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RESEARCH

Lumpy skin disease is circulating among sheep pox vaccinated cattle in Egypt 2017

Akram I. Aboelkhair[1], Ayman H. El-Deeb[2], Momtaz A. Shahin[1] and Hussein A. Hussein[2]

ABSTRACT

Background: Lumpy skin disease is an infectious viral disease caused by Lumpy skin disease virus (LSDV) affecting cattle causing skin lesions. It`s a member of Poxviridea family, genus Capri-pox, and closely related to sheep and goat pox. In Egypt, the protection from lumpy skin disease (LSD) among cattle population was carried out using sheep poxvirus vaccine. Last year (2016-2017) revealed many cases of LSD in sheep pox vaccinated cattle in Egypt.
Objective: In this study LSDV was isolated from sheep pox vaccinated cattle showing LSD signs to confirm LSD viral infection even in sheep pox vaccinated cattle.
Methods: 130 Nodular samples were collected from 150 vaccinated cattle showing signs of LSD, Then Molecular detection of LSDV with PCR using G-protein-coupled chemokine receptor (G-PCR) gene primer revealed positive result for LSDV, So classical Isolation of LSDV was started by inoculation of nodules on Chorioallantoic membrane (CAM) 9-11 day old SPF eggs, then adaptation on Madin-Derby bovine kidney cell line (MDBK); confirmation of LSD isolated virus was done by using Electron microscopy (EM) on suspension of cell culture passage of the virus, Serological identification was done by serum neutralization test (SNT) using reference antisera which confirm presence of LSDV . By nucleotide sequences and genetic characterization was conducted on (G-PCR) gene segment of these isolates.
Results: 93 samples were positive for LSDV by using conventional PCR direct from nodular lesion, 4 positive samples were inoculated on CAM showed characteristic pock lesion, formerly after passaging of samples on MDBK cell line revealed characteristic cytopathic effect of LSDV, as well as tissue culture suspension were positive using conventional PCR . For antigenic identification SNT were applied. Sequencing of positive sample reveled identity of LSDV by targeting G-protein coupled chemokine receptor gene (GPCR) which confirm that there is no genetic changes in LSDV.
Conclusion: phylogenetic analysis of LSDV at summer 2017 revealed that recent isolates has no genetic difference compared by other isolates from Egypt or Africa during previous few years, as LSDV affected sheep poxvirus (SPV) vaccinated cattle,

Keywords: LSDV; MDBK; CAM; SNT; IFAT; SPV; PCR; Egypt.

BACKGROUND

Lumpy skin disease (LSD) is an infectious disease, caused by Lumpy skin disease virus (LSDV), characterized by eruptive nodules on cattle hide (Abutarbush et al. 2015), (LSDV) is a member of Capripoxvirus genus (Mafirakureva, Saidi, and Mbanga 2017). Capri-poxviruses including sheep pox virus (SPV) , goat pox virus and lumpy skin disease virus, all cause economic losses in domesticated ruminants (Aspden et al. 2003), LSD is a notifiable disease to World Organization for Animal Health (OIE) (Armson et al. 2017), (LSD) is a pox viral disease of cattle (Agianniotaki *et al.* 2017), characterized by fever, skin nodules, mouth lesions, pharynx and respiratory tract nodules, enlarged lymph nodes, emaciation, and skin edema are present, sometimes death (Abutarbush *et al.* 2015). Important of the disease is due to loss of condition in infected animals also because of permanent damage of hides. The route of transmission is

Correspondence: husvirol@cu.edu.eg
Full list of author information is available at the end of the article

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mechanical through mosquitos bite (Carn and Kitching 1995). The incidence of LSD increases during the mosquito seasons, decreases at dry seasons (Mafirakureva, Saidi, and Mbanga 2017) .

In Egypt, first report of LSDV was in Suez and Ismailia in May and October 1988 (Awad *et al.* 2010), then spread throughout Egypt to infect 50,000 cattle and cause 1,449 mortalities in 1998 (Armson *et al.* 2017; Shalaby *et al.* 2016) During epizootics, LSDV is transmitted mechanically by blood feeding flies e.g. Aedes aegypti (Chihota *et al.* 2003). Due to the rapid spread and the severe economic losses caused by LSD, the Office International des Epizooties (OIE) listed LSDV in the notifiable disease of cattle. (Shalaby *et al.* 2016)

Diagnosis of LSD depends on signs of skin nodules on infected animal hide, isolation of virus (Awad *et al.* 2010), serological tests and histopathology. Conventional diagnostic methods are time consuming. In addition, they have low specificity. Polymerase Chain reaction (PCR) is the simple, rapid and specific diagnostic method (Awad *et al.* 2010; El-Tholoth and El-Kenawy 2016).

Tissue culture adapted LSDV, LSDV/Ismailyia88 strain and skin isolate of SPV were antigenically identical. And is more closely related to field skin isolate of LSDV than SPV/Kenyan vaccinal strain (El-Tholoth and El-Kenawy 2016). Thus, further study should be applied on the advantage of a LSD vaccine prepared from LSDV in protection of cattle against LSD compared to the commonly used sheep pox vaccine (El-Kenawy and El-Tholoth 2010) .

Several reports have described methods for diagnosis LSDV following either natural or experimental infection (Awad *et al.* 2010). To diagnose LSDV diseases in Egypt, Histology on nodular lesions cannot definitively confirm lumpy skin disease (Awadin *et al.* 2011). So Electron microscope used in these study to confirm the outer layer of virus for rapid diagnosis , serological identification also used for confirmation of LSDV infection. Even though experienced veterinarians diagnose sheep pox and cattle pox in acute forms easily, still low virulence strains, exanthemas, Herpes virus infection and ORF (scabby mouth – *Parapoxviridae*) may present causing problems for differential diagnosis (Tian *et al.* 2010). Laboratory confirmative diagnosis depends on classical virological techniques as electron microscopy for identification of virus, virus isolation in cell culture, molecular characterization and sequencing of samples as we showed in this study (Tian *et al.* 2010).

MATERIALS AND METHODS

Animals:

One hundred and fifty cattle from different governorates in Egypt were clinically examined. The clinical examination includes examining temperature, skin, mucous membrane and superficial lymph nodes (Elhaig, Selim, and Mahmoud 2017).

Samples: Tissue samples:

One hundred and thirty nodules and lymph nodes were taken from infected animals vaccinated with SPV vaccine for virus isolation (Agianniotaki et al. 2017) , identification and molecular detection, nodules transported in phosphate-buffered saline (Elhaig, Selim, and Mahmoud 2017) (PBS P.H. 7.2) and preservation were at -80 Co .

Virus Isolation:

Isolation of Lumpy Skin Disease Virus revealed the characteristic pock lesion on Choroallantoic membrane of 9-10 (SPF) ECE , then 200ul from each prepared CAM lesion were inoculated for three passages into MDBK (Gelaye *et al.* 2015) tissue culture cells according to the method described by (Carn and Kitching 1995) (Bagla *et al.* 2006).

Detection of LSDV antibodies:

Standard reference LSDV (Neethling strain) and standard reference LSDV antiserum was supplied by Pox department Veterinary Serum & Vaccine Research Institute (VSVR-Abbasya), for application of PCR control positive and SNT. standard reference LSDV antiserum were used for detection of antibodies against LSDV isolate using virus neutralization test according to (Kitching 2003),(OIE 2017).

All viruses in the genus Capripoxvirus have common major antigen for neutralizing antibodies, so it is not possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological techniques (Gari *et al.* 2008). The neutralization index is the difference between log of virus titre and log of serum virus titer in the test NI = 2. Neutralization test were performed for identification of LSDV isolate using alpha procedure. The techniques were performed according to the method described by (OIE, 2017) (Davies 1991) .

Molecular identification of LSDV isolates by PCR:

Viral DNA extraction:

Extraction of viral DNA was applied on 4 positive infected MDBK cell line showing CPE after lysis of cells by rabid freezing and thawing using liquid nitrogen and stored at -80°C until using for PCR. DNA was extracted from tissue samples by using a Thermo Scientific™ K0701 extraction kit (Thermo Scientific™ GeneJET™ PCR Purification Kit), according to the manufacturer's instructions. The extracted tissue was then transferred into 1.5-ml micro-centrifuge tubes. (Mafirakureva, Saidi, and Mbanga 2017).

PCR Assay:

The reaction mixture (50 ul) contained 250 mg of total DNA. 2 ml M MgCl2. 5P mol of each primer (forward primer was 5′- GGG TGA ACT ACA GCT AGG TAT C -3′ and reverse primer was 5′- AGT ACA GTT AGT AGC GCA ACC-3′), 200 uM of each dNTP and 2U of DNA polymerase (Biotool, USA) in a reaction buffer containing 75 mM Tris-HCl (ph 9), 2mM MgCl2, 50 mM KCl, 20 Mm (NH4)2SO4 and 0.001% BSA. Amplification was carried out in a MJ thermal cycler (MJ incorporation USA) programmed to perform a denaturation step at 94 Co for 2min. followed by 35 cycles for 1 min. at 94 Co for denaturation, 1 min at 51 Co for primer annealing and 1 min at 72ºC for extension. The last extension step was 5min. longer*. A 10 ul PCR products were mixed with 2 ul gel lading buffer (Sigma- Aldrich) and electrophoresed in 1.5% agarose gel, containing 1 ug/ml ethidium bromide in Tris- acetate buffer (0.04 M)Tris-acetate and 0.001 M EDTA, ph 8). The resulting DNA fragments were visualized by UV transilumination and photographed (Shalaby *et al.* 2016) . A visible band of appropriate size 519bp was considered as a positive reaction (Kasem *et al.* 2017). A reference LSDV propagated on MDBK cell line was obtained from virology department, Animal Health Research Institute, Dokki, Giza and used as positive control (Binepal, Ongadi, and Chepkwony 2001).

Step	Temperature C^{o}	Time	Cycles
Initial denaturation	94	2 min	1
Denaturation	94	1 min	35
Annealing	51	1 min	
Extension	72	1 min	
Final extension	72	5 min	pause

*Used primers thermal profile for PCR thermocycler

Electron Microscopy:

One drop of infected cell culture suspension showed the virus particle on cell culture MDBK cells using the negative stain (phosphotungstic acid) appeared as ovoid shape, rounded ended bilayer, ball of wool filaments are characteristic for the virus (Kitching and Smale 1986).

Sequencing:

GPCR gene segment was generated by amplification of overlapping fragments using primers pairs described. In each reaction the DNA was initially denatured at 94 Co for 2min. followed by 35 cycles for 1 min. at 94 Co for denaturation, 1 min at 51 Co for primer annealing and 1 min at 72ºC for extension. The amplification products were visualized and assessed for size by agarose gel electrophoresis. All PCR products were purified then amplicons were sequenced, The resulting sequences were assembled with the SeqMan Pro™ program (Lasergene v.11; DNAStar Inc., USA) and aligned with each other using the CLUSTALWalgorithm *(*Tulman *et al.* 2001) in BioEdit 7.0.5.3 (Hall, 1999).

Molecular phylogenetic analyses were performed using MEGA 5.2, the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and confidence on branching was assessed using bootstrap resampling (1000 replicates).

RESULTS

Clinical findings:

From 150 examined cattle, 130 showed typically LSDV symptoms It includes salivation, nasal and lacrimal discharges accompanied by swelling of the superficial lymph nodes was observed. Most of the affected animals revealed obvious coetaneous nodules all over the body (Fig. 1).

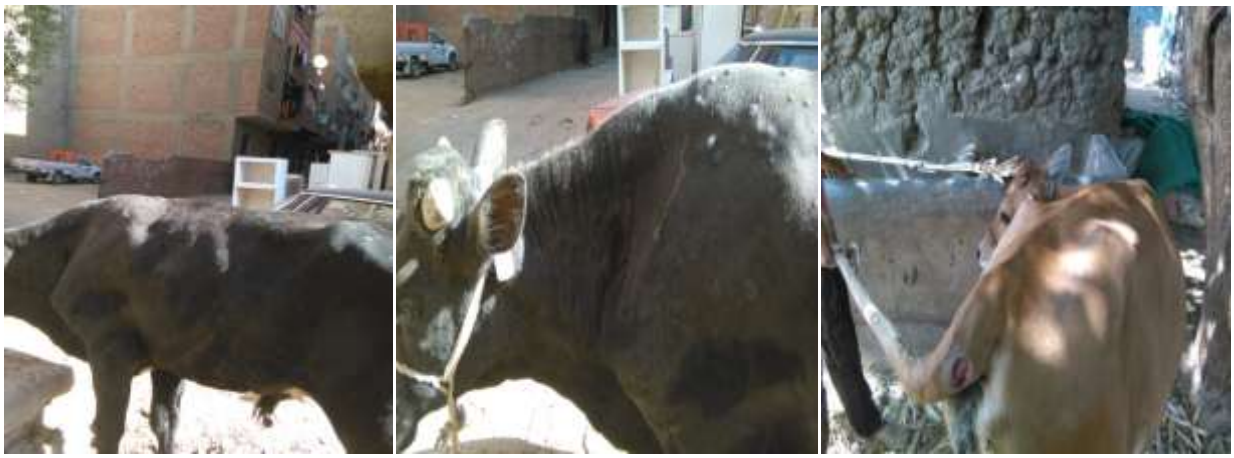
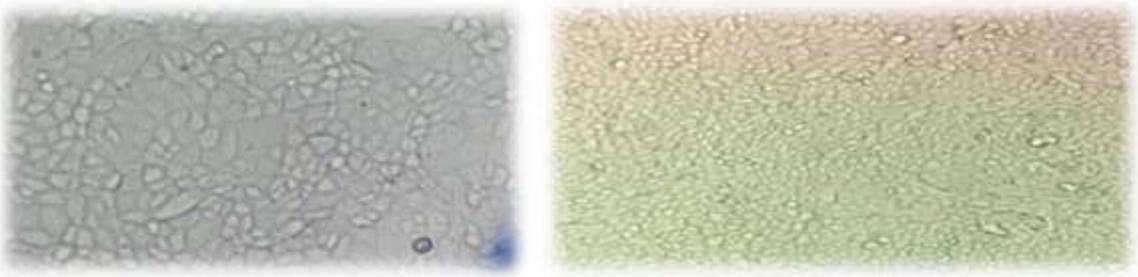


Fig. 1: showed nodules on the body of infected cattle and buffalo

Virological findings:

Ten prepared skin nodules inoculated on MDBK cells for three successive passages, 4 of them showed clear cytopathic effect CPE, which characterized by cell granulation followed by cell rounding and aggregation in a separated manner. This occurred 3-4 days post inoculation (PI), followed by completed sheet detachment after 5-7 days PI (Fig. 2).

Fig. 2: characteristic CPE of LSDV, PI on MDBK cells (X 40).



The normal MDBK monoconfulant layer

Isolation on CAM:

Isolation of Lumpy Skin Disease Virus revealed the characteristic pock lesion on Choroallantoic membrane of ECE Fig. (3).

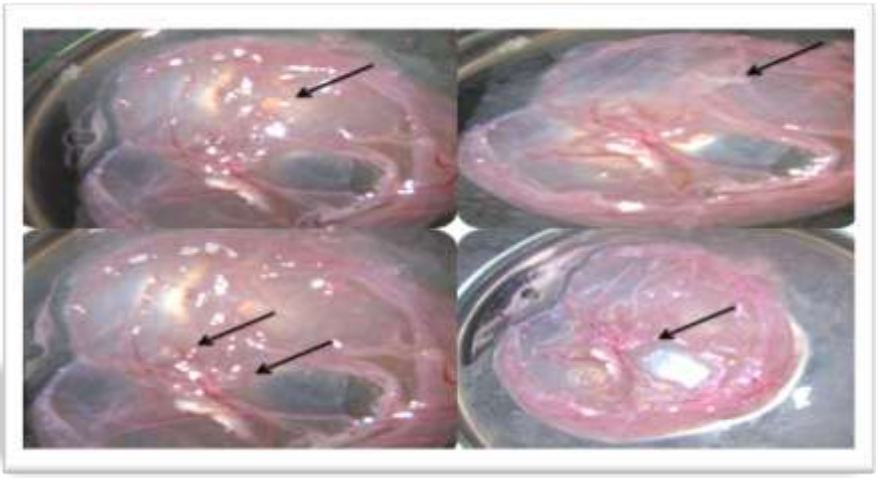


Fig. 3: lesion of LSDV on CAM varied from thickening of membrane in first passage to numerous white foci more pronounced by second and third passage

PCR findings: The results indicated that PCR assay revealed a high incidence of LSDV in 100% of positive samples (Fig. 4).

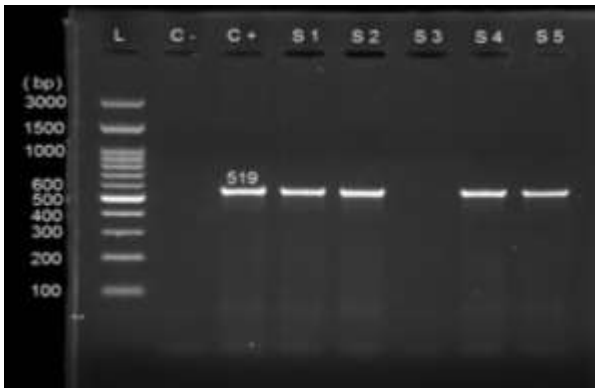


Fig. 4: Detection of DNA of LSDV by PCR (agarose gel electrophoresis of the PCR products) Lanes: (L) 100 bp DNA ladder (Fermentas); (C -) negative control only primers without DNA;(C +) Reference of LSDV "Neethling" strian; (1, 2, 4, 5) +ve samples; (3) negative sample

Electron Microscopy:

One drop of infected cell culture suspension showed the virus particle on cell culture MDBK cells using the negative stain (phosphotungstic acid) appeared as ovoid shape, rounded ended bilayer, ball of wool filaments are characteristic for the virus

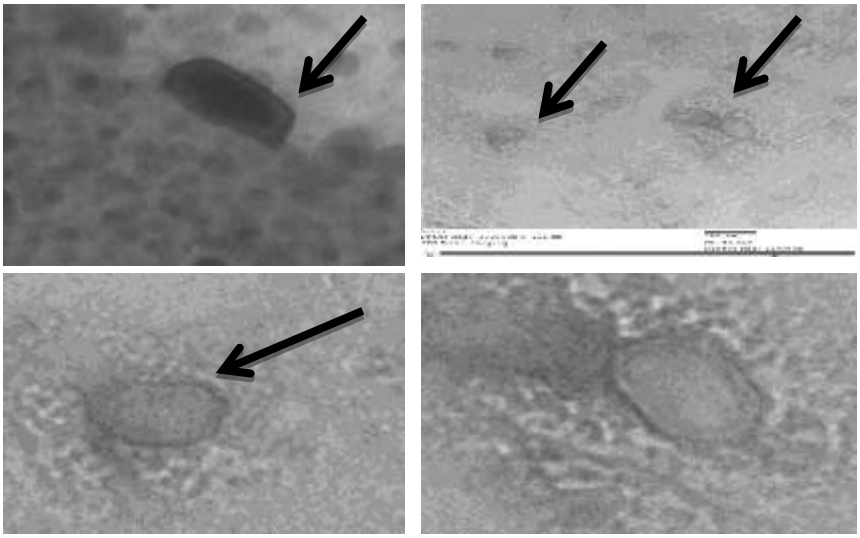


Fig. 5: Electron Microscopy showed the virus particle on cell culture MDBK cells using the negative stain (phosphotungstic acid) appeared as ovoid shape, rounded ended bilayer, ball of wool filaments are characteristic for the virus (Kitching and Smale 1986)

Nucleotide sequencing of amplified fragment:

The obtained nucleotide sequence of 519 bp PCR fragments representing the GPCR gene segment of LSDV, LSDV isolate (LSDV-GPCR-Behera-Egypt; accession no. MH289759, MH289760, MH289761, MH289762) (Egypt/Behera,) were analyzed and compared with the

published sequences of : LSDV isolate and SPV , alignment of nucleotide of LSDV isolate, tissue culture adapted LSDV with published sequences was created using ClustalW (http://www.ebi.ac.uk/clustalw/).The multiple alignment revealed that The field skin isolate of LSDV was showed to have 100% identity at the nucleotide level with LSDV of previous years (Tulman et al. 2001), The Phylogenetic tree pattern for the alignment is shown in Fig. (6):

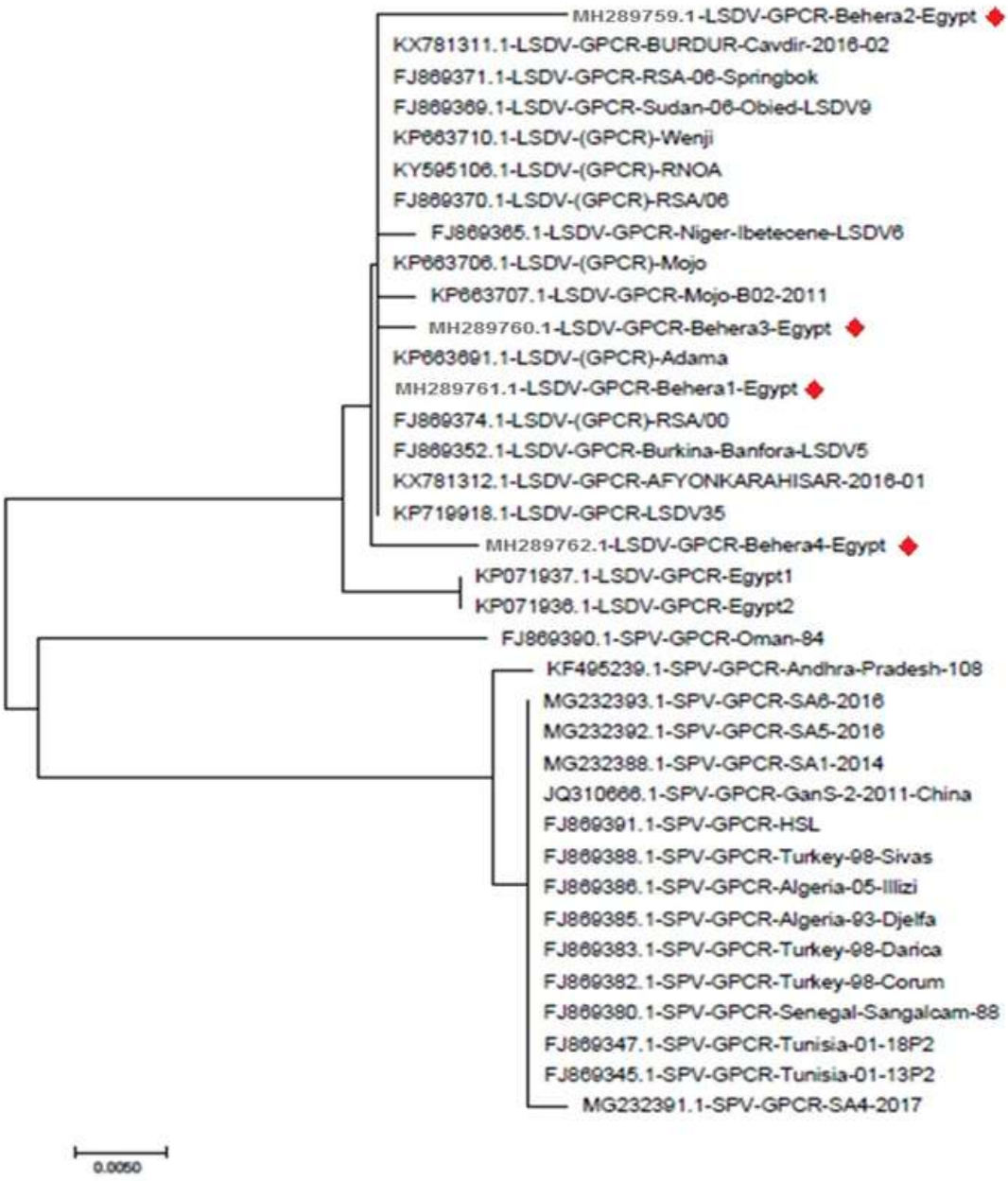


Fig. 6: Phylogenetic tree of field skin isolate of LSDV, SPV (El-Kenawy and El-Tholoth 2010; Tulman et al. 2001)

DISCUSSION

LSD is an infectious disease of cattle characterized by rapid spread and sudden appearance of lumps in skin after fever. The control of the disease to decrease the economic loss is depending on rapid and accurate diagnosis (Aspden *et al.* 2003) . In the present study, Clinical findings in suspected lumpy skin diseased cows were reported in Behera and all over Egyptian governorate. began with fever, anorexia, skin lesions in a form of nodules all over the body, which disappeared spontaneously or gathered to form large lumps. It was complicated with respiratory manifestation, corneal opacity, mastitis, dehydration and later on recumbence, such as those signs reported in 2017 (Abdalla and Gawad 1992) . Also similar to signs previously reported in buffalo infected with LSD that showed nasal discharge, lacrimation with oedema in dewlap and enlargement of lymph nodes were in according to those reported by (Elhaig, Selim, and Mahmoud 2017) . Most of the affected animals were seen in obvious cutaneous nodules all over the body (Fig.1) and in advanced cases the nodules converted to scaps and then leaving scars. The associated clinical findings were like to those previously described by (El-Tholoth and El-Kenawy 2016) .The PCR is superior in detecting LSD virus from blood and skin samples. However, virus isolation is still required when the infectivity of the LSD virus is to be determined (Tuppurainen, Venter, and Coetzer 2005) Virus isolation from blood and skin samples was sensitive and reliable, but as a single test it may be too time-consuming to use although this depends on how rapidly the diagnosis must be confirmed , consequently the results of this study indicated that PCR assay found to be rapid, sensitive, specific and could be applied on tissue samples (Fig. 4), the same conclusion was previously reported by (Kitching 2003) On conclusion PCR assay should be applied beside classical techniques of diagnosis for any cases with skin lesions as early as possible to apply adequate control measures. In the current work, LSDV was isolated by inoculation prepared skin nodules on MDBK after three successive passages producing a clear CPE at 3-4 days PI (Fig.2) (House *et al.* 1990) .The PCR-positive samples for LSD were passaged on MDBK cells for trials of isolation the current virus in the field, results of virus isolation revealed characterisitic CPE of LSDV as previously descriped (House *et al.* 1990) Identification of the Virus by SNT using specific LSD antiserum (Kitching 2003) . 4 LSD-positive samples were sequenced and the result of phylogenetic analysis indicate that the LSDV is the same affect the cattle and buffalo in previous few years with no genetic changes , which confirm infection is due to incomplete vaccinal coverage or vaccination failer (Kasem *et al.* 2017) (Abutarbush *et al.* 2016). From the previously discussed results, we can conclude that Lumpy Skin Disease is one of the major cattle diseases in Egypt. So that the animals should be quarantined and preventive measured must be done. As well may be further study must be done to determine the efficacy of SPV vaccine, to protect cattle population in Egypt against LSDV.

This study reports the circulation of LSDV in cattle population in Egypt as we isolated 4 strains of the virus which have identical nucleotide sequences closely related to Capripox viruses and in SNT these viruses characterized as LSDV.

AUTHOR DETAILS

[1]Animal health research institute, Agricultural Research Center, Egypt.
[2]Faculty of veterinary medicine, Cairo University, Egypt.

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