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Molecular and phylogenetic analysis of Sheep pox virus isolate from Kaliobyia province in Egypt

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ABSTRACT

Sheep pox virus (SPPV) is one of members of genus *Capripoxviruses* in the *Poxviridae* family. It is considered a threat affecting sheep and causes significant financial losses in agriculture economy all over the world. This study was designed for isolation and molecular detection for SPPV from skin nodules of clinically suspected sheep in Kaliobyia province. Direct detection of the virus was carried out also on milk samples (n=25) as good entrants for early detection of SPPV before the onset of clinical signs. Molecular detection and phylogenetic analysis of SPPV were performed based on open reading frame (ORF) 103 gene. Suspected skin nodules were used for virus isolation on Chorioallantoic membrane (CAM) of specific pathogen free embryonated chicken eggs (SPF-ECEs) for three successive passages. Two virus isolates and two milk samples were identified from the tested samples by polymerase chain reaction (PCR) using primers targeting ORF 103 (with an amplicon size of 570bp). Furthermore, one milk sample PCR product was sequenced and phylogenetically analyzed based on ORF103 gene and revealed that our new isolates was identical to Sheep pox virus isolated from Minufiya governorate with 97.1% homology. Also, it showed quite identity with other reference sequences of SPPV strains retrieved from the GenBank database. This study may afford to fresh insights about the epidemiological aspect of the circulating SPPV in Egypt.

1. INTRODUCTION

Sheeppox is one of the most endemic diseases in sheep and reported in many countries worldwide including North Africa and Middle East and also, sporadic outbreaks occurred in South-Eastern Europe (OIE, 2017). It causes significant financial losses in agriculture economy owing to the disease's consequences that vary from damage of wool to losses in sheep flocks.

Sheeppox virus (SPPV) is one of genus Capripoxviruses, subfamily Chordopoxvirinae in the Poxviridae family (OIE, 2017). Capripoxviruses also compromising from Lumpy skin disease virus (LSDV) virus and Goat pox virus (GPV) (Das et al., 2012).

SPPV Genome consists of double-stranded DNA of about 150-kbp. It has an envelope and cubic structure (Yilmaz et al., 2016). The extracellular enveloped virion (EEV) and the intracellular mature virion (IMV) are the two main infectious formulas of Poxviridae family members (Olga et al., 2016).

Sheeppox virus causes disease in sheep characterized by fever, generalized papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death (OIE, 2017).In Egypt sheep pox been known for many years. Several outbreaks of Sheeppox virus (SPPV) have been occurred in Kafr El-Sheikh province (Tawfik et al., 2001), Giza province (Mervat et al., 2006) and at different Governorates of upper Egypt (Amal et al., 2008; Mahmoud and Khafagi, 2016) and Kaliobyia governorate (Selim et al., 2016).

Despite of locally produced vaccine against sheep pox in Egypt and an applicable strategy for vaccination, the virus is still causing a problem among small ruminants, that limits the new breeds trade and sheep wool industry. the prodigious financial harms mainly resulted from mortalities in new lambs, low production rate, and trade limitations (Aldaiarov et al., 2016).

SPPV diagnostic strategy is usually established on highly specific clinical signs, virus isolation, virus neutralization test (VNT) and molecular identification via polymerase chain reaction (PCR). Also, other procedures such as ELISA and western blot technique (OIE, 2008). PCR established on (ORF) 103 genes for genotyping and phylogenetic analysis was used to detect and differentiate SPV from other similar infections affecting sheep, due to its fast result, specificity and sensitivity (Zhu et al., 2013).

PCR is considered as sensitives, rapid, and powerful technique to identify capripoxviruses including SPPV from skin samples without need to 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).

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The objective of this work was to highlight the clinical signs of the disease, to confirm the presence of the nodular form by PCR method and deemed the milk samples as decent entrants for SPPV detection before the beginning of SPPV clinical manifestations, also studying the utility of ORF 103 genes for molecular detection and phylogenetic analysis of SPPV local strains that may afford different evidence on the SPPV epidemiological aspect in Egypt.

2. MATERIAL AND METHODS

2.1. Sheep pox virus (SPPV) strain

Reference Romanian strain of SPPV adapted on Vero cells was Kindly obtained from (VSRI, pox department), Abassia, Cairo, Egypt. The virus had a titer of 10^6 $TCID_{50}$ / ml. The viral strain was stored at – 70 ˚C. It was used as positive control in PCR detection methods for SPPV.

2.2. Clinical Samples

Skin nodules (n= 25) were collected from different localities in Kaliobyia province during 2018. Samples were collected from unvaccinated sheep showing clinical signs as nodular skin lesions and crusts on the affected sheep that shown in Figure (1). The samples were transferred in transport medium (PBS) containing antibiotics and under chilled conditions and stored at -20 ºC till being prepared and analyzed for virus isolation and molecular analysis (Rashid *et al.*, 2017).

Milk samples (n=25) were collected during 2018 from infected and apparent healthy sheep in the same location from Kaliobyia province. These sheep were unvaccinated against SPV. Samples were collected under aseptic condition and frozen until analysis (Sharawi and Abd El-Rahim, 2011).

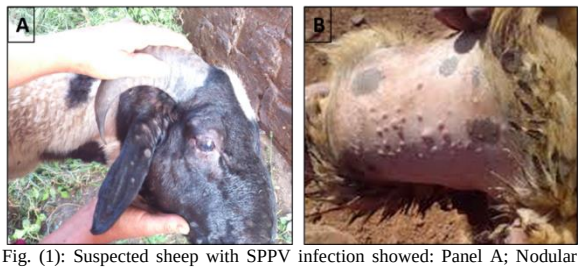

Fig. (1): Suspected sheep with SPPV infection showed: Panel A; Nodular and crusted lesions on the head of suspected SPPV infected sheep. Panel B; Nodular lesions on the tail of suspected SPPV infected sheep

2.3. Virus isolation

Isolation of suspected SPPV from tissue samples was applied on Chorioallantoic membrane (CAM) of SPF-ECEs at 11 days' age for three passages (Rovozzo and Burke, 1973).

2.4. DNA extraction and molecular amplification

Extraction of viral genome from samples was performed by QIAamp extraction Mini kit (Qiagen, Germany; Cat.no; 51304) following the manufacturer's recommendations. Briefly, 200µl of each prepared sample preparation was incubated at 56ºC for 10 min, mixed with 10µl of proteinase K and 200µl of lysis buffer. 200µl ethanol (96%) were added to the lysates and mixed again by pulse vortexing for 15 seconds. 100 µl of elution buffer delivered in the kit was used for DNA elution. The used Primer set was synthesized by Metabion (Germany) as outlined in Table (1).

A total of 25µl reaction mixture encompass 12.5µl of Emerald Amp-GT master mix (Takara, Japan; Cat no. RR310A), 1 µl of 20pmol concentrations forward and revers primer, 4.5 µl of Nuclease-free water, 6 µl of DNA sample and DNA T3- Thermal cycler (Biometra®) was used for amplification with cycling condition according to Zhu et al., (2013).

2.5. Analysis of the amplified products

The PCR products were separated by electrophoresis on 2% agarose gel (Bioline, Japan, Cat. No. BIO-41025). The fragment sizes of amplified products in the gel were determined by Gelpilot 100 bp DNA Ladder of 6 size range bands: 100-600 bp (Qiagen, Germany; Cat No. 239035). 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 the amplified products and analysis of sequencing data

The 570bp obtained specific PCR band from milk sample was purified using QIAquick® PCR purification Kit (Qiagen Inc., Valencia CA, Cat. No, 28104),followed by forward and reverse sequencing on an Applied Biosystems Sequencer (ABI, 3130, USA).The obtained sequence data were aligned using CLUSTAL W (multiple sequence alignment program) of Lasergene DNA Star software (Thompson et al., 1994).

Phylogenetic analysis and pairwise distance analysis were performed by neighbor-joining method in MEGA6 TBE buffer according to Tamura et al. (2013)

Table (1): Oligonucleotide primers sequences targeting ORF 103 gene of SPV

Gene	Sequence	PCR product	
Sheeppox ORF 103	F. ATGTCTGATAAAAAATTATCTCG R. ATCCATACCATCGTCGATAG	570 bp	Zhu et al., 2013

3. RESULTS

3.1. Isolation of suspected SPPV on CAM:

It was found that 10 out of 25 inoculated samples induced lesions on CAM in form of typical pock lesions, death of embryo, thickening, edema, and hemorrhage on the membranes as shown in Figure (2).

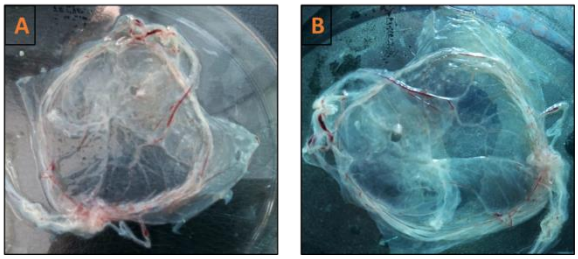

Fig. (2); Representative pictures for the viral isolation on CAMs of SPF-ECEs. Panel A; non inoculated control negative. Panel B; CAM showing typical pock lesions, death of embryo, thickening, edema, and hemorrhage on the inoculated membranes

3.2. Molecular detection by PCR:

A total number of 25 milk samples and 10isolated samples were tested for SPPV DNA using PCR. The amplification and running of characteristic 570bp fragments of amplified Sheeppox viral DNA were shown in Figure (3). SPPV DNA were detected from 2 out of tested 25 milk samples and 2 out of 10 viral isolates.

3.3. Sequencing and construction of phylogenetic tree:

The 570-bp-DNA sequenced data from positive milk sample analyzed and showed identity to SPPV. Moreover, BLAST program analysis showing that sequence

alignment of the amplicons attained from all kind of samples revealed 97.1% homology to the nucleotide sequence of the detected SPPV isolate in Minufiya governorate in Egypt (MF443334.1), Sheeppox virus isolate of Saudi Arabia (MN072630.1), 96.7% Sheeppox virus isolate GanS-JQ/2010/China ORF 103 gene (MG458404.1) and 96% with Sheeppox virus isolate GanS-ZY/2012/China ORF103 gene (MG458406.1) available in GenBank as shown in Figure (4).

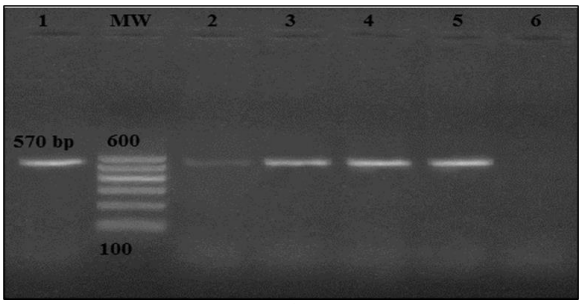

Fig. (3): The Electrophoretic pattern of the PCR products based on ORF 103 of SPV to the viral isolates and milk samples on the gel electrophoresis (1.5%). Amplicon size was detected at 570 bp which considered positive results for this size. Positive and negative controls (1& 6) are included

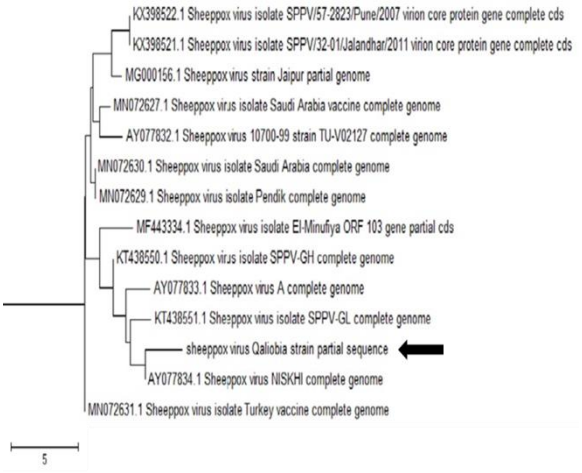

Fig.(4): Phylogenetic tree using neighbor-joining method based on ORF 103 gene nucleotide sequences of our new SPV isolate (indicated by black arrows) with other sequences of SPV retrieved from the GenBank database. The scale bar means nucleotide changes or substitutions per site.

4. DISCUSSION

Sheeppox virus is the most listed endemic disease disturb small ruminants' production and the foremost effect on the trade of intensive livestock production, recorded with worldwide distribution (OIE, 2017), So in endemic countries, the accurate diagnosis strategy and vaccination program of sheeppox disease, is the main weapon for control and eradication. Consequently, screening affected sheep flocks depends on using sensitive investigative tool (Bhanuprakash *et al.*, 2011).

SPPV shed in discharges of diseased sheep (saliva, secretions, feces, milk, or scabs) and also from carriers of the virus (OIE, 2017). Maximum shedding level of infectious virus and viral DNA of infected animals be fallen between 1-2 weeks post infection and persistent for up to an extra 3-6 weeks (Bowden *et al.*, 2008).

In the current study, trials for sheep pox virus isolation were conducted on 25 skin lesions on CAM of 11 SPF-ECEs. Ten samples showed typical pock lesions, death of embryo, thickening, edema, and hemorrhage of the membrane in agreement with (Mahmoud and Khafagi 2016). Viral isolation is a 'gold standard' for numerous viruses and is essential to elude misdiagnosis and to distinguish it from other related diseases (Chana etal., 2007). the best viral samples for isolation on cell culture are skin tissue lesions due to great tropism of poxvirus in skin cells (Nawal etal.,2006; Amal etal.,2008; El-Sabbagh etal.,2014; Sharawi et al., 2011).

The most sensitive and rapid assay, comparable to virus isolation and identification, is PCR technique and from the tests that mentioned by the OIE (ELISA, FAT assay, agar gel immunoprecipitation test) and have by this time presented is prior studies (Carn, 1995). PCR can detect virus within 24 hours, less time consuming, can be easily preset to large numbers of samples, and more effective in studying the identity between SPPV and LSDV based on unique restriction sites in the consistent PCR fragments (Bhanuprakash et al., 2006).

Milk samples from apparently healthy sheep revealed positive results by PCR in 2of 25 total samples. These results agreed with results by (Zhu et al., 2013; Zro et al.,2014; Khameis et al., 2018; and Abd-Elfatah et al., 2018). For field application, molecular identification of SPPV in clinical sample, such as milk samples are ideal, they do not need definite transport media contrasting skin tissues. While, for qualitative molecular diagnosis milk samples were deemed as decent applicants for SPPV earlier detection.

5. CONCLUSION

In this study, a field capripoxvirus was successfully isolated from diseased sheep in 2018 in Kaliobyia Governorate, Egypt. Milk samples were good candidates for early detection of sheep pox virus before onset of clinical symptoms. The fast identification of sheep pox virus infection depends on using ORF103 gene-based PCR assay with sequence analysis. In Egypt, this outcome may afford a new epidemiological aspect about SPPV circulation.

CONFLICT OF INTEREST

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

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