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Diagnosis of Sheep Pox Viral Infecion in Al wadi Aljadid governorate, Egypt

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Abstract

Pox is one of the listed and notifiable disease affecting cattle, sheep & goat. Sheep pox causes a significant loss of reduced productivity and lower quality of wool and leather. This study, was conducted to identify 49 scabs skin samples from clinically diseased sheep was investigated by different laboratory techniques by using specific pathogen free (SPF) Embryonated chicken egg (ECE) chorio allantoic membrane (CAM) inoculation, electron microscope Examination (EM), Indirect fluorescent antibody test (IFAT), polymerase Chain Reaction (PCR) & partial ORF 103 gene sequence analysis. Positive samples obtained from ECE, EM and FAT were used for molecular characterization. The virus was identified through sequence and phylogenetic analysis of the RPO30 gene, open reading frame (ORF 103) genes. A phylogenetic analysis was performed using partial sequencing of the ORF103 gene and comparing with reference sheep pox viruses isolates obtained from Gene Bank. The results of the sequence analysis were similar among themselves (99-100% identity), they were found different from the other sheep pox viruses around the world. This study provides firstly molecular analysis (phylogenetic) of sheep poxviruses from Aldaakhiluh Alwadi Aljadid governorate, Egypt.

Keywords: *Sheep pox, ECE, IFA, EM, PCR, ORF 103 gene & RPO30 genes*

Introduction

Sheep pox virus (SPPV) and goat pox virus (GTPV) belong to Capripox virus genus (CaPV), Poxviridae family which is the causative agent of sheep and goat pox respectively **(Zhou *et al*., 2012** and **Babiuk *et al*., 2008)**. Sheep pox and goat pox classified as notifiable animal diseases by the World Organization for Animal Health (OIE) and considered endemic in Africa, Middle and Far East, and the India **(Zhou *et al*., 2012).** Sheep pox (SPPV) is a highly contagious, host specific, viral infection with high morbidity (75-100%) and mortality (5-50%) in sheep**.** Due to cross-protection within Capripoxviruses genus, sheep pox virus (SPPV) vaccines have been widely used for cattle against lumpy skin disease virus (LSDV) **(Tuppurainen *et al*., 2014).** SPPV and GTPV genomes are approximately 150 kbp and are strikingly similar to each other, exhibiting 96% nucleotide identity over their entire length **(Tulman *et al*., 2002).** Transmission of SPPV and GTPV occurs by direct and indirect contact to aerosols, respiratory droplets or contact with oronasal secretions produced by acutely infected animals **(Kitching and Taylor, 1985 and Verma *et al*., 2011**). Transmission through contact exposure with abrasions or mechanical transmission by arthropod vectors may also occur (**Sherry *et al*., 2014).** The disease is characterized by fever, generalized skin and internal pox lesions, lymphadenopathy and death **(Varshovi *et al*., 2009)**. The virus neutralization test is the most specific serological test, but because immunity to Capripox infection is predominantly cell mediated, the test is

not sufficiently sensitive to identify animals that have had contact with the virus and developed only low levels of neutralizing antibody. Agar gel immuno-diffusion (AGID) and indirect immuno-fluorescence tests (IFA) are less specific due to cross-reactions with antibody to other poxviruses **(Kitching and Carn, 2010).** However, prophylactic immunization of all susceptible animals with a potent and efficacious vaccine was the best way to control the disease especially in areas where these diseases are endemic. The diagnosis of sheep and goat pox has traditionally used characteristic clinical signs with polymerase chain reaction (PCR) for virus isolation. PCR has provided a sensitive and powerful technique for identifying this infection **(Mangana-Vougiouka *et al*., 2000; Hosamani *et al*., 2004; Zheng *et al*., 2007**). PCR-based diagnostic methods were effective in diagnosis of goat pox and sheep pox in suspected biopsy samples in the field **(Rao and Bandyopadhyay, 2000**). In this study, we used published molecular tools to identify the sheep pox virus, and 2 genes (RPO30 and ORF 103) were used for the first time to characterize sheep pox virus (SPPV) from Aldaakhiluh Alwadi Aljadid governorate, Egypt.

Materials and Methods

Samples

In the present study, 49 pock tissues samples were collected from Wadi el-gidid, Egypt in years 2017-2018 to be processed in a trial for sheep pox virus isolation and characterization. 50 % glycerin buffer saline, 10% suspension in phosphate buffer saline (PBS) containing 100 IU/ml pencillin, 100 ug/ml streptomycin and 50 units mycostatin, the mixture was incubated at 25 C 1 hour then frozen and thawed 3 times at –20˚C and centrifuged at 3000rpm /10 minutes, the supernatant fluid was used as inoculums for virus isolation in embryonated chicken egg (ECE).

Virus isolation: Supernatant fluids of scabs injected on chorio allantoic membrane (CAM) according to **Sabban, 1957**. Samples were isolated in 11 days old in specific pathogen free (SPF) embryonated chicken eggs(ECE) Koum Oshiem SPF, Fayoum, Egypt) and harvesting of CAMs.

Indirect fluorescent antibody test:

Antisheep IgG conjugated with fluorescent isothiocyanate (developed in rabbits and supplied by sigma). Sheeppox virus: Reference virus and antiserum were kindly supplied by veterinary serum and vaccine Research Institute. Abbasia, Cairo.

Tissue Culture:

VERO cell culture propagated with Eagle's minimum essential medium supplemented with 10% fetal calves serum kindly obtained from the Department of virology, Animal health Research Institute, Dokki, Giza.

Electron microscope examination:

Samples showed positive pock lesions on CAMs, were prepared for transmission electron microscopy in EM unit by **Mahmoud *et al*., 2010.**

, National Research Centre and examined as described (Agriculture faculty)

Agar gel immune-diffusion test: It was applied according to **OIE 2012**

Polymerase chain reaction (PCR)

DNA Extraction

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in kit. Extraction step for viral DNA directly from processed CAMs was done using a commercial kit (QIAamp. viral DNA Mini Kit).

Oligonucleotide Primers :

Primers used were supplied from Metabion (Germany) are listed in table (1).

PCR : Primers were utilized in a 25 µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler**.**

Table (1). Primers sequences, target genes, amplicon sizes and cycling` conditions.

Target gene	Primers	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
RPO30	TCTATGTCT TGATATGT GGTGGTAG AG-TGATTAGG TGGTGTATT ATTTTCC	151	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Santhamani *et al*., (2013)**
ORF 103	ATGTCTGA-TAAAAAAT TATCTCG ATCCATAC CATCGTCG ATAG	570	94˚C 5 min.	94˚C 30 sec.	52˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Zhu *et al*., (2013)**

Analysis of PCR Product

The product of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Purification and sequence of PCR product

PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul *et al*., 1990)** was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of Lasergene DNAStar **Thompson *et al*., (1994)**.

Analysis of sequenced isolate:

Nucleotide sequences were analyzed with **DNA Star program** (version 7.0, CA, USA) and Phylogenetic tree based on 570 nucleotide sequences ORF 103 gene constructed by the neighbor-joining method of **(DNA Star software)**.

Result

to determine existence of virus particles; we inoculated the samples on to CAMs of SPF ECE 11 day old . Characteristic pock lesions for poxviruses were seen in 35 out of 49 suspected samples, After the samples underwent 7passages in CAMs of SPF ECE , large number of pock lesions were prominent **Figure (1).** The supernatant of positive CAMs displayed the characteristic capripoxvirus virions on electron microscopic examination **Figure (2)**. The positive results represented by the presence of negatively stained oval-shape virus particles. The virions were approximately 200 nm longand 150 nm wide **Figure (2).**

Agar gel immune-diffusion test results were negative**.**

Tissue culture results also negative

IFAT on CAM-positive ECE Virus-specific green fluorescence was found in the infected ECE- None of the control CAM was stained.

To gain further information from the viral sequences, we implemented a PCR to diagnose CaPV infection from the supernatant of positive CAM. To confirm the causative agent, we amplified of RPO30 and ORF 103 genes via PCR **Figure (3 & 4).** Two specific products with the expected size **(Figure 3,4),** including the RPO30 (151bp) and ORF 103 (570 bp) genes, were amplified from the sample extracted from the supernatant of positive CAM. The purified of ORF 103 genes via PCR 570 bps Specific product size isolate was sequenced, subjected to similarity analysis, analyzed phylogenetically & sequence analysis was submitted to gene bank and identified under accession number (MK256477), subjected to similarity analysis, analyzed phylogenetically **Figure (5).**

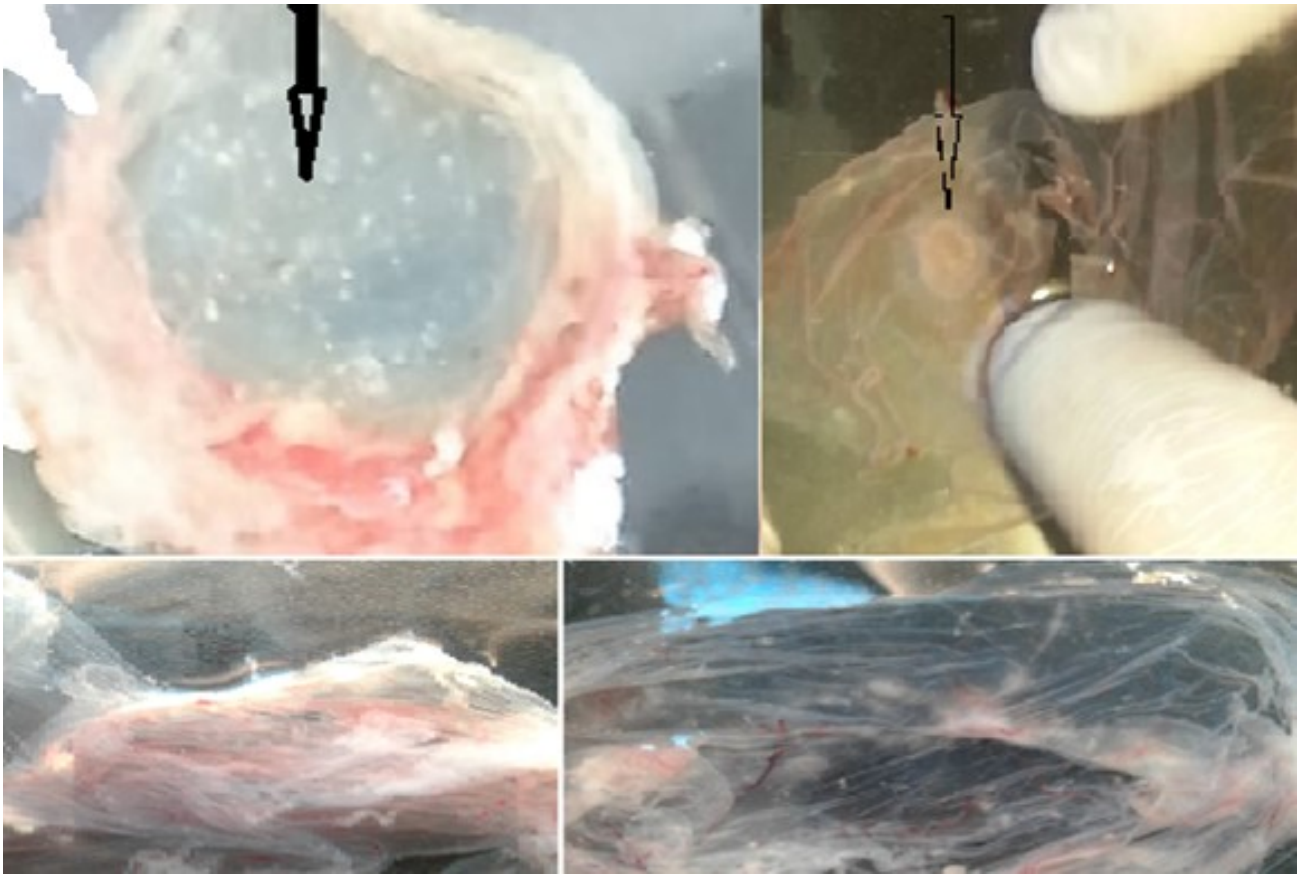

Figure (1). Large number of pock lesions diffused on CAMs

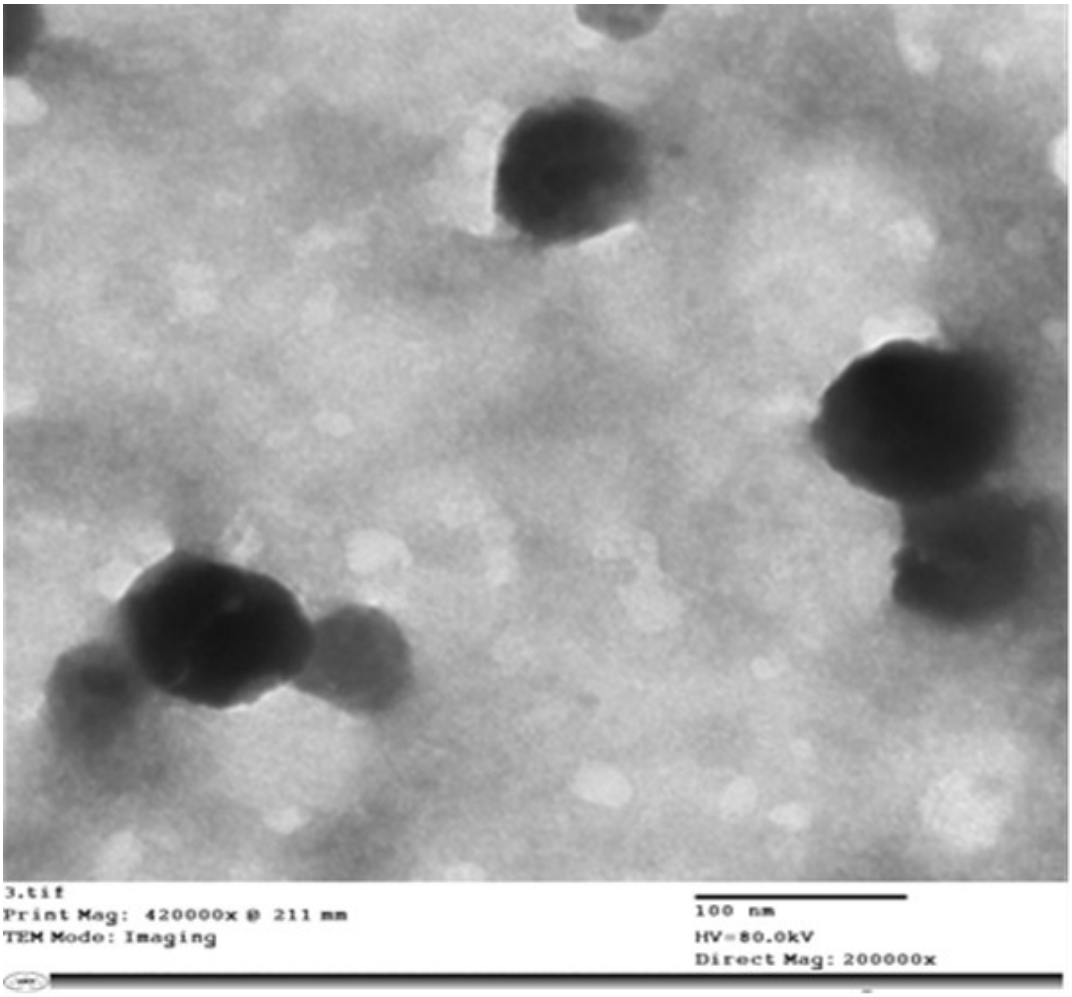

Figure (2). Electron microscopic image of capripoxvirus

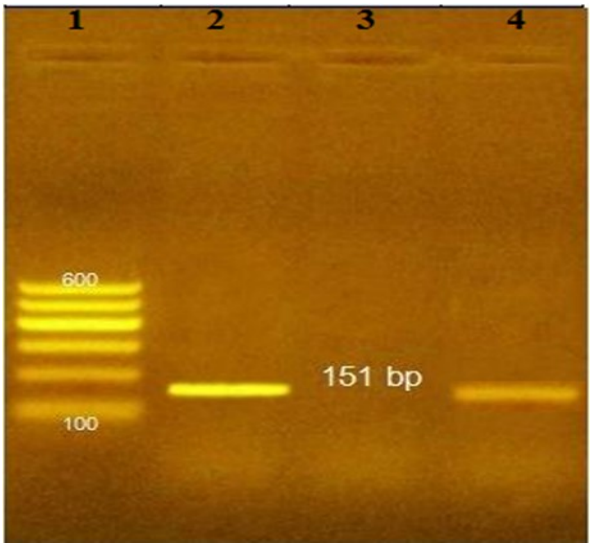

Figure (3). Detection of sheep pox viral DNA after amplification by PCR.
Lane1: Ladder, Lane2: positive control, Lane 3: negative control &Lane 4: sheep pox Partial DNA suspected isolate.

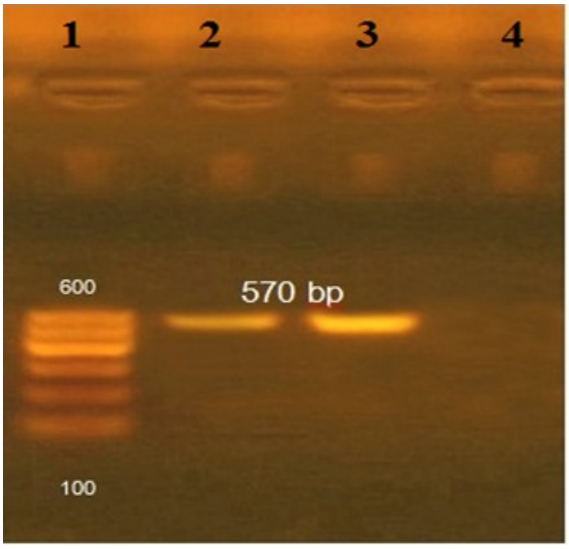

Figure (4). Amplification of ORF 103 gene by PCR
Lane1: Ladder, Lane2: positive control,Lane 3:sheep pox Partial DNA suspected isolate &Lane 4: negative control.

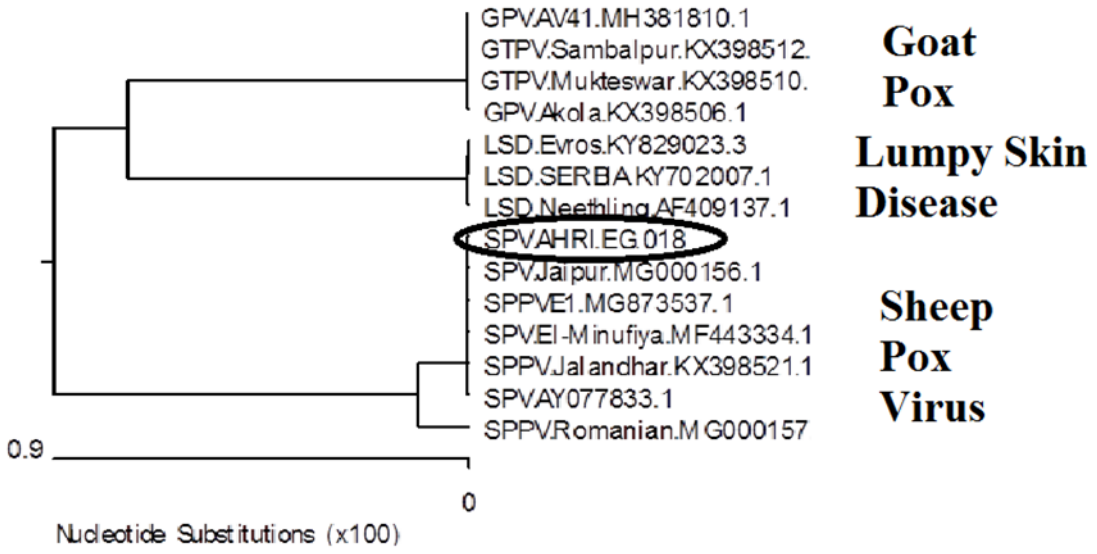

Figure (5). Phylogenetic tree of Sheep pox virus isolate (SPV-1.EG.AHRI.Wady El-gidid) based on 570 nucleotide sequences of ORF 103 genes drawn by (DNA-Star version 7.0) & Sequences of other strains were obtained from GenBank.

Discussion

In the present work, we could detect 35 CAM pock lesions out of 49 initial screened samples but not accurate identification, so we used other confirmatory tests as Agar gel immunodiffusion (AGID) test were negative may be this test need the concentrated virus and antiserum. AGID test cannot be recommended as a serological test for the diagnosis of capripox because of the cross-reaction with antibody to contagious pustular dermatitis virus, which is the main differential diagnosis.

A consequence of this cross-reaction is many false-positive results **(OIE, 2012).** The tissue culture were negative owing to need of specific cells - Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most susceptible **(OIE, 2012)**.

Indirect fluorescent antibody test were positive but Cross reactions can occur with orf, bovine papular stomatitis virus and perhaps other poxviruses **(OIE, 2012).**

Although electron microscopy has been clinically used for a long time in the detection of sheeppox virus, different strains of viruses within the family Poxviridae sheeppox, goatpox, Lumpy Skin Disease virus cannot be morphologically discriminated **(Babiuk *et al*., 2008).** CaPVs are strictly host-specific **(Babiuk *et al*., 2008))** However, there are several reports indicating the SPPV could infect goats and GTPV could infect sheep **(Bhanuprakash *et al*., 2010)** and In very rare cases, isolation of O-240 isolate of LSD from Kenyan sheep, was reported **(Kitching, 1999).** Members of the Capripox genus cannot be distinguished using serological methods **(Kitching, 2003).** Information based only on the host an-imal species from which the strain was first isolated is inadequate to identify CaPVs, and owing to the very close antigenic relationship among CaPVs, conventional serological methods cannot distinguish SPPV and GTPV**.** Therefore, effective molecular techniques are necessary for complete and perfect strain differentiation **(Bhanuprakash *et al*., 2006 & 2010).** PCR described combines high specificity and sensitivity with speed. PCR was therefore shown to be the method of choice for sheep poxvirus diagnosis and shown to be more sensitive than antigen trapping ELISA **(Ireland and Binepal, 1998).**

Sheeppox virus infection requires rapid and correct diagnosis to prevent any outbreaks. More effective preventive precaution in addition to the early detection of infected animals should be performed to control programs **(Zro *et al*., 2014).** So we used in this study 2genes for identification of sheep pox. . RPO30 gene based PCR assay in combination with gene sequencing helps in molecular epidemiological studies of CaPV infection. Different sheep pox virus strains could not be differentiated by analysis of their proteins and serological methods **(DAVIES and OTEMA, 1981).** Sequencing analysis is a very effective method to help understand the genomic nature of the virus. Gene Sequence analysis can be used in differentiating SPPV, GTPV and LSD genetic relationship among different virus strains **(Lamien *et al*., 2011, Hosamani *et al*., 2004; Zhou *et al*., 2012).**

Sequence and phylogenetic analysis showed that there was a close relationship among 570 nucleotide sequence of ORF 103 genes of SPPV-1/Wadi el-gidid /2018/Egypt (MK256477) and Sheep pox virus strain SPPVE1 virion core protein gene (MG873537.1), Sheep pox virus strain Jaipur (MG000156.1), Sheep pox virus isolate SPPV-GL, (KT438551.1), Sheep pox virus isolate El-Minufiya ORF 103 gene (MF443334.1), Sheep pox virus isolate SPPV/57-2823/Pune/2007 (KX398522.1), Sheep pox virus strain Romanian Fenner, partial genome (MG000157.1), Sheep pox virus isolate SPPPV/30-02/Ahmedabad/2008 (KX398520.1) & Sheep pox virus isolate SPPV/Ahmedabad/2009/P5 virion core protein gene (KX398517.1) shared identical relationships with other Capripoxviruses isolated from different regions (the nucleotide sequence identities were 100%) as shown **Figure (5)**. ORF 103 genes of SPPV/A1/Wadi el-gidid /2018/Egypt show 98% identity with Lumpy skin disease virus isolate Evros / GR/15 (KY829023.3), Lumpy skin disease virus isolate SERBIA/ Bujanovac/2016 (KY702007.1), Lumpy skin disease virus NW-LW isolate Neethling Warmbaths LW (AF409137.1), Goat pox virus isolate AV41 (MH381810.1), Goat pox virus isolate GTPV/ Sambalpur /2001/P6 virion core protein gene (KX398512.1), Goat pox virus isolate GTPV 143/ Mukteswar /2012 virion core protein gene (KX398510.1) & Goat pox virus isolate GTPV/ Ladakh /2001/P3 virion core protein gene (KX398508.1).

Conclusion the study presents molecular characterization of SSPV isolate in Alwadi Algidid governorate, Egypt. Capripox case was detected and identified as SPPV-1.EG.AHRI.Wadi-Elgidid. Based on sequence and phylogenetic analysis, genetic relationship, as well as viruses isolated from other regions. The results showed that molecular techniques based on the PRO30 and ORF 103 genes are efficient for the characterization and differentiation of CaPVs.

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