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Adaptation and comparative growth behaviour of Egyptian SPV isolates in Vero cells

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ABSTRACT

Although primary lamb testis (LT) cell culture seemed to be sensitive and the most suitable for adaptation of recently isolated sheep poxvirus (SPV), giving rapid and complete Cytopathic Effect (CPE), these cells were difficult to be maintained as a monolayer cell line. Moreover, their primary and secondary cultures still have many of the detrimental, inherent characteristics and contaminating elements. Therefore, the use of more stable cell lines (Vero cells) becomes a necessity for an economic preparation of SPV seed vaccine. Three SPV isolates, which previously passaged and adapted in lamb testis cell culture (isolates with titer reaching to 6.5 Log10 $TCID_{50}$ / ml), were chosen to be transferred and propagated on Vero cell line**.** With further studies on its growth curve and growth kinetics to identify the growth behavior of these isolates, followed by detection of the virus antigen in the infected Vero cells by indirect florescent antibody technique (IFAT) as the first step for antigenic identification of the isolated adapted virus. The chosen optimum multiplicity of infection (MOI) was (0.01) with the optimum virus harvestation time was 4 days post-inoculation (DPI) and virus titre was equal to $10^{5.5}$. Finally, we recommend the use of Vero cells as a continuous cell line for preparation of seed of SPV prepared from an Egyptian isolate.

1. INTRODUCTION

Sheep pox is considered as one of endemic notifiable disease in Egypt and has vaccination program, as recorded by WHO with worldwide distribution as widely found in India, China, Egypt, Saudi Arabia, Greece, Iran, Iraq, Bangladesh, and Pakistan (Maksyutov *et al.,* 2013; Hani *et al.,* 2015; Constable *et al.,* 2017; OIE, 2017; Tuppurainen *et al.,* 2017). It is caused by sheep pox virus (SPV) which is an enveloped and double-stranded DNA virus belongs to Genus *Capripoxvirus* of the Family *Poxviridae*with goat poxvirus (GPV) and lumpy skin disease virus (LSDV) (Buller *et al.,* 2010).

Sheep pox disease is characterized by pyrexia, skin lesions especially on hairless areas, internal pox lesions, and lymphadenopathy following an incubation period of 4–8 days, with elevation in body temperature, followed by the formation of macules in the skin (Bhanuprakash *et al*., 2006; Babuik *et al.,* 2008). Mortality rates of SPV and GPV range from 5% to 10% in local breeds of endemic regions to 100% in naive populations (Bhanuprakash *et al.,* 2006; OIE 2017a).

SPV and GPV are highly contagious diseases transmitted via aerosol (Carn, 1993), direct contact of infected and non-infected animals (Kitching and Taylor 1985), and contact with contaminated feed, wool, and other objects (Bhanuprakash *et al.,* 2006). Insects do not seem to play a major role in the transmission of SPV and GPV (Carn, 1993).

Primary cultures prepared from lamb testes (LT) and lamb kidneys (LK) are used from ancient times for isolation of capripox viruses (Amal et al., 2008) adapted SPV and GPV on Vero cells which gave CPE for 3 successive passages.

Abdi Assefa *et al.,* (2019) could isolate SPV and GPV from outbreak Cases in Ethiopia using Vero cells, and performed genotyping to them using RPO30 gene.

Multiplicity of infection (MOI) is a critical factor that must be carefully determined as it influences the virus growth dynamic and virus yield (Genzel *et al.,* 2006; Trabelsi *et al.,* 2012; Trabelsi *et al.,* 2014) succeeded to adapt SPV (RM65 strain) firstly on lamb kidney cells, then on Vero cells after nine passages with MOI of 0.005, yielded a higher virus titer when compared to that achieved at a MOI of 0.001.

For determination of virus particles inside the culture, Zhang *et al.* (2018) applied indirect immunofluorescence assay when infected Lamb Testis (LT) cells with AV41(a strain of GPV) or its recombinants at an MOI of 0.05.

In our study, we aim to use the lowest MOI which provides the highest titer with the optimum virus harvestation time.

2. MATERIAL AND METHODS

2.1. Propagation and titration of the selected sheep pox virus isolates on African Green Monkey Kidney Cells (Vero):

The 5th passage of sheep pox virus of three different Egyptian governorates (GVS1, GVS2 and GVS3; El Wadi Al Gadid, Marsa-Matrouh and Giza, respectively) on LT cell culture, which previously recorded the highest titer ($10^{6.5}$ $TCID_{50}$/ ml), was transmitted to grow on Vero cell line according to OIE (2017) and examined daily for (CPE).

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The titer was calculated according to Reed and Muench (1938).

2.2. Study the growth and cytopathic changes of three virus isolates:

Multiplication, cytopathic changes, harvestation time of cell free and cell associated virus were studied and performed according to Tozzini (1987).

Vero cells were grown in 8 plates (12 well plates). They were used for virus inoculation and the plates were incubated. The inoculated plates were observed, the culture supernatant of each virus sample was collected as a cell free virus fluid (CFV), then a new 0.5 ml of M.M was added to each empty well and plates were frozen to achieve a cell associated virus fluid (CAV). This process is repeated at 2, 12, 24, 48, 72, 96, 120 and 144 hours post inoculation (PI).

2.3. Growth Kinetics of 3 SPV isolates in Vero cells:

This technique was performed using different multiplicity of infection (MOI) of the selective SPV isolates (virus dilutions from 10^{-1} to 10^{-3}). (Babuik *et al.,* 2007; Trabelsi *et al.,* 2014) Vero cells were seeded in a 12 well plates, they were infected in triplicate pattern with 0.5 ml/well of different (MOI) (0.1, 0.01, 0.001) of each virus isolate (GVS1, GVS2 and GVS3) with control wells and incubated at 37 °C. In the following days, plates were observed, and the titer was estimated.

2.4. Indirect Florescent Antibody Technique (IFAT):

Cells with CPEs were subjected to an IFA test with rabbit anti-GTPV polyclonal antibodies. The secondary antibody used was fluorescein isothiocyanate conjugated goat anti-rabbit immunoglobulin G. After immunostaining, the control and inoculated cells were observed under a fluorescence microscope according to Aboulsoud (1995), Zhao *et al.* (2013) and OIE (2018a).

3. RESULTS

3.1. Propagation and titration of SPV isolates in Vero cell cultures: Inoculated Vero cells were examined daily for detection of cytopathic effect (CPE). The positive samples showed the characteristic granulation of cells followed by cell rounding and aggregated separately; this occurred after 3 days post inoculation (DPI) and gradually increased till 70-80% of the sheet that was completely detached in some samples after 5 days. The control Vero cell culture spindle shape cells in monolayer confluent sheet. Results are showed in Table (1) and Fig (1).

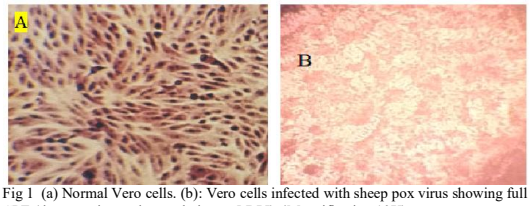

Fig 1 (a) Normal Vero cells. (b): Vero cells infected with sheep pox virus showing full CPE (degeneration and vacuolation at 5 DPI). (Magnification 10X).

Table 1 Passages and titers of selective 3 SPV isolates in Vero cells:

Virus passages	Virus titers ($TCID_{50}$/ml) of governorate virus samples		
	GVS1	GVS2	GVS3
1	5.2	5.2	5.0
2	5.5	5.5	5.0
3	6.0	6.0	5.5
4	6.0	6.0	5.5
5	6.0	6.0	5.5

$TCID_{50}$/ml: Tissue Culture Infective Dose 50/ ml. GVS1 to GVS 3: Pooled Governorates Virus Samples of three governorates. N.B. Control positive: Reference Romanian SPV with titer = $10^{6.0}TCID_{50}$/ ml.

3.2. Growth curve of cell free and cell associated viruses of a selective SPV isolate (CFV &CAV):

Results of infectivity titration of CFV & CAV at various hours' intervals PI (2,12,24,36,48,72, 96 and 120 Hrs PI) are presented in table (2) and figure (2).

which indicated that the titer of CFV of GVS1 and GVS2 decreased from $_{10}{}^{6}$ $TCID_{50}$/ml to $_{10}{}^{5}$; $_{10}{}^{3.5}$ and $_{10}{}^{2.5}$ $TCID_{50}$/ml at 2, 12 and 24 h PI respectively. The titers of CAV were $_{10}{}^{3.0}$, $_{10}{}^{3.5}$ and $_{10}{}^{4.0}$ TCID $_{50}$/ml at the same hours. The significant infectivity virus titers of both CFV &CAV were $_{10}{}^{5.5}$ $TCID_{50}$/ml at 96 Hrs PI. At 120 Hrs PI, the CFV titers increased and reached $_{10}{}^{6}$ $TCID_{50}$/ml while CAV were $log_{10}{}^{5}$ TCID $_{50}$/ml for G1 and G2.

Table 2 The growth curve of CFV &CAV of 3 SPV isolates in Vero cells (5^{th} passage):

Hours post Inoculation (HPI)	Titers of CFV &CAV ($TCID_{50}$/ml) of 3 governorates samples					
	GVS1		GVS2		GVS3	
	CFV	CAV	CFV	CAV	CFV	CAV
0	*6.0	*6.0	*6.0	*6.0	*5.5	*5.5
2	5.0	3.0	5.0	3.0	5.0	3.0
12	3.5	3.5	3.5	3.5	3.0	3.0
24	2.5	4.0	2.5	4.0	2.5	3.5
36	2.5	4.0	2.5	4.0	2.5	3.5
48	3.0	4.5	3.0	4.5	2.5	4.0
72	4.5	5.0	4.5	5.0	4.0	4.5
96	5.5	***5.5	5.5	***5.5	5.0	***5.0
120	**6.0	5.0	**6.0	5.0	**5.5	4.5

CFV: Cell Free Virus. CAV: Cell Associated Virus. GVS1, GVS2 &GVS3: 3 governorates virus samples. *: Titer of the original virus (whole culture virus). **: maximum titer of CFV. ***: maximum titer of CAV.

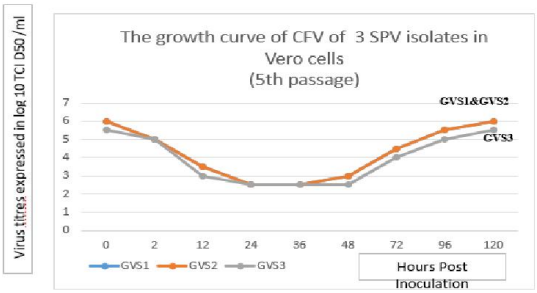

Figure 2 The growth curve of CFV of 3 SPV isolates in Vero cells (5th passage):

3.3. Growth kinetics of whole infected culture of a selective SPV isolate:

Results of MOI and titers of the selective three virus isolates are presented in Table (3). The selective optimum titers were $log_{1}{}^{6}$, $log_{10}{}^{6}$ and $log_{10}{}^{5.5}$ $TCID_{50}$/ml using 0.1 MOI for the three viruses isolates at 4 DPI respectively.

Table 3 Growth kinetics (GK) of a selective governorates SPV isolates.

(DPI)	Titer of a three governorates SPV isolates ($TCID_{50}$/ ml) with different MOI								
	GVS1			GVS2			GVS3		
	0.1	*0.01	0.001	0.1	*0.01	0.001	0.1	*0.01	0.001
2	4.0	3.5	2.5	4.0	3.5	2.5	3.5	3.0	2.0
**4	6.0	5.5	4.5	6.0	5.5	4.5	5.5	5.0	3.5
5	6.0	5.5	4.5	6.0	5.5	4.5	5.5	5.0	3.5

DPI: Days post Inoculation. MOI: Multiplicity of Infection (virus dilutions from 10^{-1} to 10^{-3}). GVS 1 to GVS 3: 3 Governorates Virus Samples. $TCID_{50}$/ml: Tissue Culture Infective Dose $_{50}$/ ml. *: optimum MOI (10^{-1}, 10^{-2} virus dilutions). **: optimum virus harvestation time (4 DPI).

3.4. Indirect Fluorescent Technique (IFAT):

The infected Tissue culture cover slips with a selective high titer SPV isolate (GVS1) showed a specific greenish yellow fluorescent color at 72 hrs PI using heterologous positive rabbit SPV antisera of reference Romanian SPV. The fluorescent reaction varied from fine granular perinuclear to diffuse fluorescent masses in the cytoplasm of infected cells. The intracytoplasmic fluorescence at 72 h PI with SPV isolate was specific and considered a guide for virus

antigenicity and identification. Fig. (3.A) illustrate pattern of fluorescent reaction infected Vero cells at 72 h PI, while Fig. (3.B) is done for the non-infected Vero cells.

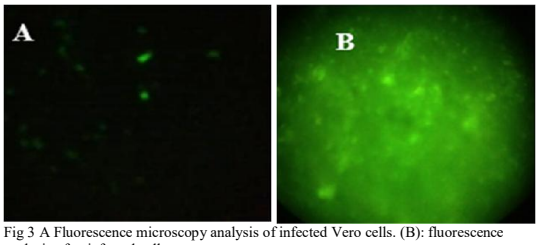

Fig 3 A Fluorescence microscopy analysis of infected Vero cells. (B): fluorescence analysis of uninfected cells

4. DISCUSSION

Vero tissue culture was commonly used to propagate the SPV isolates because of their ability to support the replication of a variety of isolates (Olfat, 2000; Hosmani *et al*., 2004 ; Mikhael *et al*., 2012). Generally, the use of T.C minimize animal use as a primary and secondary cultures which still have many of the detrimental, inherent characteristics and contaminating elements.

The propagated SPV on LT cells was transmitted to grow on Vero cell line. The CPE was slower in the first two passages represented by only cell aggregation, but after serial passages, CPE increased gradually to become complete and rapid cell degeneration and vacuolation at the 4th and 5th DPI. The cellular changes were visible and characteristic cytopathic effects (CPE) were seen from 3rd passage and appeared as rounding, clumping of cell, intracytoplasmic inclusion bodies, vacuolation and degeneration. Table (1) showed that the titer of the first passages on Vero cell line was rather lower than that of the LT propagated virus (which final titer was $10^{6.5}TCID_{50}$ /ml), but the titer increases gradually during the different subsequent passages on Vero. This rise in virus titer with increasing passages gave hope that by more passages, more adaptation may be obtained with increase in the virus titer. This actually happened and the average titers increased gradually during five successive passages till reach the maximum titer by the 4th and 5th passages and became fixed. The end point of virus titers was 6 $\log_{10}$, 6 $\log_{10}$, 5.5 $\log_{10}TCID_{50}$/ml of GVS 1, GVS 2 and GVS 3 respectively. This progress in the infectivity titer and different stages of the CPE was dissimilar to those results previously reported formerly (Singh and Rai, 1991; Hosmani *et al.,* 2004; Mahmoud *et al.,* 2016; OIE, 2017; Abd-Elfatah *et al.,* 2018). These results are in the same line with those reported by Chaudhary *et al.* (2009), who adapted (RF virus strain) in lamb testicle cells then to Vero cells after nine passages. Also, agree with Yogisharadhya *et al.* (2011). In contrast, some strains as ranipet strain of ovine Poxvirus resist growing on Vero cells (Davis, 1976; Jassim and Keshavamurthy, 1982).

To optimize the replication of the newly adapted virus isolates to Vero cells, we studied the effect of two factors which are the growth curve including the cell free and cell associated virus and growth kinetics including virus input or (MOI) and time of harvestation.

Growth curve of the 5th passage of three viruses was performed in Vero cells to evaluate the replication cycle characters and production of excessive new viruses in Vero cells at different time intervals post infection. Also, estimation the titers of intracellular virus (CAV) and extra cellular virus (CFV) at specific time of virus replication to select the best time for virus harvestation and determination which titer is higher; (CAV or CFV) to be used as an inoculum for later seed virus preparation.

The obtained results in table (2) and figure (2) indicated that a logarithmic increase in virus titers started from 48 hours post inoculation (PI) with maximum titer at 120 hours PI which indicated that the best time for virus harvestaion was the 5th day post inoculation. The titer of both the cell free virus (CFV) and cell-associated (CAV) is quite high, so it is preferable to include both cell-associated and cell-free virus in the harvested material for vaccine preparation to increase the final titer of the virus used. These findings were in agreement with (Rhizkallah,1994; Olfat, 2000; Mikhael, 2012) who studied the growth characters of SPV in Vero cell culture.

The growth behaviour of SPV isolates (GVS1, GVS2 and GVS3) were investigated also with different (MOI) at 2, 4 and 5 DPI. MOI is a critical factor that has to be carefully determined which can influence virus growth dynamic and virus yield (Genzel *et al.,* 2006; Trabelsi *et al.,* 2012) as well as influence the production process if needed in the future, because it determines the best titer of the virus, the optimum harvestation time and the most suitable dose used for Vero cells inoculation.

In our study, we aim to use the lowest MOI which provides the highest titer. As SPV is considered as a lytic virus, so the chosen optimum MOI was (0.01) with the optimum virus harvestation time was 4 days' post Inoculation (DPI) and virus titer equal to $10^{5.5}$. Despite the MOI (0.1), has virus titer reaching to 10^{6}, but it is not economic to use it ,because when we use 0.1 MOI we can produce 10 doses only with titer of each dose 10^{6}, while when we use MOI 0.01 we produce 100 doses with virus titer reaching to $10^{5.5}$ regarding that the protection titer is $10^{2.5}$ only, so it is economic to use MOI (0.01) as the optimum MOI. While Trabelsi *et al* (2014) used 0.005 as the most suitable MOI in their study for SPV vaccine preparation. Previous studies revealed that cells infected at high MOIs maximum titers were attained earlier than in cultures infected at lower MOIs (Maranga *et al.,* 2003; Audsley, *et al.,* 2005). In addition, cell infection at high MOIs increases the amount of defective virus (non-infective) produced by the cells. The optimal MOI is largely dependent on the virus to be produced. We noticed that the results of growth kinetics come similar to that obtained by growth curve as the best harvestation time was 4-5 DPI.

This previous kinetics and Growth curve come in agree with Kumar *et al.* (2021) whose kinetics of the viral DNA synthesis and one-step growth curve analysis suggested that Vero cell-adapted LSDV initiates synthesizing its genome at ~24 post-infection (hpi) with a peak level at ~96 hpi whereas evidence of progeny virus particles was observed at 36–48 h with a peak titer at ~120 h.

To detect whether the virus antigen existed, we performed an IFA test on CPE-positive Vero cells. Virus-specific green fluorescence was found in the cytoplasm of infected cells (Fig. 3. A). None of the control cells was stained (Fig. 3. B). This was the same observed by Zhu*etal.* (2013) and Zhang *et al.* (2018). Finally, after adaptation of SPV isolates, and investigation of some characters including the growth curve and growth kinetics of these SPV isolate, further studies are recommended for SPV vaccine preparation, that not only used for giving protection to sheep, but also can be used to vaccinate cattle under stress factors against LSDV.

6. REFERENCES

1. Abd-Elfatah E.B., El-Mekkawi M.F. and Aboul-Soud E.A. (2018): Capripoxviruses of small ruminants: control and evaluating the future update efficacy of a current vaccine in Egypt. Adv. Environ. Biol. 12(12): 11-16..
2. Abdi Assefa, Barbara Weilnda and Fufa Abunna. (2019): isolation and characterization of pox virus circulating in sheep and goats from outbreak cases of Adea Berga District, West Shoa Zone, Oromia, Ethiopia. Int. J. Curr. Res. Aca. Rev. 7(6), 1-21.
3. Aboul Soud, E.A. (1995): Studies on the adaptation of lumpy skin disease virus (LSDV) in cell cultures. Ph. D. Thesis, (Microbiology), Fac. Vet. Med., Alexandria Univ., Egypt.
4. Audsley J.M. and Tannock G.A. (2005): The growth of attenuated influenza vaccine donor strains in continuous cell lines, J. Virol. Methods 2: 187–193.
5. Babiuk S, Parkyn G, Copps J, Larence J.E., Sabara M.I., Bowden T.R., Boyle D.B., Kitching R.P. (2007): Evaluation of an ovine testis cell line (OA3.Ts) for propagation of capripoxvirus isolates and development of an immunostaining technique for viral plaque visualization. J Vet. Diagn. Invest. 19: 486-491.
6. Babiuk S., Wallace D.B., Smith S.J., Bowden T.R., Dalman B., Parkyn G., Copps J. and Boyle D.B. (2008): Detection of antibodies against capripoxviruses using an inactivated sheeppox virus ELISA. Transboud Emerg. Dis. 56: 132–141.
7. Bhanuprakash V., Hosamani M., Juneja S., Kumar N. & R. K. Singh (2006): Detection of goat pox antibodies: comparative efficacy of indirect ELISA and counter immune electrophoresis. J. Appl. Anim. Res. 30(2): 177-180.
8. Bhanuprakash V., Indrani B.K., Hosamani M., Singh R.K. (2006): The current status of sheeppox disease. Comp. Immunol. Microbiol. Infect. Dis. 29: 27–60.
9. Buller R.M., Arif B.M., Black D.N., Dumbell K.R., Esposito J.J., Lefkowitz E.J., McFadden G., Moss B., Mercer A.A., Moyer R.W., Skinner M.A. and Tripathy D.N. (2010): Family Poxviridae. in: C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger and L.A. Ball (Eds): Virus Taxonomy: 8th Report of the International Committee on Taxonomy of Viruses, San Diego. Elsevier Academic Press. Pp. 117 – 133.
10. Carn V.M (1993): Control of capripox viruses infections. Vaccine 11: 1275 - 1279.
11. Chaudhary S.S., Pandey K.D., Singh R.P., Verma P.C. and Gupta P.K. (2009): A Vero cell derived combined vaccine against *sheeppox* and *Peste des Petits* ruminants for sheep. Vaccine J. 27: 2548-2553.
12. Constable P., Hinchcliff W.K., Done S. and Gruenberg, W. (2017): Veterinary medicine (11th ed) A textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats.
13. Davies F.G. (1976): characterization of virus causing a pox disease in sheep and goats in Kenya, with observations on the epidemiology and control. J. Hyg. 79 (2): 163-171.
14. Genzel Y., Fischer M. and Reichl U. (2006): Serum-free influenza virus production avoiding washing steps and medium exchange in large-scale microcarrier culture. Vaccine 24: 3261–3272.
15. Hani B., Thang T., Charles N., Timothy R.B., Volker G., Suresh T., Lorne A.B., Pravesh K., Arshad M., David B.W., and Shawn B. (2015): A lumpy skin disease virus deficient of an IL-10gene homologue provides protective immunity against virulent *capripoxvirus* challenge in sheep and goats. Antivir. Res. 123: 39–49.
16. Hosamani M., Mondal B., Tembhurne P.A., Bandyo-Padhyay S.K., Singh R.K., and Rasool T.J. (2004): Differentiation of *sheeppox* and *goatpox viruses* by sequence analysis and PCR-RFLP of P32 gene. Virus Genes J. 29: 73-80.
17. Jassim F.A. and Keshavamurthy B.S. (1982): Propagation of sheep pox virus in cell cultures. Acta. Virol. 26 (3): 190–192.
18. Kitching R.P. and Taylor W.P. (1985): Transmission of capripoxvirus. Res. Vet. Sci. 39: 196–199.
19. Kumar N., Chander Y., Kumar R., Khandelwal N., Riyesh T., Chaudhary K., Shanmugasundaram K., Kumar S., Kumar A., Gupta M.K., Pal Y., Barua S., and Tripathi B.N. (2021): Isolation and characterization of lumpy skin disease virus from cattle in India. PLoS One 16(1): e0241022.
20. Mahmoud A.Z., Khalafalla A.I. and Abdellatif, M.M. (2016): An epidemiological study of sheep and *goatpox* outbreaks in the Sudan. Food Biol. J. 5: 1-5.
21. Maksyutov R.A., Gavrilova E.V., Agafonov A.P. and Taranov O.S. (2013): An outbreak of *Sheeppox* in Zabajkalskij kray of Russia. Transbound. Emerg. Dis. 62(4): 453-456.
22. Maranga L., Brazão T.F., and Carrondo M.J.T. (2003): Virus-like particle production at low multiplicities of infection with the baculovirus insect cell system. Biotechnol. Bioeng. 2: 245–253.
23. Mikhael C.A. (2012): Trials for preparation of improved bivalent vaccine for sheeppox and goatpox viruses. PhD. Thesis. Faculty of Vet. Med. Cairo University.
24. Mikhael C.A., El-Sanousi A.A., Reda I.M. and Soliman S.M. (2012): preparation of univalent and bivalent Capripox vaccines. 5th Sci. Conger. of Egypt. Soc. For Anim. Manag. Pp. 55-69.
25. OIE.(2017): Sheep Pox and Goat Pox. OIE Terrestrial Manual 2017, Chapter 2.7.13. Available online:https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.13_S_POX_ G_POX.pdf.
26. Olfat, E.N. (2000): Studies on goat pox vaccine. Ph.D. Thesis (Virology), Fac. Vet. Med., Cairo University.
27. Reed L.J. and Muench H. (1938): A simple methods of estimating fifty percent end point. Amer. J. Hygiene 27: 493-497.
28. Rizkallah S. S. (1994): Further studies on sheep pox disease in Egypt. Ph.D. Thesis. Fac. Vet. Med., Cairo University.
29. Singh, B. and Rai, A. (1991): Adaptation and growth of sheep pox virus in Vero cell culture. Indian Vet. Med. J. 15 (4): 245-250.
30. Tozzini F., Salim H.A., Abdiljabar H.D., and Abulcadir A.F. (1987): Growth characteristics of vaccine strains of rinderpest virus and ovine pox virus on subcultures of bovine fetal lung fibroblasts. Arch. Vet. Ital. 38 (1): 9-12.
31. Trabelsi K., Majoul S., Rourou s., Kallel H. (2012): Development of a measles vaccine production process in MRC-5 cells grown on Cytodex1 microcarriers and in a stirred bioreactor, Appl. Microbiol. Biotechnol. 93: 1031–1040.
32. Trabelsi K., Majoul S., Rourou S., Kallel H. (2014): Process intensification for an enhanced replication of a newly adapted RM-65 sheep pox virus strain in Vero cells grown in stirred bioreactor. Biochem. Eng. J. 90: 131–139.
33. Tuppurainen E.S.M., Venter E.H., Shisler J.L., Gari G., Mekonnen G.A., Juleff N., Lyons N.A., De Clercq K., Upton C., Bowden T.R., Babiuk S., and Babiuk L.A. (2017): Review Capripoxvirus Diseases: Current status and opportunities for control. Transbound. Emerg. Dis. 64: 729–745.
34. Yogisharadhya R., Bhanuprakash V., Hosamani M., Venkatesan G., Balamurugan V., Bora D.P., Bhanot V., Prabhu M., and Singh R.K. (2011): Comparative efficacy of live replicating sheeppox vaccine strains in ovines. Biologicals J. 39: 417-423.
35. Zhang M., Sun Y., Chen W., and Bu Z. 2018. The 135 gene of goatpox virus encodes an inhibitor of NF-κB and apoptosis and may serve as an improved insertion site to generate vectored live vaccine. J. Virol. 92: e00190-18.
36. Zhu X.L., Yang F., Li H.X., and Dou Y.X. (2013): Identification and phylogenetic analysis of a *Sheeppox virus* isolated from the Ningxia Hui Autonomous Region of China. Genet. Mol. Res. 12 (2): 1670-1678.