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 passed and adapted in lamb testis cell culture (isolates with titer reaching to 6.5 Log10 TCID50 / 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 fluorescent 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 (DI) and virus titre was equal to 10^{15.3}. 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; Ham 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 (Bhanaprkash 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 (GV1, GV2, and GV3; El Wadi Al Gadid, Marsa-Matrouh and Giza, respectively) on LT cell culture, which previously recorded the highest titer (10^6.5 TCID50/ ml), was transmitted to grow on Vero cell line according to OIE (2017) and examined daily for (CPE).
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 10⁻⁷). (Babuk 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 Fluorescent 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 Aboulouad (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 shown in Table (1) and Fig (1).

![Figure 1](image1.png)

Figure 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>
<thead>
<tr>
<th>Virus passages</th>
<th>Virus titers (TCID₅₀/ml) of governator virus samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>GVS1</td>
<td>GVS2, GVS3</td>
</tr>
<tr>
<td>1</td>
<td>5.2, 5.5, 5.0</td>
</tr>
<tr>
<td>2</td>
<td>5.5, 5.5, 5.0</td>
</tr>
<tr>
<td>3</td>
<td>6.0, 6.0, 5.5</td>
</tr>
<tr>
<td>4</td>
<td>6.0, 6.0, 5.5</td>
</tr>
<tr>
<td>5</td>
<td>6.0, 6.0, 5.5</td>
</tr>
</tbody>
</table>

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, 40, 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 10⁵ TCID₅₀/ml to 10², 10¹ and 10⁻¹ TCID₅₀/ml at 2, 12 and 24 h PI respectively. The titers of CAV were 10⁻³, 10⁻² and 10⁻¹ TCID₅₀/ml at the same hours. The significant infectivity virus titers of both CFV & CAV were 10⁻¹ TCID₅₀/ml at 96 Hrs PI. At 120 Hrs PI, the CFV titers increased and reached 10⁵ TCID₅₀/ml while CAV were log₁₀ TCID₅₀/ml for G1 and G2.

<table>
<thead>
<tr>
<th>Hours post Inoculation (HPI)</th>
<th>Titers of CFV &amp; CAV (TCID₅₀/ml) of 3 governator samples</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GVS1</td>
</tr>
<tr>
<td>0</td>
<td>%-0</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
</tr>
<tr>
<td>12</td>
<td>3.5</td>
</tr>
<tr>
<td>24</td>
<td>2.5</td>
</tr>
<tr>
<td>36</td>
<td>2.5</td>
</tr>
<tr>
<td>48</td>
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<tr>
<td>72</td>
<td>4.5</td>
</tr>
<tr>
<td>96</td>
<td>5.5</td>
</tr>
<tr>
<td>120</td>
<td>**6.0</td>
</tr>
</tbody>
</table>

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

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 titer of CFV & CAV were log₁₀, log₁₀ and log₁₀ TCID₅₀/ml using 0.1 MOI for the three viruses isolates at 4 DPI respectively.

<table>
<thead>
<tr>
<th>Hours post Inoculation (HPI)</th>
<th>Titer of a three governator virus samples (TCID₅₀/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GVS1</td>
</tr>
<tr>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>5.5</td>
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<tr>
<td>4</td>
<td>6.0</td>
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<td>6</td>
<td>6.0</td>
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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
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 harvestation 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; Mikhail, 2012) who studied the growth characters of SPV in Vero cell culture.

The growth behaviour of SPV isolates (GVSI, GV32 and GV33) 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^{5}, 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^{5}, 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^{5.5} 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 et al. (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.
REFERENCES


