

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ABSTRACT

In early 2006, a lumpy skin disease (LSD) outbreak has invaded cattle in different localities of Egypt, exerting severe economic losses to livestock industry. Representative specimens (skin biopsies) were collected form nodular skin lesions of infected foreign (imported from Ethiopia, at Ismailia private quarantine) and local cattle (at Fayoum, Menofia and Sharquia governorates). A polymerase chain reaction (PCR) assay was used, as a basic step, for rapid diagnosis of the causative agent in clinical specimens to control spread of infection in the rest of Egypt. The PCR assay, utilizing a LSDV P32 based primer set, could identify LSDV in all outbreak clinical specimens. The specific PCR amplification products (amplicons) were purified and subjected to direct nucleotide sequencing. Blast search, multiple alignments and phylogenetic analyses of the nucleotide sequence data revealed that outbreak LSDV is closely related to other capripoxviruses of LSD, sheep pox and goat pox. Selection and processing of clinical specimens, methods of DNA isolation, and PCR assay applied in this endeavor, presented a reliable laboratory diagnostic tool for LSDV.

Key words: *Lumpy skin disease, LSDV, PCR - Nucleotide sequence, P32 gene, Phylogeny.*

INTRODUCTION

Lumpy skin disease virus (LSDV) is a member of the capripoxvirus genus, together with sheep pox virus (SPV) and goat pox virus (GPV), within the chordopoxvirus (ChPV) 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 (*Tulman et al.,* 2001**).** The molecular basis of CaPV host range restriction and virulence remains to be elucidated.

LSDV is the etiologic agent of an economically important disease of cattle in the Middle East and Africa (*Davies,* 1991**;** *Fenner,* 1996). LSD is a subacute to acute OIE "List A" bovine illness, characterized by extensive cutaneous lesions and signs typical to generalized poxvirus diseases **(***Coetzer et al.,* 1994). Natural infection has not been reported in any other ruminant species with the exception of a report of five cases in water buffalo (*Bubalus bubalis*) in Egypt (*Ali et al.,* 1990) and Arabian Oryx (*Oryx leucoryx*) in Saudi Arabia (*Greth et al.,* 1992).

Capripoxviruses (CaPVs) infections are generally host specific with explicit geographic distributions, however, are serologically indistinguishable, able to induce heterologous cross-protection, and experimentally may cross-infect (*Carn,* 1993*; Davies,* 1991). Transmission of LSD among cattle is inefficient, and arthropod-vectored transm-ission may be significant in epizootic outbreaks and in the spread of LSD into nonenzootic regions **(***Carn***,** 1993*; Davies*, 1991). In affected cattle, LSDV exists in skin nodules, crusts of skin lesions, blood, saliva, nasal discharge, semen and milk (*Weiss,* 1968).

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 (*Carn,* 1993*; Heine et al.*, 1999). The PCR has been proved more sensitive and specific compared to immunoassays (*Ireland and Binepal,* 1998).

In Egypt, LSDV was first isolated and identified from cattle during two outbreaks in Suez and Ismalia governorates on 1989 (*House et al.*, 1990; *Davies,* 1991). The possible introduction of new strains of LSDV by the uninterrupted movement of animals across borders is a major constant threat. In early 2006, a severe LSD outbreak struck foreign (imported from Ethiopia) and local cattle in different Egyptian governorates, causing enormous economic losses. Many confusing arguments have arisen about this invasive LSDV that needed to be unraveled.

The key objective of this endeavor is to molecularly detect the causative outbreak LSDV from clinically suspected specimens using PCR assay, as a basic step for rapid and accurate diagnosis. That would secure proper vaccine formulation to control spread of this LSDV incursion to Egypt through cattle imported from Ethiopia.

MATERIALS AND METHODS

Virus and cells

The Egyptian strain of LSDV (*House et al.,* 1990) was used as a control virus in this study. Viral stock was prepared by infecting Madin Darby Bovine Kidney (MBDK) cells at a multiplicity of infection (MOI) of 0.1 from plaque-purified virus and was subsequently titrated on MDBK cell cultures. The MDBK cells were grown and maintained in minimum essential medium with Earle's salts (MEME) supplemented with heat-inactivated 10% bovine calf serum (BCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Prior to experimental work, both MDBK cells and BCS were tested free of LSDV by indirect immunofl-uorescence (*Tuppurainen et al.,* 2005).

Clinical Specimens

In May 2006, skin biopsies, comprising epidermis, dermis and subcutis of the nodular skin lesions, were collected from foreign (n=27; imported from Ethiopia at Ismailia private quarantine) and local (n=53; at Fayoum, Menofia and Sharquia governorates) cattle for virus detection by PCR. These cattle suffered biphasic fever (40°C-41.5°C), depression, inappetence, salivation, and ocular-nasal discharges. Superficial lymph nodes were markedly enlarged especially prescapular and precrural lymph nodes. Biopsy specimens were taken aseptically and the incisions were sutured. Samples were collected in 15 ml sterile tubes and stored at -20°C until used.

Extraction of viral DNA

The LSDV genomic DNA was extracted following the procedure described in *Sambrook and Russell* (2000) after some modifications as follows:

1-From virus stock

Briefly, a 25 ml of each crude virus in culture supernatant from the LSDV infected MDBK cells was clarified by centrifugation at 6000 rpm at 4ºC for 20 min. The clarified viruses were ultracentrifuged at 40,000 rpm at 4ºC for 2 hrs then, the supernatants were discarded. The virus pellets were dissolved in 0.5 ml of 2% SDS, mixed with 40 µg of Proteinase K and incubated at 56ºC for 1 hr with intermittent shaking. The mixture was then extracted with equal volume of phenol: chloroform: isoamyl alcohol reagent (25:24:1, vol/vol/vol, equilibrated to pH 8.0 with 10 mM Tris. HCl). DNA in the aqueous phases was precipitated with 2 volumes of cold absolute ethanol and 1/10 volume of 3 M sodium acetate. The DNA was pellted by 30 min centrifugation at 14000 rpm and 4ºC. The DNA pellets were washed in cold 70% ethanol, re-precipitated by centrifugation, dried, dissolved in 25 µl of nuclease-free water and stored at -20ºC until used. Concentration and purity of DNA was measured by spectrophotometer.

2-From skin biopsy

Skin biopsy specimens were cut into fine pieces using sterile scissors and forceps, then ground in a sterile mortar and liquid nitrogen were used to disrupt the cells. Each sample was suspended in 1.5 ml lysis buffer containing 40 % guanidine solution (Sigma) and 200 µg Proteinase K (Gibco) then, incubated at 56°C for overnight. The mixture was then extracted with equal volume of phenol: chloroform: isoamyl alcohol reagent (25:24:1, vol/vol/vol, equilibrated to pH 8.0 with 10 mM Tris. HCl). This step was repeated as needed. DNA in the aqueous phase was precipitated with 2.5 volumes of cold absolute ethanol and 1/10 volume of 3 M sodium acetate. The DNA was pelleted by 30 min centrifugation at 14000 rpm and 4ºC. The DNA pellets were washed in cold 70% ethanol, re-precipitated by centrifugation, dried, dissolved in 25 µl of nuclease-free water and stored at -20ºC until used.

3-Reference genomic DNA of LSDV "Neethling" type strain 2490

Originally isolated from Kenya in (1958), it was used as a standard for optimizing the PCR reaction conditions. It was kindly obtained from Dr. *Eeva Tuppurainen* at the Institute for Animal Health, Pirbright Laboratory, Pirbright, U.K. (extracted from virus infected primary lamb testicle cells).

Polymerase chain reaction (PCR) assay

The PCR primers were chosen from unique LSDV sequences within the gene for viral attachment protein (*Ireland and Binepal*, 1998). Forward primer: 5'-AAATTATATACGTAAATAAC -3' Reverse primer: 5'-ATAGTAAGAAAAATCAGGAAA -3' 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 $MgCl_2$; 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 ^{o}C for 2 min; 40 cycles of: 94^{o}C for 50 sec, 50^{o}C for 50 sec and 72^{o}C for 1 min; followed by one final cycle of 72^{o}C for 10 min.

Analysis of PCR amplification products (amplicons)

The resulting PCR amplicons (10-15 µl) were analyzed by 2% agarose gel electrophoresis as described by *Sambrook and Russell,* (2000). The DNA bands were visualized using ultraviolet transilluminator after gel staining with ethidium bromide (0.5 µg/ml). The PCR amplicons of proper predicted size (about 192 bp) were gel purified using DNA gel purification kit (ABgene, Germany) and quantitated according to the procedure described by *Sambrook and Russell* (2000).

Direct sequencing of PCR amplicons

Three PCR amplicons (n = 3) derived from genomic DNAs of skin biopsies (one represented each governorate) were purified using Microcon columns (Amicon, USA) and directly sequenced in both directions with the same primers used to generate the PCR amplicons. Sequencing was done in an ABI PRISM system (Faculty of Veterinary Medicine, University of Munich, Munich, Germany) using the dideoxy chain-termination method (*Sanger et al.,* 1977), based on the incorporation of fluorescent-labeled dideoxynucleotide terminators. The identity of each nucleotide was verified at least twice.

Computer-assisted sequence and phylogenetic analyses

The resulted nucleotide sequence data of the selected PCR amplicons (n=3) were compiled and compared to each other. Further, nucleotide sequence majority, representing the most identical sequence among Egyptian outbreak LSDVs, namely Egy/2006 (accession number EU807974) was compared to those of other related viruses accessed by blast search for sequence homology via the GenBank database. The multiple nucleotide sequences were aligned by the Clustal W (1.82) program (*Thompson et al.,* 1994) of European Bioinformatics Institute (EBI, EMBL). Clustal W is a fully automatic program for global multiple alignment of DNA sequences. Phylogenetic correlation and tree construction were done using the PHYLIP and Tree view 32 (1.6.6) programs (*Page*, 1996; *Felsenstein*, 2001). All programs used in this study were accessed through their interactive web services.

RESULTS AND DISCUSSION

The viral DNA was successfully extracted from specimens of skin biopsies and local vaccinal virus culture supernatant and was utilized for polymerase chain reaction (PCR). The specific primers set amplified a DNA fragment of 192 bp equivalent to the expected amplification product (amplicon) size from LSDV. All of the 80 skin biopsy samples (100 %) were positive for this PCR assay. None of the negative controls produced any amplicon (Fig.1). Subsequently, it was certain that these specimens contained DNA of LSDV.

Representative outbreak DNA amplicons (n = 3), resulted from specific direct PCR amplification, were purified and analyzed for their nucleotide sequences (Fig.2). The nucleotide sequence data were comparatively aligned to each other and revealed close sequence identity (Table 1). All sequences in the alignment of the Egyptian outbreak LSDV isolates were subjected to blast search versus the GenBank database. The closest sequences to the Egyptian outbreak LSDVs were those of isolates/strains of LSDV, SPV and GPV. The nucleotide sequence that was most identical among all sequences in the alignment (n = 3) was extracted and utilized as sequence majority of the outbreak LSDV Egypt 2006 (Egy/2006). The multiple sequence alignments between the outbreak LSDV nucleotide sequence majority and its counterparts of other LSDV, SPV and GPV isolates/strains revealed variable similarity percentages as presented in

(Fig.1 and Table1). The phylogenetic tree produced confirmed the results obtained from both nucleotide sequence alignments and blast search as illustrated in figure (3). Phylogenetic analysis strongly indicated that Egypt 2006 outbreak isolates were identical to LSDV and closely related to SPV and GPV (Fig.3).

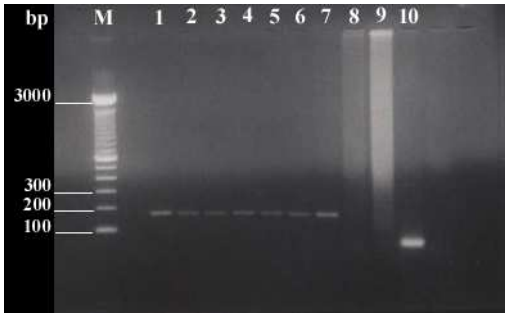

Fig.(1) : Agarose gel electrophoresis of the PCR amplicons (~ 192 bp) of genomic DNA derived from Egypt 2006 outbreak bovine skin biopsies and reference strains of LSDV utilizing primers specific for LSDV viral attachment gene (P32), separated on 2% agarose gel and stained with ethidium bromide. Lanes: (M) 100 bp DNA ladder (Fermentas); (1) Reference of LSDV "Neethling" strian; (2) Old Egyptian LSDV strain; (3 –7) Outbreak samples; (8) Negative control (no primers); (9) Negative control DNA of MDBK cells; (10) Negative control (only primers without DNA template).

A severe LSD outbreak has struck cattle population in Egypt on early 2006. Effective control of LSD requires rapid and accurate laboratory diagnosis supported by clinical findings (*Tuppurainen et al.*, 2005). Accordingly, this work was conducted in the process of rapid and accurate diagnosis to control severe losses of this outbreak among livestock. Infected animals from which the specimens were collected, exhibited typical signs of LSD. The collected specimens (skin biopsies) were used for viral genomic DNA isolation followed by a PCR assay.

The PCR assay detected LSDV in 80/80 (100%) skin biopsies from representative infected cattle. The PCR result was fully correlated to field diagnosis based on clinical symptoms. It has been reported that although many other sources for virus detection such as blood, semen and milk were determined, skin biopsies were the best as they contain much more viral particles to be detected by PCR (*Tuppurainen et al.*, 2005). Therefore, the current approach presents a suitable applicable diagnostic tool for both endemic countries and those in which the live virus is not available. The PCR assay used in this work showed high specificity as a unique band of the expected size (~ 192 bp) was obtained for all DNA samples derived from skin biopsies; Neethling reference strain and old Egyptian strain of LSDV. Also, the confirmatory results were obtained within 24 hours of sample collection. While, virus isolation takes several weeks as the LSDV grows slowly in tissue cultures and may require several passages (*Alexander et al.*, 1957; *Prydie and Coackley*, 1959). Bacterial and fungal contaminations are frequently encountered in biopsy samples and these may infect cell cultures, particularly those ones that require prolonged incubation (*Van Rooyen et al.*, 1969). Examinations using immunofluorescent assays may indicate the presence of LSDV antigens especially in the early stages of the disease; however nonspecific fluorescence may cause diagnostic infidelity

(*Davies et al.*, 1971). Moreover, the direct or indirect immuno-fluorescence can not distinguish different members of the capripoxvirus group (*Davies and Otema*, 1981).

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Egy_2006_LSDV/P32          AAATTATATACGTAAATAACATACCTGCTTAAAACCATAGTAATTTAGAA
NI-2490_LSDV/P32           AAATTATATACGTAAATAACATACCTGCTAAAAACCATAGTAATTTAGAA
NW-LW_LSDV/P32             AAATTATATACGTAAATAACATACCTGCTAAAAACCATAGTAATTTAGAA
SPV_Makhdoom-2007_/P32     AAATTATATACGTAAATAACATACCTGCTAAAAACCATAGTAATTTAGAA
GPV_G20-LKV/P32            AAACTATATACGTAAATAACATACCTGCTAAAAACCATAGTAATTTAGAA
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Egy_2006_LSDV/P32          TTCAAATCCAAAATTATCATTATTATAATAAATAAAATAATAAGTGCTCC
NI-2490_LSDV/P32           TTCAAATCAAAAATTATCATTATTATAATAAATAAAATAATAAGTGCTCC
NW-LW_LSDV/P32             TTCAAATCAAAAATTATCATTATTATAATAAATAAAATAATAAGTGCTCC
SPV_Makhdoom-2007_/P32     TTCAAATTAAAAATTATCATTATTATAATAAATAAAATAATAAGTGCTCC
GPV_G20-LKV/P32            TTTAAATTAAAAATTATCATTATTATAATAAATAAAATAATAAGTGCTCC
**  * * * *    * * ** ** * ******* *** ***** * ** ********** **  **** *** ****


Egy_2006_LSDV/P32          TATTATACTAATATCAAATATACCAAAAATTGAAACCAATGGATGGGATA
NI-2490_LSDV/P32           TATTATACTAATATCAAATATACCAAAAAATGAAACCAATGGATGGGATA
NW-LW_LSDV/P32             TATTATACTAATATCAAATATACCAAAAAATGAAACCAATGGATGGGATA
SPV_Makhdoom-2007_/P32     TATTATACTAATATCAAATATACCAAAAAATGAAACCAATGGATGGGATA
GPV_G20-LKV/P32            CATTATACTAATATCAAATATACCAAAAAATGAAACCAATGGATGGGATA
                           ******************* ** * *** *  *******  **  ************** **** ** *


Egy_2006_LSDV/P32          CATAGTAAGAAAAATCAGGAAA
NI-2490_LSDV/P32           CATAGTAAGAAAAATCAGGAAA
NW-LW_LSDV/P32             CATAGTAAGAAAAATCAGGAAA
SPV_Makhdoom-2007_/P32     CATAGTAAGAAAAATCAGGAAA
GPV_G20-LKV/P32            CATAGTAAGAAAAATCAGGAAA
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Fig.(2): Alignment of the majority nucleotide sequence of the outbreak LSDV isolates Egy/2006 (accession number EU807974) versus those of other related capripoxviruses, generated from the most identical nucleotide sequences encoding from the viral attachment protein (P32 gene) of the analyzed viral genomes, using CLUSTAL W (1.82) program. Stars indicated that nucleotides in that column are identical in all sequences in the alignment.

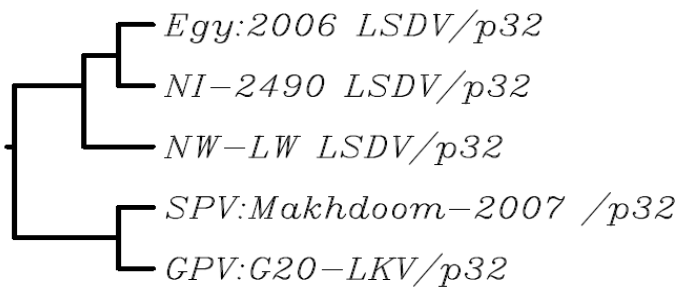

Fig.(3): Phylogenetic tree of the majority nucleotide sequence of the Egyptian outbreak LSDV isolates Egy/2006 (accession number EU807974) versus nucleotide sequences of the related capripoxvirus genus, generated from nucleotide sequences encoding for the viral attachment protein (P32 gene) of the analyzed viral genomes, using CLUSTAL W (1.82) program. The phylogenetic analyses were performed using the PHYLIP package and Treeview programs.

Serological methods are useful for confirming retrospectively LSD but are too time consuming to be used as primary diagnostic methods (*Davies,* 1991 and *Heine et al.,*1999). 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 (*Kitching and Hammond*, 1992). Therefore, PCR was the test of choice for rapid detection and identification of the LSD outbreak causative agent.

Multiple sequence alignments showed high homology percentage (≥ 99 %) of the nucleotide sequences among local isolates of LSDV. Nevertheless, blast searches over the Genebank database together with the phylogenetic analyses and sequence alignments revealed that local isolates of LSDV are highly related (≥ 95 %) to not only other LSDV strains but also other Capripoxviruses (goat and sheep pox). These results coincide with the theory of that all capripoxviruses are genetically related and originated from one ancestor lineage **(***Black et al.,* 1986, *Fenner et al.,* 1987 and *Tulman et al.,* 2001). Capripoxviruses are not readily neutralized and neutralization studies cannot differentiate LSDV from SPV and GPV (*Davies and Otema,* 1981). Cattle in contact with sheep or goats infected with SPV and GPV usually do not develop antibodies to capripoxviruses. However, cattle that have been vaccinated with SPV will develop neutralizing antibodies to LSDV (*Capstick and Coackley,* 1961). These results support the justified use of sheep pox virus vaccine for control of LSD **(***Kitching and Taylor,* 1985**).**

In conclusion, the virus caused Egypt 2006 outbreak is surely a LSDV which is closely related to other capripoxviruses of LSDV, SPV and GPV as revealed by the high nucleotide sequence identity and close branch distances in the phylogenetic tree. This conclusion is supported by history of importing live cattle from Ethiopia, shortly before the outbreak beginning, as both imported and local breeds expressed the disease. Selection and processing of clinical specimens, methods of DNA isolation, and PCR assay applied in this endeavor, presented a reliable laboratory diagnostic tool for LSDV.

Direct sequencing of PCR amplicons and comparative genetic analyses were useful not only to trace the new outbreak LSDV, but also to establish genetic tools for national-wide epidemiological studies and development of novel efficient LSDV vaccines. In order to prevent the introduction of LSDV, it is strongly recommended to utilize this PCR assay in examination of live domestic and wild bovine species and bovine semen from endemic countries at quarantines.

Table (1): Identity score % of multiple alignment of the representative majority nucleotide sequence of Egyptian outbreak LSDV isolates (Egy/2006) versus nucleotide sequences of related capripoxviruses, generated from sequences encoding for the viral attachment protein (P 32 gene) of the analyzed viral genomes, using CLUSTAL W (1.82) program.

Seq. A Name	Len. (nt)	Seq. B Name	Len. (nt)	Score (%)
Egypt2006 outbreak	172	Lumpy skin disease virus NI-2490 isolate Neethling 2490	169	98
Egypt2006 outbreak	172	Lumpy skin disease virus NW-LW isolate Neethling Warmbaths LW	169	98
Egypt2006 outbreak	172	Sheeppox virus isolate Makhdoom-2007	168	97
Egypt2006 outbreak	172	Goatpox virus G20-LKV	165	95

Seq. = sequence **Len. (nt)** = length in nucleotides **Score (%)** = sequence identity percent.

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الملخص العربي

تفاعل البلمرة المتسلسل للكشف السريع عن فيروس مرض الجلد العقدى حديث الدخول إلى مصر

*** علاء عبد المنعم الخولى ، * حاتم محمد توغان سليمان ، ** خالد عبد الرحمن أبو المعاطى عبد الرحمن**
* معهد بحوث الأمصال واللقاحات البيطرية بالعباسية – ص . ب ١٣١ ، القاهرة.
** المركز القومى للبحوث – الدقى .

فى أوائل عام ٢٠٠٦ ، تعرضت قطعان الأبقار فى مناطق عديدة بمصر الى وباء مرض الجلد العقدى مسبباً خسائر إقتصادية فادحة فى صناعة الماشية . تم تجميع عينات ممثلة (نسيج حى من الجلد) من إصابات الجلد العقدية للحيوانات المصابة (المستوردة من أثيوبيا) وتم حجرها بيطرياً فى محاجر خاصة بالاسماعيلية ، وحيوانات محلية فى مناطق الفيوم والمنوفية والشرقية. تم إجراء إختبار تفاعل البلمرة المتسلسل على الحمض النووى الجينى المستخلص من العينات الإكلينكية بأستخدام بادئات خاصة بالبروتين ٣٢ (P32) من جينوم الفيروس .وتم الكشف عن الفيروس فى جميع العينات الوبائية . وقد تم تنقية ناتج تفاعل البلمرة المتسلسل من جيل الأجاروز والتحليل المباشر للتتابع النيوتيدى لها . وبإجراء المسح البحثى عن مشابهات التتبع النيوتيدى فى بنك الجينات ، وإجراء تحليل مقارن فيما بينهم وكذلك إجراء تحليل التشعب الوراثى الفيلوجينى ، وأثبتت نتائج هذة التحاليل أن الفيروس LSDV المسبب للوباء وثيق القرابة بالفيروسات الاخرى من عائلة جدرى الماعز Capripox virus والتى تشمل فيروسات الجلد العقدى ، جدرى الاغنام وجدرى الماعز . وقد قدمت هذه الدراسة تطبيقاً لأختيار وتجهيز العينات الإكلينيكية وطرق إستخلاص الحمض النووى وأختبار تفاعل البلمرة المتسلسل كوسيلة تشخيص فعالة لعدوى فيروس الجلد العقدى معملياً .