**Original Paper**

Molecular detection and phylogenetic analysis of ORF103 and P32 genes of *Capripoxviruses* isolated from naturally infected cattle and sheep from Kaliobya province in Egypt

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ABSTRACT

Although each capripoxvirus members including lumpy skin disease virus (LSDV), sheeppox virus (SPPV) and goatpox virus (GTPV), predominantly affect their specific host species and cause generous annual financial losses, they are no longer absolutely host-specific. The aim of the current study is to isolate and genetically characterize the CaPV strains from clinically affected sheep and cattle in Kaliobya province, Egypt during an outbreak in 2017-2018. A total fifty samples of skin lesions and nodules were obtained from clinically suspected field cases of sheep and cattle, respectively. They were prepared and isolated on chorioallantoic membrane (CAM) of embryonated chicken eggs. A typical pock lesion was seen in seventeen of the positive samples. PCR detection targeting ORF103 and P32 genes were used to identify the isolated samples. Two from each sheep and cattle samples as well as the sheep vaccinal strain used in Egypt were further sequenced, and phylogenetic analysis performed to validate the viruses. The phylogenetic analysis revealed that the field isolates from sheep were more closely related to the LSDV field isolates than to sheeppox virus. Consequently, molecular techniques based on the ORF103 and P32 genes can be used to classify and distinguish capripoxviruses. This finding could shed light on the LSDV epidemiology in the Kaliobya governorate. Cross-species infection by LSDV in sheep may have occurred in this outbreak. So, further research on the comparative study of ANK gene sequences, host range factors, of the isolate strains is required to confirm this suggested cross-species infection by LSDV in sheep.

1. INTRODUCTION

Capri pox-viruses are main cost-effective pathogens affecting global trade besides economy in endemic areas. Lumpy skin disease (LSD), sheep pox (SPP) and goat pox (GTP) viruses are members of genus Capri poxviruses in family Poxviridae. This taxonomy is established on the animal species from which the virus was first isolated, respectively, cattle, sheep, and goat, that posing non-tariff barriers on global trade initiating extraordinary morbidity and mortality in addition to reproductive performance reduction and low wool and meat quality (Madhavan et al., 2016). The brick shaped capripoxviruses are enveloped with complex symmetry of capsid enclosing double stranded DNA genome which is about 150 kb (SPPV, and GTPV) and 151 kb LSDV (Madhavan et al., 2016). SPPV and GTPV strains are not in consideration host-specific, owing to both sheep and goats can be infected by a single strain. Sheeppox virus strains cause severe disease in sheep while goats may become mildly infected with the same strains. Likewise, Sheep may be infected with virulent goatpox virus strains (Amal et al., 2008).

Whereas LSDV can cause infection in sheep and goats experimentally (Capstick, 1959). The natural infection of sheep and goats with LSDV has not been termed previously. Nevertheless, in very sporadic cases, isolation of O-240 isolate of LSD from Kenyan sheep (Kitching, 1999). Similarly, LSDV was reported in domestic water buffaloes (Ali et al., 1990; El-Nahas et al., 2011; Sharawi and Abd El-Rahim, 2014).

SPPV and GTPV are widespread in great parts of Asia, North Africa, whereas LSDV is widespread in Middle East, Africa, Asia, and Balkan Area in Europe (Toplak et al., 2017; Badhy et al., 2021). Capripoxviruses are occasionally spread into other bordering countries through animal movement trade (Babiuk et al., 2008). Transmission of LSDV is possible to be through mechanical transmission of the virus by blood-feeding vectors particularly by *Aedes aegypti* mosquitoes (El-Bagoury et al., 2018). Furthermore, direct and indirect contact to aerosols, respiratory droplets or contact with secretions and excretions (saliva, feces, milk, scabs or oronasal secretions) of infected animals can transmit the virus (Sozdutmaz and Bulut., 2010).

Diagnosis of capripoxviruses including LSDV and SPPV is usually based upon the pathognomonic clinical signs in their respective hosts and different laboratory techniques such as virus isolation on ECEs as well as identification by several PCR -and serological techniques. Serological diagnosis is time-consuming and cannot differentiate among capripoxviruses (El-Bagoury et al., 2009).

PCR is considered as sensitive, fast, and powerful tool to identify capripoxviruses from skin samples without need for further culture in addition to the useful and easily use of ORF103 gene to identify and differentiate Capripoxviruses (Abd-Elfatah et al., 2018; Khameis et al., 2018).

Capripoxviruses are phylogenetically distinct and can be differentiated by molecular tools, so the characterization of these viruses needs molecular detection targeting Capripoxviruses specific genes such as RPO30 and P32 genes (Yan et al., 2012).

Because of capripoxvirus antigenic homology in all strains, which affords immunity against all other strains protect cattle, sheep, and goats (Kitching, 2003), The Egyptian authorities (Veterinary Serum & Vaccine Research Institute [VSVRI], Egypt) produce mass annual vaccination with sheep pox vaccine, but almost every summer outbreaks of LSDV still reported. For example, a widespread LSD outbreak in summer of 2006, affecting sixteen governorates knockout Egypt (Awadin et al., 2011) and it recurred in (2011, 2014) (Amin et al., 2015, El-Tholoth and El-Kenawy, 2016), and in (2017, 2018) (Zeedan et al., 2019; Allam et al., 2020).

Consequently, the key objective of this work was to study the genetic relationship between field isolates of SPPV and LSDV from clinically suspected sheep and cattle during occurrence of LSDV in Kaliobya governorate, Egypt during 2018. Established on trials for viral isolation and molecular basis to improve novel insights into the biology and aid the development of a new method for capripoxviruses control.

2. MATERIAL AND METHODS

2.1. SPPV vaccinal strain and Reference LSDV

Reference Egyptian SERVAC CAPRI-S vaccine, the virus was cultivated on Vero cells according to (Rizkallah 1994), Kindly obtained from Pox vaccines Research Department, (VSRI), Abbassia, Cairo, Egypt. The virus had a titer of 10^6 TCID₅₀ / ml. The viral strain was stored at -70° C and was used for PCR and sequencing. Lumpy skin disease virus (LSDV) Neethling reference strain was kindly obtained from the Department of virology, Animal health Research Institute, Dokki, Giza. It was used as positive control in PCR.

2.2. Clinical history and sample collection

For the period of 2018, epidemics of LSDV hit cattle in Kaliobya Province, Egypt, and were reported to have developed skin nodules besides sporadic clinical cases in sheep. Most of cattle cases had severe clinical symptoms with some mortalities beside scattered nodules and scabs all over their body parts including vulva, teat, and forelimbs (Fig. 1), While sheep showing rise of body temperature (range between 40° C- 40.8° C), low food intake, skin lesions in different sites include all face, inside lips, eyes, nostrils, mammary glands, Inner side of the thigh and under the tails (Fig. 2). Based on history exposed, the cattle were vaccinated against LSDV the local vaccine (SERVAC CAPRI-C) produced by VSVRI while sheep were unvaccinated against sheep poxvirus. Total 50 skin

nodules skin nodules were aseptically collected from cattle and contacting sheep (Table1) and prepared for virological examination (Rao and Bandyopadhyay, 2000).



Fig. (1): Suspected cattle with LSDV infection showed: Panel A& B; Nodular and crusted lesions scattered all over their body parts



Fig. (2): Suspected sheep with SPPV infection showed: Panel A& B; Pox lesions at the head of sheep, inner side of the thigh and under the tail

2.3. Trials for Virus isolation on CAM of SPF-ECEs

Prepared Skin nodules suspension in phosphate buffered saline (PH 7.4) with 100 U/ml penicillin and 100 mg/ml streptomycin was lysed by freezing and thawing for three times then centrifuged at 2000 g for 30 minutes. The supernatant was purified and filtrated through a 0.45μ m pore-size cellulose acetate filter. SPF-ECEs after 9-10 days of incubation were inoculated with 0.1 ml of the supernatant by CAM route according to (Mahmoud and khafagi, 2016). The examination of eggs was done daily, terminating with 5–7 days post-inoculation. The embryos that died within the first 24h were considered as nonspecific deaths.

Table (1): Virus isolation on chorioallantoic membrane (CAM) of embryonated chicken eggs (ECEs)

Skin nodules	Number of inoculated samples	Number of positive samples	Number of negative samples
Sheep	25	10	15
Cattle	25	7	18
Total	50	17	33

2.4. Nucleic acid extraction and identification of viral isolates using PCR

Viral genome extraction from samples was performed by Thermo scientific @ Gene JET Genomic DNA Purification Kit (Catalogue No. K 0702), (Thermo Scientific; EU, Lithuania). It is designed for rapid and efficient purification of high-quality genomic DNA. It yields purified DNA of more than 30 kb in size. Isolated DNA can be used directly in PCR following the manufacturer's recommendations. The used Primer set was synthesized by Metabion (Germany) as outlined in Table (2).

A total of 25 μ l reaction mixture encompassing 12.5 μ l of Using 2x AmpliTaq Gold™ 360 Master Mix kit (Thermo Fisher Scientific, Waltham, MA, USA; Catalog No. 4398876), 1 μ l of 20 pmol concentrations forward and revers primer, 4.5 μ l of Nuclease-free water, and 6 μ l of DNA sample. A DNA T3 thermal cycler (Biometra®) was used for amplification with cycling condition targeting

ORF103 gene according to (Zhu et al., 2013) , and according to (Al-Shabebi et al., 2014) for targeting fusion protein P32 gene.

Table (2): Oligonucleotide primers sequences targeting ORF 103 and P32 genes

Gene	Sequence	product	Reference
ORF 103	F. 5'ATGTCTGATAA- AAAATTATCTCG3'	570 bp	Zhu et al., 2013
	R. 5'ATCCATACCAT- CGTCGATAG3'		
P23	F. 5'CTAAAATTAGA- GAGCTATACTTCTT 3'	390 bp	Al-Shabebi et al., 2014
	R. 5'CGATTTCCATA- AACTAAAGTA 3'		

2.5. Gel electrophoreses analysis of the amplified product

The PCR products were alienated by electrophoresis on 2% agarose gel (Bioline Company, Japan, Cat. No. BIO-41025) in 1x Tris-Borate EDTA buffer containing ethidium bromide at room temperature. The fragment sizes of run amplified products in the gel was determined by GeneDirex50bp Ladder RTU of 17 size range bands: 50-1500 bp (Cat No. DM012-R500) . The gel was photographed by Molecular Imager Gel Doc™ XR+ Imaging system (BIO-RAD, USA) and the data was explored through computer software.

2.6. Sequencing of amplified products and Phylogenetic analysis of sequencing data

The obtained specific PCR bands from the viral isolates on CAMs were purified using QIAquick® PCR purification Kit (Qiagen Inc.; Valencia, CA, USA; Cat. No. 28104), followed by forward and reverse sequencing on an Applied Bio-systems Sequencer (ABI, 3130, USA) using a ready reaction BigDye Terminator V3.1 cycle sequencing kit (Cat. No: 4336817). The obtained sequence data were aligned using A Basic Local Alignment Search Tool analysis (Altschul et al., 1990) .CLUSTAL W (multiple sequence alignment programs) of Laser gene DNA Star software was established for A comparative analysis of sequences (Thompson et al., 1994). Phylogenetic analyses were done using maximum likelihood, neighbor- joining and maximum in MEGA6 software according to (Tamura et al., 2013).

3. RESULTS

3.1. Virus isolation on chorioallantoic membrane (CAM) of embryonated chicken eggs (ECEs):

Isolation of capripoxviruses from skin lesions and nodules on CAMs of ECEs up to 3rd passages (Table 1) showed congestion and clotting of blood in CAM blood vessels, death of embryo, thickening, edema, presence of pock lesions on the inoculated CAMs (Fig. 3; Panel B).

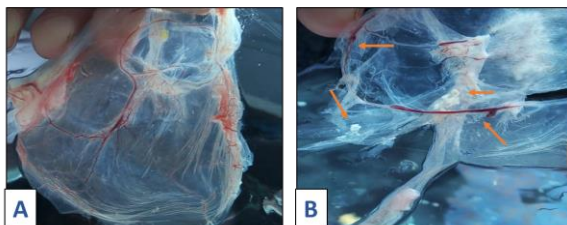


Fig. (3): Representative pictures for the viral isolation on CAMs of SPF-ECEs. Panel A; non inoculated control negative. Panel B: CAM showing typical pock lesions, death of embryo, thickening, and edema on the inoculated membranes (indicated by arrows)

3.2. Polymerase chain reaction (PCR):

Detection of nucleic acids in harvested CAMs using conventional PCR was performed in 6 positive CAM prepared samples from sheep and cattle (Three samples for each). CAM samples were selected for pursuing PCR because of their higher titer of viruses than the original samples from skin nodules. The amplification and running of characteristic 570 bp fragments of ORF 103 genes were shown in Figure (4). For further confirmation, the amplification of 390 bp of P32 gene from the same selected positive isolates (Fig 5).

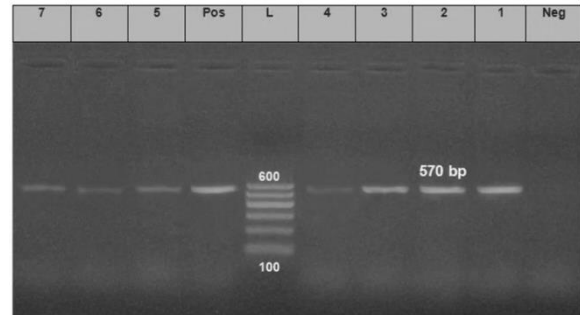


Fig. (4): The Electrophoretic pattern of the PCR products based on ORF 103 of capripoxvirus to the viral isolates of CAMs on the gel electrophoresis (1.5%). Amplicon size was detected at 570 bp which considered positive results for this size. L: DNA ladder, Pos: Positive PCR products from LSDV reference strain, Neg: negative PCR results, Lanes (1, 2, 3): positive PCR products of field isolates from sheep, Lanes (5, 6, 7): positive PCR products of field isolates from cattle nodules, Lanes (4): Positive PCR products from sheeppox virus vaccinal strain

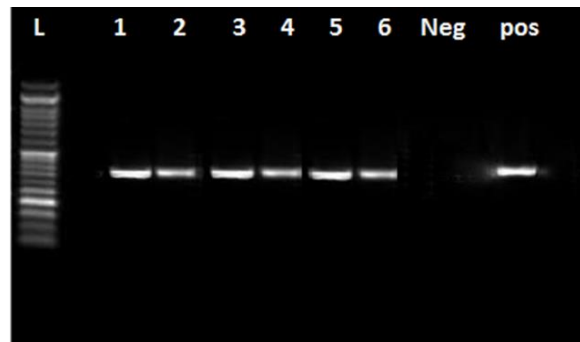


Fig. (5): The Electrophoretic pattern of the PCR products based on P32 of capripoxvirus to the viral isolates of CAMs on the gel electrophoresis (1.5%). Amplicon size was detected at 390 bp which considered positive results for this size. L: DNA ladder, lanes (1, 2, 3): positive PCR products from field skin isolates of SPPV from sheep. lanes (4, 5, 6): positive PCR products from field skin isolates of LSDV from cattle, Pos: Positive PCR products from LSD virus reference strain, Neg: Negative PCR results

3.3. Nucleotide sequencing of amplified ORF103 and P32 gene fragments:

The obtained nucleotide sequence of 570 bp PCR fragments representing the virion core protein ORF103 gene of LSDV isolates from cattle and sheep pox isolates from sheep (two selected samples for each) as well as the sheeppox vaccinal strain were analyzed and compared with the published sequences of capripoxviruses on the GenBank database.

The multiple alignment of nucleotide sequences revealed that LSDV isolates were closely identical (94.29 %). The sheeppox isolates were identical to each other and with sheeppox vaccinal strain (98.68%) identity. On comparing with other sequences of capripoxviruses, the field skin isolate of LSDV from cattle were showed to have more than 98% identity at the nucleotide level with Egyptian LSDV isolates from neighboring governorates in Egypt. Surprisingly, the field skin isolates of sheeppox virus from

sheep revealed higher identity percent with LSDV isolates than to SPPV and sheeppox vaccinal strains (Fig 6). Sequence analysis of amplified attachment gene P32 (390 bp) of field skin isolate of LSDV, and field skin isolate of SPPV was performed. Comparison of the two sequences showed that they share 92.33% similarity with each other. On the same pattern of ORF103 gene sequence analysis, P32 gene sequence analysis showed that both field isolates of LSDV from cattle and SPPV from sheep were more closely related to LSDV (Fig 7).

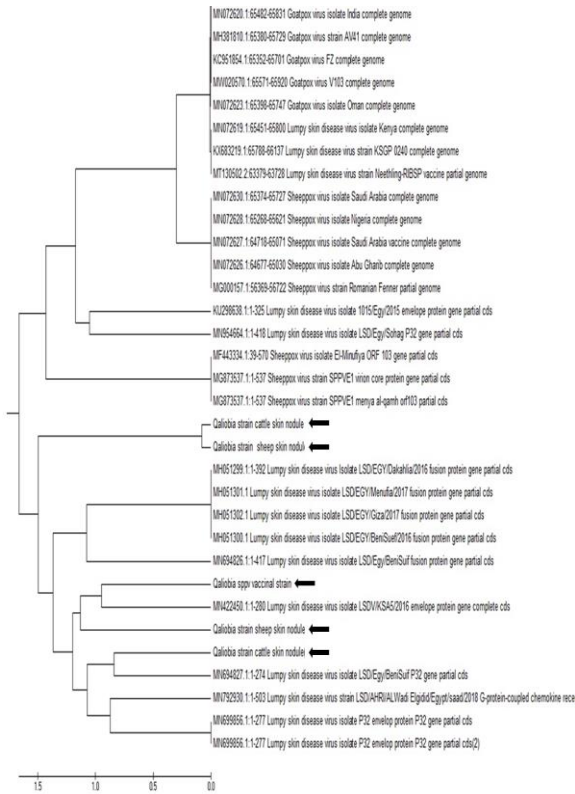


Fig. (6): Phylogenetic tree using neighbor-joining method based on ORF 103 gene nucleotide sequences of our new capripoxviruses isolates (indicated by arrows) with other sequences of retrieved from the GenBank database. The scale bar means nucleotide changes or substitutions per site

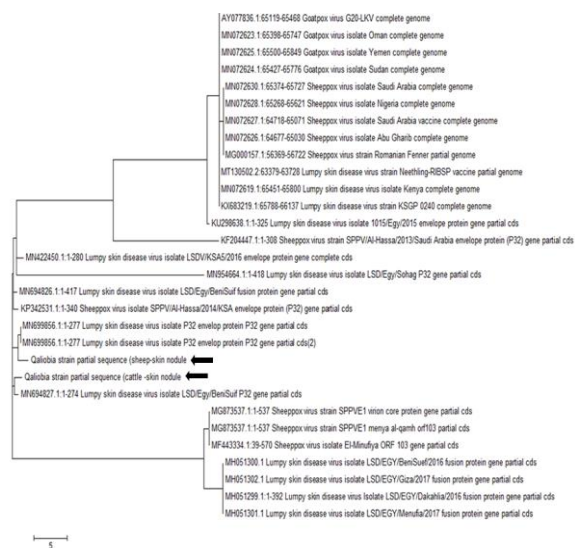


Fig. (7): Phylogenetic tree using neighbor-joining method based on P32 gene nucleotide sequences of our new capripoxviruses isolates (indicated by arrows) with other sequences of retrieved from the GenBank database. The scale bar means nucleotide changes or substitutions per site

4. DISCUSSION

Regardless of the annual strategy of vaccination against capripoxviruses, the epidemics of members are hit the Egypt practically every summer causing financial losses in agriculture Egyptian economy leaving small land farmers with sore economic harms and veterinary authorities with insufficient control actions .

Quick and exact diagnosis of the disease followed by hasty implantation of control methods such as(slaughtering ,program of ring vaccination and restriction movement) are essential to control borderline transmission and spread of the infection (El- Tholoth and El-Kenawy 2016).

Many outbreaks of LSDV in Egypt during 2017 and 2018 along with presence of sporadic cases of sheeppox in area that affected by LSDV have been reported, hence study the genetic homology between the SPV field isolates and LSDV isolates was performed.

In the current study, Trials for isolation of capripoxviruses showed characteristic pock lesions, edema and thickening on CAMs of SPF-ECES and these results came in agreement with (Awad et al., 2010; El-Nahas et al., 2011; El-Tholoth and El-Kenawy, 2016; Lamya et al., 2017) who stated the similar characteristic lesions and mentioned that both LSDV and SPPV were present at great levels in skin epithelial cells which reflected the predilection site for these viruses.

Confirmation of the harvested CAMs by conventional PCR targeting ORF103 virion core protein and P32 genes (attachment protein) demonstrating the extraordinary sensitivity of PCR as a specific method for laboratory corroboration of capripoxviruses which acclaimed by (Amal et al., 2010; El-Kenawy and El-Tholoth, 2010 and 2011). Even though the fact of host species specificity of capripoxvirus, these viruses are not exactly host specific and have ability to infect different animal species, for instance SPPV and GTPV strains can experimentally and naturally cause diseases in both sheep and goats (Bhanuprakash et al., 2010; Yan et al., 2012; Hani et al., 2015). Data constructed only on the host animal species from which the strain was first isolated is insufficient to categorize CaPVs and owing to the identical close antigenic homology among CaPVs, serological methods cannot distinguish SPPV and GTPV. Consequently, molecular methods constructed on the P32, ORF 095, and ORF 103 genes followed by sequencing actions are proficient for categorizing the strain and phylogenetic analysis to compare the relations between field strain and other capripoxviruses recorded in NCBI (Zhu et al., 2013).

The analysis of aligned sequences of amplified ORF 103 and P32 genes of field isolates of LSDV and SPPV from cattle and sheep respectively revealed relative homology to each other this may indicate that field SPPV isolate may be LSDV that altered its host to infect sheep. Our findings came in agreement with the results recorded previously (El-Kenawy and El- Tholoth 2010), and with kitching 1999, who isolated O-240 strain of LSDV from Kenyan sheep and also Tuppurainen et al., 2014; Vandebussche et al., 2016, who proved that KS-1 strain isolated from sheep and goat with pox lesion in Kenya was LSDV.

Phylogenetic analysis of nucleotide sequences retrieved from GenBank confirmed that capripoxviruses originated from a common ancestor LSDV and categorized into three distinctive clusters dependent on the surveillance of disrupted LSDV genes between GTPV and SPV (Tulman et al., 2002; El-Kholy et al., 2008). On the other hand, while LSDV can experimentally infect sheep and goat (Capstick, 1959), Our isolates either from cattle or from sheep were integrated into LSDV group and were more

genetically identical, signifying possible transmission of cattle LSDV to sheep under natural conditions.

5. CONCLUSION

Phylogenetic analysis constructed on nucleotide sequence of the P32 and ORF103 genes of different positive PCR products displaying high genetic similarity between the isolated strain from (sheep, cattle) and LSD virus. These molecular techniques are proficient for Capripoxviruses characterization and differentiation. This finding may afford different information on the epidemiological aspect of LSDV in Kaliobia governorate. Thus, further studies should be implemented and assessed on efficacy of frequently used sheep pox vaccine in applicable vaccination strategy for protection of cattle against LSDV and sheep against sheep pox virus in Egypt.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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