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Isolation, identification, and characterization of Capripox virus among clinically infected cases of small ruminants in some Egyptian governorates

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ABSTRACT

Sheep pox virus (SPV) is a member of the genus Capripoxvirus, of family Poxviridae, which affect sheep and goats causes significant economic losses. The present study was applied for isolation and identification of Sheep pox virus from clinically affected sheep during 2017 to 2019 in five Egyptian governorates, El Wadi El Gedid, Marsa Matrouh, Giza, Kafr El-Sheikh and Menofia. Despite the obligatory use of sheep pox vaccine for vaccination of sheep and goats in Egypt, but some factors hinder the mass vaccination for all sheep and goats' population like the grazing nutritional behavior, poor management, scare feeding and inadequate veterinary services. The virus was isolated from skin lesions and lymph nodes on chorio allantoic membrane of Embryonated Chicken Eggs (ECE), with typical pock lesions, followed by isolation on lamb testis culture (LT) showing the characteristic cytopathic effect. Similarly, the conventional PCR depending on P32 gene followed by 13 L gene, which give the same results proving that the isolated virus is SPV. This proves that SPV is still circulating in some Egyptian governorates.

1. INTRODUCTION

Capripoxviruses (CaPVs) of small ruminants are one of the most severe diseases of domestic small ruminants, as they affect the animal production and quality of meat, hair, wool and leather and may leads to animal abortion and death. (OIE 2017 a). These viruses have a direct effect on the agricultural economy causing financial losses (Mangana-Vougiouka *et al.,* 2000; Hosamani *et al.,* 2004; Parthiban *et al.,* 2005; Oguzoglu *et al.,* 2006). They are listed in ovine notifiable diseases of Office International des Epizooties (USDA, 2002; OIE, 2017b).

SPV and GPV infections are endemic and recorded in different regions of the world including Africa, Bangladesh, Iran, Afghanistan, India (Parthiban *et al.,* 2005), the Middle East, including Egypt, and Turkey (Oguzoglu *et al.,* 2006). All ages are affected; despite the disease is more dangerous in young than older animals as mortality ratio may reach 100% (Bhanuprakash *et al.,* 2006).

SPV is a highly contagious disease, spread through aerosols and/or close contact with infected animals, in directly by contamination of cuts and abrasions (Kitching and Carn, 2004). Poor conditioned animals, overcrowding, poor feeding, and abnormal uses of vaccination considered the main causes for distribution of sheep pox disease (Sheikh-Ali *et al.,* 2004; Zangana and Abdullah, 2013).

The systemic signs include fever, conjunctivitis, rhinitis, lymphadenopathy, popular and vesicular eruption which develops to pustule and finally scabs on parts of the body lack wool such as checks, lips, inner aspect of thighs, groins and under the tail. Also, the mucous membranes may be necrosed (Mersha, 2011).

Various cell types such as lamb testes, kidney and skin tissues and continuous cell lines (BHK-21, Vero) have been used for virus isolation, attenuation, and replication of SPV (and Sadri and Fallahi2010; Boshra et al., 2013).

Diagnosis of capripoxvirus disease is based upon clinical signs with laboratory confirmation by virus isolation and (PCR) because there are no serological methods that can differentiate between capripoxvirus isolates (Carn, 1995).

So, In this study, the disease agent was confirmed as SPV by clinical signs, post-mortem examination, isolation, and identification of the causative agent on ECE, LT cells, followed by (PCR) technique.

2. MATERIAL AND METHODS

2.1. Clinical investigation of a natural outbreak of typical clinically diseased flock of sheep with SPV and sampling

The natural outbreaks were recorded in five Egyptian governorates from April 2017 to February 2019. The disease spread through different regions of El Wadi El Gedid, Marsa Matrouh, Giza, Kafr El-Sheikh and Menofia Governorates. All animal handling procedures and sampling were approved by Egyptian Veterinary Authorities and according to the guidelines of the European Community Council Directions (1986 (86/609/EEC) and Constable et al. (2017).

Twenty skin nodules, biopsy and scabs were collected from infected sheep form each governorate, these skin lesions

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were transported in Hank's medium containing antibiotics (Penicillin 100 µg/ml, streptomycin 100 µ g/ml, neomycin 2.5 mg/ml and nystatin 50 µg/ml) till used.

2.2. Sheep pox virus control: Sheep pox Romanian and Kenyan Strain.

2.3. Virus isolation in embryonated chicken eggs (ECE):
Fertile specific pathogen free, SPF-ECE (11-12) days old, were obtained from Quem Ochem Company, Fayoum, Egypt. Eggs were inoculated by the prepared samples via the chorio-allantoic membrane route (CAM). The protocol was described by Cunningham (1973), Mahmoud, et al (2016) and Sharma, et al. (2019) by the artificial air sac route. Embryos were candled for embryo viability.

2.4. Cell culture:
Primary LT cell cultures were prepared from prepubertal lamb as described by Babuik et al. (2007).

2.5. Virus propagation and adaptation on lamb testis (LT):
The pock lesions collected from the CAMs (4th passage of each sample separately) were ground, then frozen and thawed 3 times. Homogenized samples were centrifuged, and 0.5 ml of the clear supernatants were used to inoculate a confluent monolayer of (LT) cells grown on tissue culture flasks. Then the confluent monolayer was covered with Growth Earle's minimum essential medium (GMEM) supplemented with 10% fetal bovine serum. Incubated cells were kept at 37 °C. Normal control cells were maintained in a similar manner. The cells were observed daily for any cytopathic effects (CPE).

2.6. Extraction of genomic DNA:
Extraction of genomic DNA was done by use of QIAamp DNA Mini Kit. Material of kits were described according to manufactures instruction for PCR primer sequence for (partial P32 gene) is:

Forward primer (F) CTAAAATTAGAGAGCT ATACTTCTT
Reverse primer (R) CGATTTCCATAAACTAAAGTA.

The amplified product is 390 bp according to Heine et al., 1999. While for the sequence of I3L gene primers, a pair of three primers was designed (two forward and one common reverse) for detection and differentiation of SPPV and GTPV. They are:

F1.5'-GCCAGGAACTTTATATTCGATG-3'
F2.5'-ATATAGAATAGGGCTAGTTGCAG-3'

with

R.5'-CATCAAAAATGACATCTACATATATAGC-3'

The amplified products 293 and 133 for SPV and specific amplification of 133 bp fragment for GPV at the same conditions, according to Venkatesan *et al*. (2014).

3. RESULTS

3.1. Clinical investigation of sheep pox cases in Egypt during 2017-2019:
Clinical manifestation of capripox in the affected sheep were variable in two phases. The acute febrile phase within the first few days (5-6 days) with developing cutaneous lesions and a less severe and prolonged phase associated with healing of cutaneous scars and possible self-recovery up to 5-6 weeks. Respiratory signs were characterized by considerable dyspnea and signs of labored breathing. Diseased animals appeared lethargic, and often emaciated in prolonged illness. In severe cases, death occurred within a few days with impairment of the respiration.

Postmortem examination (PM) of dead lambs showed edema, nodules, also ulcers on tongue, trachea, and lungs, beside skin lesions of SPV figure (1). Morbidity and mortality results are illustrated in table (1).

Fig 1 Papules and nodules on face and udder on sheep suffering from sheep pox disease.

Table 1 Morbidity and mortality % of SPV outbreaks in Egypt (2017-2019)

Clinical record	Egyptian infected governorates (various localities, 100 animals/group)				
	G1	G2	G3	G4	G5
Morbidity%	35%	30%	23%	12%	10%
Mortality%	8%	5%	1-3%	1-3%	1-3%

G1: El Wadi El Gedid. G2: Marsa-Mattrouh. G3: Giza. G4: Kafr El Sheikh. G5: Menofia

3.2. Propagation of the isolated virus on CAM of ECE:
Inoculation of each pooled skin lesions virus fluid of each governorate on CAM of ECE for successive 4 passages revealed the development of Pock lesions. The obtained results showed that the virus replication and pock lesions formation were not clear for the first passage of the virus in ECE, while there was only thickening and oedema of CAM till the 5th day post inoculation (DPI). From the 2nd to the 4th virus passages, there was obvious fine greyish yellow discrete lesions on the CAM of a live embryo of the inoculated eggs. These pock lesions increased in number and size by successive serial passages with survival of the inoculated chicken embryos post inoculation till harvesting time (5th DPI) (Table 2 & Fig 2).

Table 2 Results of propagation of SPV of governorates virus samples in ECE:

Virus passages	Virus titers (log10 EID50/ml) of governorate virus samples				
	GVS1	GVS2	GVS3	GVS4	GVS5
1	4.5	4.5	4.0	4.0	4.0
2	5.0	5.0	4.5	4.5	4.5
3	5.0	5.5	5.2	4.5	4.5
4	5.5	5.5	5.0	5.0	5.0

EID_{50}/ml: Egg Infective Dose $_{50}$/ ml.GVS1 to GVS 5: Pooled skin samples of five governorates.

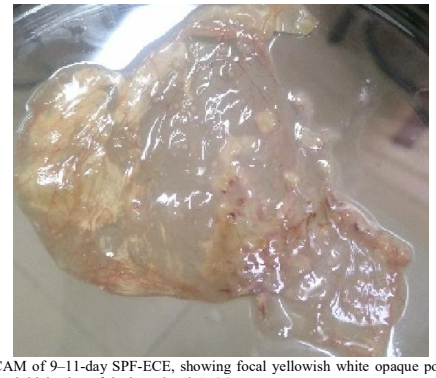

Fig. 2 CAM of 9–11-day SPF-ECE, showing focal yellowish white opaque pocks with generalized thickening of the inoculated CAM.

3.3. Propagation and titration of SPV isolates in primary lamb testis (LT) cell cultures:
Inoculation of 0.5 ml of supernatant of CAM virus product (4^{th} passage of each sample separately)/small sized prescription flask, showed CPE in the monolayer cells after 4 days PI from 3^{rd} to the 5^{th} passage. Cellular changes and

characteristic CPE was not clear in the 1st and 2nd blind passages and CPE development began to appear in the following three successive passages at 48 Hrs PI and he completed at the 4th DPI. Viruses were characterized by formation of focal lesions in the cell sheet composed of groups of shrunken, round and granular cells. Inoculated cells appeared shrunken and may contain one to several of Intra-cytoplasmic inclusion bodies (ICIB) of variable size. At the 4th DPI, cells were lost leaving an irregular empty batches and vacuolation to be completed at the 5th DPI, which is the suitable time for virus harvestation (Table 3, Figure 3).

Table 3 Passages and titers of SPV virus isolates in LT cells.

Virus passages	Virus titers ($log_{10}TCID_{50}$/ml) of governorate virus samples				
	GVS1	GVS2	GVS3	GVS4	GVS5
1	5.0	5.0	4.5	4.5	4.5
2	5.0	5.0	4.5	4.5	4.5
3	5.5	5.5	5.0	5.0	5.0
4	6.2	6.2	5.7	5.2	5.2
5	6.2	6.2	5.7	5.2	5.2

$TCID_{50}$/ml: Tissue Culture Infective Dose $_{50}$/ml.GVS1 to GVS 5: Pooled pock lesions of five governorates.

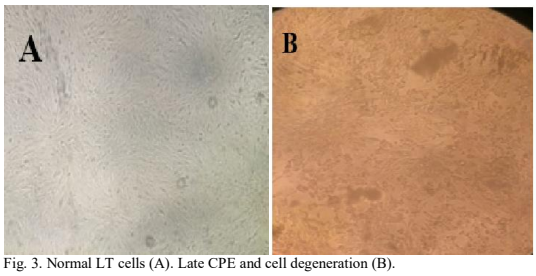

Fig. 3. Normal LT cells (A). Late CPE and cell degeneration (B).

3.4. Genomic identification of SPV isolates:

It is done for the five SPV isolates, the Romanian and Kenian strains of SPV, depending upon three genes which are P32 (Fig 4 & 5).

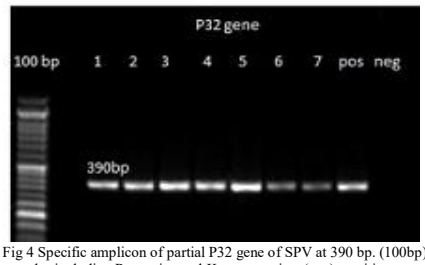

Fig 4 Specific amplicon of partial P32 gene of SPV at 390 bp. (100bp) ladder, (1-7): 7 samples including Romanian and Kenyan strains. (pos): positive control. (neg): Negative control.

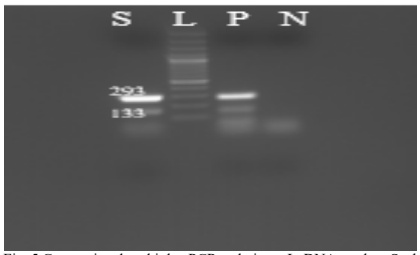

Fig. 5 Conventional multiplex PCR technique. L: DNA marker. S: sheep pox sample. P: Positive SPPV band at 133 and 293bp. N: Negative control.

4. DISCUSSION

Capripoxvirus of small ruminants found widely distributed and causes death of lambs and immune suppression of affected animals of both sexes and all ages (Mersha, 2011; Constable *et al.,* 2017). In Egypt, sheep pox is an endemic disease despite the application of a control program based on sanitary prophylaxis depends upon vaccination by live attenuated vaccine (Abd-Elfatah *et al.,* 2018). The Sheep pox virus infection is one of the common diseases among those causing economic losses occur in the form of high mortality rate in young, productivity reduction and poor wool and leather quality (Parthiban *et al.,* 2005).

In this study typical sign of sheep pox were observed, as the affected sheep showed signs varied from acute signs in young lambs to mild infection in adult sheep as previously described by Shawn *et al.* (2009) and Zro *et al. (*2014), who described 2 forms of the sheep pox infection in adults and lambs. These obtained results from clinical investigations of infected lambs and adult sheep in five Egyptian governorates from 2017 to 2019 were the same was reported by El-kattan and Bassiouny (2016), Mahmoud, and Khafagi (2016), Egyptian Veterinary Authorities, Ministry of Agriculture and OIE (2017b) and Khameis et al. (2018).

Most of the affected sheep were living in free grazing conditions. This is believed to contribute the widespread of capripox infections in the area of study. In addition to the vaccination history in the infected governorates was also recorded and revealed that the infected lambs (1-3 months' age) and adult sheep (2-4 years' age) in El Wadi El Gedid, Marsa Matrouh governorates with history of non-vaccination, while some sheep flocks were vaccinated and others non-vaccinated in the rest three governorates, this explain the higher morbidity and mortality rates among sheep in GV1 and GV2.It was higher than the results obtained by Mondal *et al.* (2004) in India in which the percentage of death was 6.3% and Hamoda and Zaghloul (1996) when they described the SPV outbreak in Egypt in 1995. And Zangana and Abduilah (2013) but less than recorded by Ammar et al. (1999) detected mortality rate was 68.4%. Here morbidity and mortality rate were highest in lambs less than 6 months' age, because of absence of maternal immunity as a result of lack of vaccination. Similarly, the results obtained formerly (Mersha, 2011; Zro *et al.,* 2014; El-kattan and Bassiouny, 2016; Abd-Elfatah *et al.,* 2018) due to decreasing in the level of maternal immunity and absence of vaccination against SPV. The infected sheep were suffering from high temperature, and typical pox clinical signs that considered as first indicator of *Poxvirus* infection, like those obtained previously (Al-Shabebi *et al*., 2014; EL-Kattan and Bassiouny, 2016, Abd-Elfatah et al., 2018; Sharma et al., 2019).

Isolation of Capripoxviruse in (CEE) is considered to be a preliminary diagnostic test (Kadam et al., 2014) associated with the development of characteristic pock lesions when inoculated on the (CAM). The titre of GVS1 and GVS2 was$10^{5.5}$ EID_{50}/ml. It was the highest one while other isolates titre was 10^{5} EID_{50}/ml. Also purification of the inoculated samples from foreign contaminants viruses, bacteria, fungi causing elevation of the titre level of the isolated virus. The observed pock lesions of SPV on CAM of SPF-ECE, come similar to those obtained by former authors (Bhanuprakash *et al.,* 2005; El-kattan and Bassiouny 2016), who observed Yellowish white pock lesion of SPV isolated from Marsa Matrouh province on CAM of ECE, come similar to those obtained by Kadam *et al. (*2014) and Aswini (2015) when they cultivated isolated SPV from Nineveh and India on CAM of ECE.

After inoculation of 4th passage of supernatant of CAM virus product on primary culture from lamb testis (LT), cellular changes and characteristic CPE was not clear in the 1st and 2nd blind passages and CPE development began to appear in the following three successive passages. Virus

cytopathogenesis of all isolates were characterized by formation groups of shrunken, round and granular cells, then formation of highly refractile cells. At 72 hrs PI the ICIB became large, acidophilic, masses and numerous in numbers. The same results recorded by Gu *et al. (*2018) followed by cell detachment leaving an irregular empty batches and vacuolation. Results of virus titration showed that virus titers increased gradually during successive passages till reaching the maximum titre by 4th and 5th passages where it became stable and fixed as $\log10^{6.2}$ $TCID_{50}$/ml. Table (3) proves that GVS1& GVS2 samples, having the highest titre, were the best isolates to be selected for further adaptation in suitable cell line. Gradual increase in titre in LT cells agree with (Sarbasov *et al.,* 2019).

For molecular identification of isolated virus, PCR assay was done by amplification of the partial P32 gene of the extracted DNA.As the size of the PCR product was 390 bp similar to those obtained by Al-Shabebi *et al.* (2014), who isolated SPV of Al-Hassa. Also, Heine *et al.* (1999), Varshovi *et al.* (2009), Kadam *et al.* (2014) and El kattan and Bassiouny (2016), meaning it was capripox virus. Capripox laboratory conformation based on serological techniques are time consuming and most of them are of low specificity, because of close antigenic relationship between capripoxvirus and parapoxvirus. Furthermore, conventional multiplex PCR technique was used because of high sensitivity in detection of capripoxvirus DNA and good specificity in differentiation of capripoxvirus from parapoxvirus.

Identifying was based on (I3L gene) of specific SPV primer, gave bands at 133 and 293bp while a single band at product size (172bp) indicates GPV in the multiplex differentiating PCR technique. These results coincided with (Abd-Elfatah et al., 2018)

5. CONCLUSION

The results revealed that the suspicious cases of capripox disease isolated in sheep in Egypt is a true sheep pox disease and its recommend to make a strict vaccination campaign by veterinary authorities using the local SPV vaccine.

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