Khameis *et al., J. of Virol. Sci.,* Vol. 3: 49- 57, 2018
ISSN: 1685-1687

Journal of Virological Sciences

RESEARCH

Molecular detection and phylogenetic analysis of sheep pox virus in El Menofiya Governorate

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ABSTRACT

Background: Sheep pox virus is a member of the Capripoxvirus genus of the Poxviridae family, which affect sheep and causes significant economic losses in El Menofiya Governorate.

Objectives: The present study was applied for isolation and molecular identification of Sheep pox virus from clinically affected sheep in El Menofiya governorate, Egypt during 2016. Additionally, the isolated agent was identified using electron microscopy (EM).

Methods: Thirty five skin lesions of nodular and crusted scab samples were collected from clinically infected SPV sheep and were prepared and isolated on chorioallantoic membrane of 9-11 days old emberyonted chicken egg for three passages. The isolated samples were identified by EM and PCR.

Results: Five samples were positive and showed a typical pock lesion., the isolated strain was identified as a sheep pox virus by EM and PCR. The virus was confirmed through sequence and phylogenetic analysis of the RPO30 gene and open reading frame (ORF) 103 genes. The sequences designated Sheep pox virus was submitted to GenBank under accession number of MF443334.

Conclusion: The molecular techniques based on the RPO30 and ORF 103 genes are efficient for the characterization and differentiation of Capripox viruses. This finding may provide new information on the epidemiology of SPV in El Menofiya governorate.

Keywords: Molecular detection; phylogenetic analysis; electron microscopy; Chorioallantoic membrane; sheep pox virus and El Menofiya, Egypt.

BACKGROUND

Sheep occupy a premier place in the livestock industry and contribute significantly to the world economy. Its populations are threatened by a number of health hazards, among the most notable of which sheep pox which inflicts substantial losses in terms of reduced productivity and lower quality of wool and leather (Rao and Bandyopadhyay, 2000).

Sheep pox is an OIE List A disease that has the potential for rapid spread and which is important in sheep production and trade (OIE, 1996). It is endemic in Africa, India, North Equator, Middle East countries including Egypt, Turkey, Iraq, Iran, Afghanistan, and India (Babiuk *et al.,* 2008).

Sheep pox virus (SPV) is a member of the *Capripox* virus genus, subfamily *Chordopoxvirinae*, in the *Poxviridae* family (Murphy *et al.,* 1995). Goat pox virus (GPV) and lumpy skin disease (LSD) virus are other members of genus *Capripox* (Das *et al.*, 2012).

The sheep pox virion is enveloped, brick-shaped particle of 270–290 nm in size and contain a linear double-stranded DNA (Oguzoglu *et al.,* 2006). The size of the genome is approximately 150 kbp and it includes at least 147 putative genes (Tulman *et al.,* 2002).

Capripox viruses are mainly host-specific, Most isolates cause disease mainly in sheep or mainly in goats, but some isolates can cause serious disease in both species (Bhanuprakash *et al.,* 2010). serological assays could not distinguish SPV and GPV due to the close antigenic and virulence relationship (Balinsky *et al.,* 2008).Recent studies showed that the viruses are phylogenetically distinct and can be differentiated by molecular tools (Bhanuprakasha *et al.,*

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2006), So the characterization of these viruses needs molecular detection targeting Capripox viruses specific genes like RPO30, GPCR and P32 genes (Yan *et al.,* 2012).

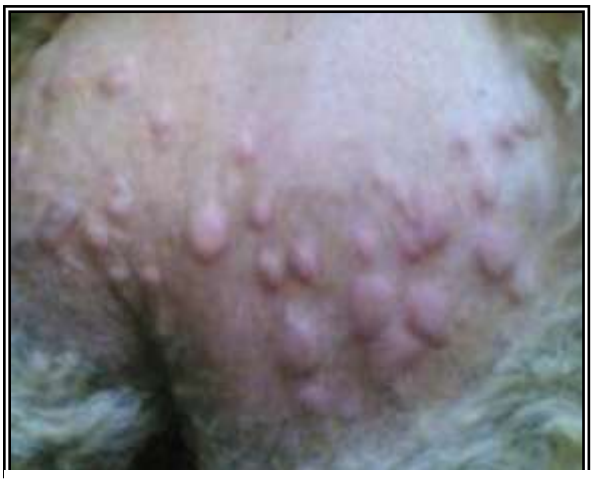
Prompt detection of sheep pox virus in the field samples is important for effective control, thereby reducing the potentially serious economic damage which can result from an outbreak (Mangana-Vougiouka *et al.,* 2000). The diagnosis of sheep pox is usually based on clinical signs followed by laboratory confirmation. Several laboratory confirmation techniques are based on virus isolation and electron microscopy (EM) (Oguzoglu *et al.,* 2006). Conventional seromonitoring techniques such as immunoflourescence, immunoprecipitation, virus neutralization and ELISA have been reported to be widely used for routine diagnosis of the disease. Formerly, a polymerase chain reaction (PCR) method based on fusion and attachment protein genes has been described for the detection of Capripox virus and has been reported to be more sensitive than antigen trapping ELISA (Ireland and Binepal, 1998).

In El Menofiya governorate, sheep pox is endemic and cause high economic losses between sheep flocks. Rapid diagnostic assay for SPV using different type of clinical samples would be useful for disease management. Therefore, the present study was carried out to isolate and identificate sheep pox virus between sheep flocks in El Menofiya governorate, Egypt during 2016 and comparing by phylogenetic analysis the field strain with Capripox virus sequences available in Gen Bank.

MATERIALS AND METHODS

Collected samples:

SPV infection leads to pyrexia, oculo-nasal discharge and pock lesions ranging from erythema to scab on the body with occasional pulmonary nodules (Rao and Bandyopadhyay, 2000), as shown in Photo (1 and 2). In the present study, 35 Clinical samples including the skin nodules and crusted scabs were collected during 2016 from unvaccinated sheep showing SPV infection symptoms in El Menofiya Governorate. The samples were transferred to the laboratory in transport medium (PBS) containing antibiotics and under chilled conditions and stored at -20 º till analyzing.



Photo(1): nodular lesions on the tail of suspected SPPV infected sheep

Photo (2): nodular and crusted lesions on the eyelid of suspected SPPV infected sheep.

sheep pox virus reference strain:

The Egyptian strain of sheep pox virus was obtained from Virology department, Animal Health Research Institute, Egypt.

Isolation:

Isolation was applied on Chorioallantoic membrane (CAM) of SPF fertile chicken eggs of 9-11 days age for three passages according to Rovozzo and Burke (1973). It was applied in Virology department, Animal Health Research Institute, Egypt.

Electron microscopy (EM):

The detection of sheep pox virus in positive isolated samples (CAM) using transmission electron microscope was done using negative staining techniques according to Gibbs *et al.,* (1980). It was applied in Faculty of Agriculture Research Park, Egypt.

Polymerase chain reaction:

It was applied in Biotechnology Department, Animal Health Research Institute, Egypt.

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 ºC 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 the kit **(Yousif *et al.*, 2010)**.

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

PCR amplification: Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler (Lamien *et al.*, 2011).

Analysis of the PCR Products: The products 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 (Biometra) and the data was analyzed through computer software.

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

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
RPO30	F: tctatgtcttgatatgtggtggtag R: agtgattaggtggtgtattatttttcc	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
ORF103	F :ATGTCTGATAAAAAATTATCTCG R: ATCCATACCATCGTCGATAG	570	94°C 5 min.	94°C 30 sec.	52°C 30 sec.	72°C 30 sec.	72°C 7 min.	Zhu et al., 2013

Sequencing:

A 570 bp of ORF 103 gene of some detected SPPV were amplified using primers (forward primer: 5'-ATG TCT GAT AAA AAA TTA TCT CG-3'; reverse primer: 5'-ATC CAT ACC ATC GTC GAT AG-3') (Zhu *et al.*, 2013) in HotStartTaq® Plus Master Mix Kit (QIAGEN, USA). The PCR master mix and thermocycling conditions were similar to that in PCR except the annealing temperature was 52 ºC as listed in table (1). The 570 bp PCR specific band was excised from the agarose gel, purified using Montàge DNA gel extraction kit (Millipore, USA) and sequenced in an automated ABI 3730 DNA sequencer (Applied Biosystems, USA).

Phylogenetic analysis:

The obtained sequences were analyzed using the online Basic Local Alignment Search Tool program (BLAST) and compared with those of capripox viruses available in the Gen Bank database. Sequence identities of nucleotides between the isolated and National Center for Biotechnology Information (NCBI) sequences were analyzed using Clustal W. The nucleotide sequences deduced were assembled into a multiple sequence alignment. A phylogenetic tree derived from the nucleotide sequences deduced was constructed for the capripox viruses by using the neighbor-joining method with the Molecular Evolutionary Genetics Analysis software (version 7.0) (Zhou *et al.*, 2012).

RESULTS

Isolation on Chorioallantoic membrane:

It was found that 5 samples from 35 inoculated samples induced lesions on CAM up to 3rd passage in form of typical lesions (pock lesion), death of embryo, thickening, oedema and hemorrhage of the membrane as shown in table (2) and Photo (3).

Table (2): Isolation of prepared collected samples on chorioallantoic membrane SPF emberyonted chicken egg.

No of inoculated samples			No of positive samples*			No of negative samples
			1st passage	2nd passage	3rd passage	
35	1	2	2	30		

*Positive result represented by thickening, oedema and hemorrhage of CAM.

Photo(3): thickening, oedema and hemorrhage of CAM typical lesions (pock lesion)

Electron microscopy:

The electron microscopic examination of the positive isolated samples (CAM) revealed that; the isolated virus was approximately 200 nm long and 150 nm wide, oval in shape, with rounded ends and characteristic ball of wool appearance as shown in Photo(4).

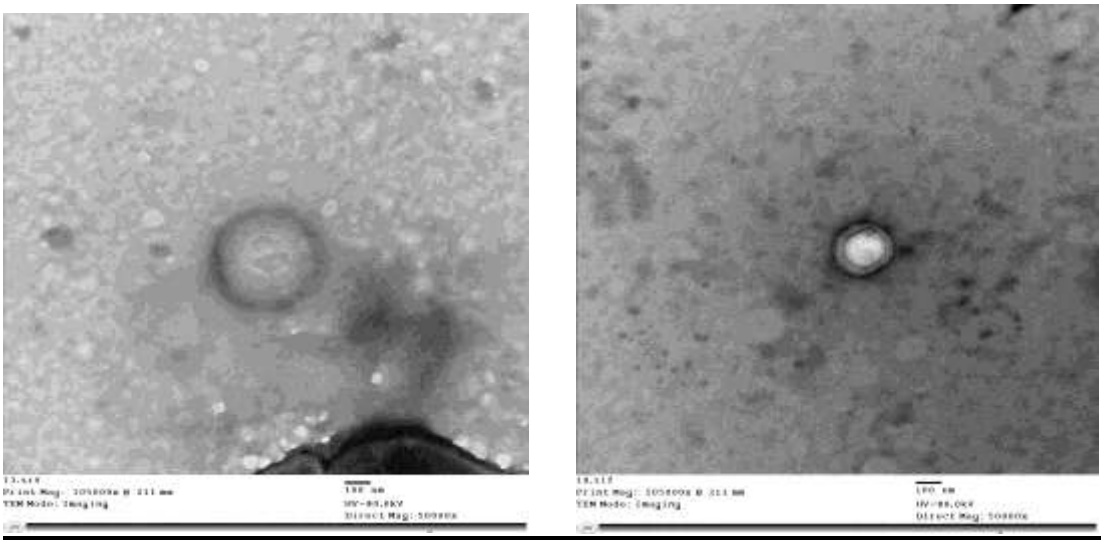


Photo (4): Electron microscopical illustration of sheep pox virus. see the oval profile and central core (bar = 200 nm). Transmission electron microscope, negatively stained, × 50000.

3.3 Polymerase chain reaction:-

Detection of nucleic acids in harvested CAM using conventional PCR was recorded in all prepared samples. The amplification and running of characteristic 151 bp fragments of sheep pox viral DNA were shown in Figure (1).

A stronger running band were produced and detected by Ethidium Bromide Stained Agarose Gel. Ethidium bromide stained agarose gel electrophoresis of PCR extracted from CAM suspensions shown in Figure (1).

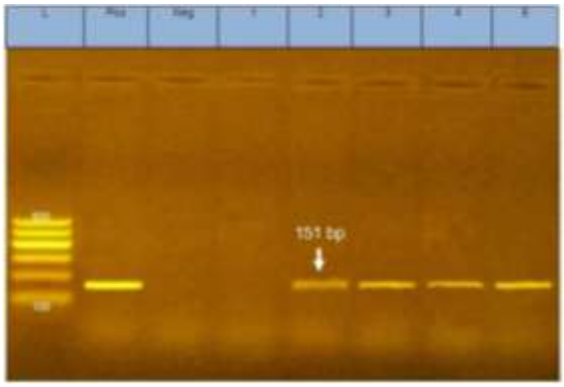


Fig. 1: Detection of sheep pox viral DNA after amplification by PCR. L : molecular marker. Pos : positive PCR products from sheep pox virus reference strain. Neg : negative PCR results. Lane (1): negative PCR products from sheep pox virus isolate (CAM). Lanes (2-5): positive PCR products from sheep pox virus isolate (CAM).

Sequencing and construction of phylogenetic tree:-

For further confirmation, the amplification of 570 bp from ORF 103 gene from three selected positive Sheep pox virus isolated samples was done. A strong running bands were produced and detected by Ethidium Bromide Stained Agarose Gel as shown in **Figure (2).**

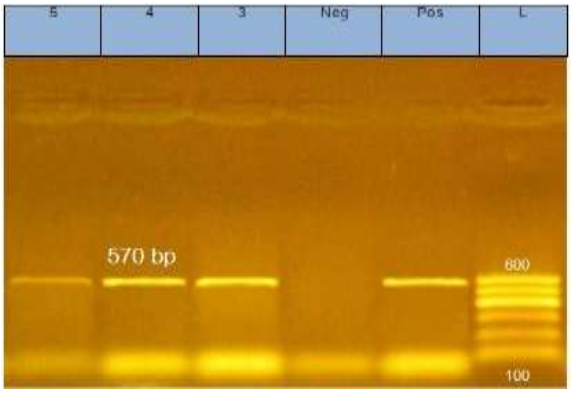


Fig. 2: Amplification of ORF 103 gene by PCR. L= DNA marker, lane 1 = positive control ; lane 2 = negative control ; lane 3 = positive isolate ; lane 4 = positive isolate ; lane 5 = positive isolate.

Sequence analysis of ORF 103 gene revealed that the three amplicons were identical. BLAST program analysis showing 100% maximum homology of this nucleotide sequence of the detected SPPV with Sheeppox virus A, complete genome (AY077833.1), 97% with goatpox virus Pellor complete genome (AY077835.1 and AY077836.1) and 97% with Lumpy skin disease virus NW-LW isolate Neethling Warmbaths LW (AF409137.1) available in GenBank as shown in Figure (3).

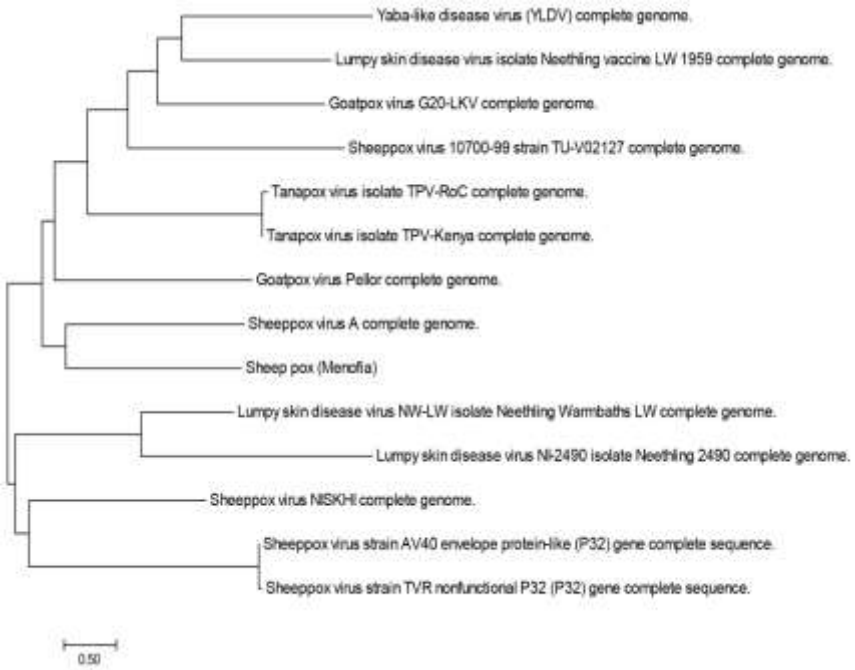


Fig. 3: Phylogenetic analysis of different Capripoxvirus based on deduced nucleotide sequence of the ORF 103 gene. The phylogenetic relationship was constructed using the Molecular Evolutionary Genetics Analysis software (version 7.0).

DISCUSSION

Sheep pox is a highly contagious viral disease of sheep characterized by fever, ocular and nasal discharges and pox lesions on the skin, respiratory and gastrointestinal mucosa. Morbidity and mortality may be high (Garner et al., 2000 and Bhanuprakasha *et al.*, 2006).

Control and eradication of sheep pox disease in endemic countries depend on accurate diagnosis and vaccination so, a rapid and sensitive diagnostic tool for screening affected sheep flocks is essential (Bhanuprakash *et al.,* 2011). The diagnosis of the capripox virus infection is successfully achieve using both ordinary (cultural isolation and EM) and newer techniques (PCR). (Bhanuprakash *et al.,* 2006).

In the present study, Trials for isolation of sheep pox virus from 35 skin lesions of nodular and crusted scab samples of clinically suspected sheep at different localities of El Menofiya governorate by three blind passages through CAM of 9-11 days old emberyonted chicken egg (SPF) showed that only 5 from 35 isolated samples induced characteristic typical lesions in the form of pock lesions, death of embryo, thickening, oedema and hemorrhage of the membrane while the other isolates (30 samples) did not showed any lesions on the CAM (table, 2 and photo, 3). Virus isolation is considered as a 'gold standard' for numerous viruses' .The most important disadvantages of this technique are that it is more time-consuming than other techniques and it needs at least 2-3 mandatory passages. Moreover, following these procedures, viruses isolated have to be identified by other techniques (Joshi et al., 1996 and Oguzoglu *et al.,* 2006).

There is no doubt that Electron microscope (EM) has the advantage of speed in the diagnosis (Gibbs *et al.,* 1980). By using EM for SPV identification in positively isolated samples, all examined samples showed the characteristic oval- or rectangular-shaped particle containing a central core (photo 4), our results agreed with those by (Oguzoglu *et al*., 2006 and McElroy and Bassett 2007).

As SPV and GPV are antigenically close, they cannot be differentiated using serological tests (Balinsky *et al.,* 2008). Currently, differentiation of these pathogens using molecular methods such as restriction endonuclease analysis (REA), whole genome sequencing, P32 gene based PCR-RFLP, PCR targeting RPO30 gene and GPCR gene based quantitative PCR are available (Hosamani *et al.,* 2004 and Lamien *et al.,* 2011). Among them, PCR targeting RPO30 (RNA polymerase 30 kD subunit gene - ORF036) gene is simple, cheap and quick to differentiate SPV and GPV (Santhamani *et al.,* 2013). RPO30 gene of SPV has a 21-nucleotide deletion in the 5' end compared to GPV.

In this study, we identified the SPV in positively CAMs using PCR by using a Primers that bind and amplify the partial RPO30 gene including the 21 nucleotide deletion, the results were strongly confirmed through presence of SPV fragments (151 bp) and were observed on gel electrophoresis (Fig. 1). These results agreed with results by Lamien et al., 2011 and Yan et al., 2012 for CaPV species identification.

A 570 bp from ORF 103 gene were successfully amplified from three detected positive Sheep pox virus isolated samples (Figure, 2). For further confirmation of the identity of the detected viruses, the amplified products were sequenced and the entire sequence of ORF 103 gene was analyzed. The results of sequence analysis revealed that the three amplicons were identical. BLAST program analysis showing 100% maximum homology of this nucleotide sequence of the detected SPV with Sheep pox 10700-99 strain TU-V02127 (gb|AY077833.1), 97% with goatpox virus Pellor complete genome (AY077835.1 and AY077836.1) and 97% with Lumpy skin disease virus NW-LW isolates Neethling Warmbaths LW (AF409137.1) available in GenBank (Figure, 3) and these results agree Tulman et al., (2002).

CONCLUSION:

In this study, a field capripox virus was successfully isolated from diseased sheep in 2016 in El Menofiya Governorate, Egypt. Using a limited number of samples indicated that sheep pox infection was associated with sheep pox virus. The RPO30 gene based PCR assay in combination with sequencing can be used for identification sheep pox virus infection.

ACKNOWLEDGMENTS

The authors extend their special thanks to Dr. Haitham Abo-Al-Ela, Animal Health Research Institute, Egypt for performing the phylogenetic assessment.

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RECEIVED: Sept. 2017; **ACCEPTED**: Oct. 2017; **PUBLISHED:** Jan. 2018

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Cite this article as:
Ashraf S. K. *et al.,* (2018): Molecular detection and phylogenetic analysis of sheep pox virus in El Menofiya Governorate. , *J. of Virol. Sci.,* Vol. 3: 49- 57, 2018