

# Comparison of Vero and a New Suspension Cell Line in Propagation of Peste des Petits Ruminants Virus (PPRV)

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**Abstract**—Peste des Petits Ruminants (PPR) is an acute, highly contagious and world organization for animal health (OIE) notifiable economically important transboundary viral disease of sheep, goat and wild ruminant species with high morbidity and mortality and caused by PPR virus. Currently PPR control is done by vaccination with an attenuated PPR strain (Nigeria 75/1) produced in monolayers of Vero cells grown in roller bottles or static flasks. Vero cell culture is very time consuming and difficult. As an alternative, we introduce a new suspension cell line as cell substrate for virus propagation. After selection the most sensitive cell clones to the virus and adaptation on suspension cell line, growth characteristics of new cell substrate was evaluated. Also, the conditions of cell culture and virus culture were optimized, and finally titer of production was measured and compared to titer of PPR vaccine available. Sensitivity of cell to the virus was stable during many passages. The Average final titer of virus production was  $6.7 \log_{10}$  CCID<sub>50</sub>/ml and may be higher in bioreactor.

**Index Terms**—PPR vaccine, Peste des petits ruminant's disease, Suspension cell line, growth curve

## I. INTRODUCTION

Peste des Petits Ruminants (PPR) is an acute, highly contagious and fatal disease of sheep and goats and is considered as one of the major constraints to the productivity of small ruminants in Africa and Asian countries [1]-[4]. The causative agent, PPR Virus (PPRV), is a member of the genus Morbillivirus within the Paramyxoviridae family and is antigenically closely related to the Rinderpest (RP) virus [5], another member of the genus Morbillivirus that causes similar diseases in wild and domestic ruminants. The PPR disease is characterized by high fever, ocular and nasal discharges,

pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro-intestinal tract leading to severe diarrhea and, in many cases, death [6], [7]. The main characteristic of the pathogenesis of PPRV infection, as for all other Morbilliviruses, is the profound but transient immunosuppression induced by this virus in its host with the consequence of increased susceptibility to opportunistic infections and increased mortality [8]. The viral replication occurs in the host cell cytoplasm and the virus is released by budding [9]. However, these mechanisms are not very efficient in this virus family and a substantial amount of the produced viruses are kept associated to the host cells membranes. Until recently a heterologous Rinderpest vaccine has been used for the control of PPR [10]; however, due to the ongoing Rinderpest eradication programs, the use of this vaccine for the control of PPR has been restricted or banned to avoid complications in RPV serosurveillance. The need for a PPR homologous vaccine led to the development of an attenuated viral vaccine based on the strain PPR 75/1, isolated in Nigeria in 1975 [11], [12]. This strain was attenuated by 74 serial passages in Vero cell cultures [10] and it is currently the only vaccine permitted for use in sheep and goats [12], the efficacy being demonstrated at a dose of  $10^3$  TCID<sub>50</sub> [1]. Nowadays this vaccine is produced in Vero cells using classical techniques, i.e. in T-flasks or roller bottles [13]. These strategies involve high efforts concerning consumables and have limited scalability, significantly increasing the bioprocess costs. Thus there is a need for better vaccine production processes for controlling future PPR outbreaks. A few cell lines are sensitive to PPRV; for example: Madin Darby Bovine Kidney (MDBK), Baby Hamster kidney clone 21 (BHK-21), CV1 and particular the African green monkey kidney (Vero) cell line [4], [13], [14]. Use of a suspension cell substrate for production of PPR vaccine can be useful in simplicity of process and improve

efficiency of production. The lymphoid tissues are major sites of morbillivirus replication because the presence of a protein receptor on the cell surface, the Signalling Lymphocyte Activation Molecule (SLAM) also known as CD150 which is used preferentially by wildtype morbilliviruses to bind to the host [7], [15]-[18]. The infection efficiency for these cells is up to 100-1000 times more than epithelial cells of other organs [15], [19]. A suspension Cell line (F9) derived from lymph nodes of cow established by Razi Institute-Iran was showed very high sensitivity to vaccinal strain of PPRV. This paper evaluates sensitivity of a suspension cell line to PPR virus for virus propagation order to vaccine production.

## II. METHOD AND MATERIAL

### A. Cell Cultures

Established Bovine Lymphoid Suspension cell line (F9 clone) obtained from Razi Institute, Iran and frozen in liquid nitrogen. So Vero cell line (ATCC, ccl-81) was used in this study.

Dulbecco's Modified Eagle Medium or DMEM (sigma, USA) supplemented with 10% (v/v) Fetal Calf Serum (FCS) was used as growth medium and DMEM with 2% (v/v) FCS as maintenance medium for all cell cultures. 0.25% trypsin-EDTA solution was used for subculture of monolayer cells. So Stoker medium was formulated in Razi institute base on reference with some modification [20].

### B. Virus

Razi Institute Vero cell substrate base Vaccinal strain of PPR virus (Nigeria 75/1) with permission was used. The PPR virus was inoculated to healthy, Vero cell culture flask. The flask was incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air (memmert, Germany). The culture fluid was harvested each time by three freeze-thaw cycles and clarified by centrifugation for 5min at 800g. The PPR virus was passaged serially for adaptation. The virus was identified on Vero cells through characteristic Cytopathic Effects (CPEs). After adaptation, the PPR virus was propagated on Vero cells. Harvest after centrifugation was aliquoted and frozen in -20 °C and a sample was titrated.

### C. Assay for Virus Yield

Virus yields from Vero and Suspension cell lines were determined in the Vero cell culture. Briefly, the serial 10-fold dilutions of each cell cultures-virus supernatants were prepared in growth medium and 50 µl of each dilution were transferred to each of the five wells of a 96 well microplate that contained the same volume of fresh Vero suspensions ( $3.0 \times 10^5$  cells/ml). Plates were incubated at 37 °C in 5% CO<sub>2</sub> atmosphere for 10 days and the Cytopathic effect was checked and virus titer (TCID<sub>50</sub>) was determined based on the statistical method of Spearman-Kärber [21].

### D. Virus Adaptation to Suspension Cell Line

For adaptation of PPRV on Suspension cell line, 0.1 MOI adapted PPRV on Vero cells was inoculated to flask

25cm<sup>2</sup> contain  $1.5 \times 10^6$  cell/ml in maintenance medium. Flask incubated in 37 °C in the presence of 5% CO<sub>2</sub> in CO<sub>2</sub> incubator. Total count and viability of Suspension cell line was calculated daily. When viability was reached under 20%, flask was frozen in -20 °C. After two times freeze and thawing, liquid was centrifuged for 10min at 600g. Supernatant was used for virus titration on Vero cells and continuing of adaptation. PPRV passaging was repeated three times.

### E. Dilution Cloning of Suspension Cell Line

For single cell cloning of F9 cells, the viable cells in the flask were counted and a cell suspension containing 100 cells was prepared in 10ml DMEM medium with 20% (v/v) Fetal Bovine Serum or FBS (Gibco, USA). 100 µL of the cell suspension was dispensed into the wells of a 96 well microplate. It was checked by visual examination on the microscope a few hours after plating and was marked and followed the wells have only one cell [20]. The plate was incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 6 days without changing the medium. The morphology, viability and growth characteristics of cell colonies were checked daily in incubation period. Selected clones were propagated in flask and frozen in Liquid nitrogen [20]. Third passage after Thawing was used for growth characteristics comparison. To ability evaluate of different cell clones to produce PPRV, during three sequential passages were adapted PPRV on cell clones and the most sensitive cell clone was selected by titration of final yields that it provides more efficiency in replication of virus and other tests were done on it.

### F. Growth Curves of Vero and Suspension Cell Line

Growth curves were carried out in a constant volume of 10ml per flask with initial inoculums of  $2 \times 10^5$  cell/ml at day 0 for Vero cell culture and selected suspension cell line. The cell count was checked daily for 7 days with hemocytometer. This process was repeated three time and the results were plotted on a log-linear scale. Also doubling time and specific growth rate ( $\mu$ ) was calculated [22]. Viability of Suspension cell line was calculated by trypan blue (Gibco, USA) staining (0.4% in PBS) on the hemocytometer [20]. Also, Suspension cell line was cultured in Cellspin (IBS, UK) system as follow 500ml DMEM contain 10% Calf Serum and  $2 \times 10^5$  cell/ml added to a special glass bottle for Cellspin with one litter capacity. Cellspin was placed in a CO<sub>2</sub> incubator. Cellspin was turned on with 70rpm for 7 days. Daily the cell count was checked.

### G. Comparison of PPRV Titers Propagated on Vero and Suspension Cell Line

PPR virus susceptibility and viral replication efficiency on Suspension cell line and Vero cell line were compared in same conditions. The Vero cell line were seeded at same concentrations in 3 numbers of 25cm<sup>2</sup> tissue culture flasks, to a final culture volume of 10 ml per flask with DMEM containing 10% (v/v) CS. After 3 days that the cells reached 90% confluence, the cells in a flask were counted and in other flask after removal of the medium, it

was inoculated with virus suspension at a MOI of 0.05. Subsequently, 10ml of maintenance medium, containing 5% (v/v) CS, was added, and the cultures were incubated. Also, the lymphoid cell line were seeded at equal concentration to Vero cell, to a final culture volume of 10 ml per flask with DMEM containing 5% (v/v) CS and the cells were infected with PPRV using a MOI of 0.05. Both of cell cultures were monitored as described above.

**H. Sensitivity Stability of Suspension Cell Line to PPRV**

Suspension cell line was passaged consecutively 20 times and in each passage three flask 25cm<sup>2</sup> was cultured. One flask for inoculation of 0.05 MOI PPRV, one for negative control and last one for cell passaging. After four days virus inoculated flask for titration of virus, was frozen.

**I. Production of PPRV on Suspension Cell Line**

**1) Effect of MOI in PPRV production**

The Suspension cell line were seeded at 0.5×10<sup>6</sup> cells/ml in 25cm<sup>2</sup> tissue culture flasks, to a final culture volume of 10ml per flask with DMEM containing 5% (v/v) CS. The cells were infected with PPRV using different MOIs (0.001, 0.01, 0.02, 0.05, and 0.1) and were monitored as described above.

**2) Effect of culture media conditions in PPRV production**

To evaluate of effect of culture medium and serum in PPRV production, the lymphoid cell line were seeded with DMEM and Stoker medium containing different values of CS and FBS (2%, 5%, 10% (v/v)). To evaluate of effect of the cells number per ml at the time of the virus inoculation, the cells were seeded at different concentrations (0.2, 0.5, 0.8 and 1×10<sup>6</sup> cells/ml) and the cell cultures were infected with PPRV using a MOI of 0.05. Also, the cells were infected with virus in two forms, same time with cell culture and 24h after cell culture and the cell cultures were monitored as described above.

**3) Production of PPRV in stirred cultures (CELLSPIN)**

After washing and sterilization of spinning vessels, the Suspension cells were seeded at 1×10<sup>6</sup> cells/ml in two vessels, to a final culture volume of 500ml per vessel with DMEM and Stoker containing 5% (v/v) CS. The cells were infected with PPRV using a MOI of 0.05. The vessels were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The appearance of CPE was assessed daily, with sampling from the cells after viral inoculation and viability of cells were obtained and when the CPE was more than 80%, the inoculated cells were frozen at -20 °C to virus titration.

**J. Statistical Analysis**

All measurements were carried out by triplicate in three different replicates. The data results of the growth curve were normalized at logarithmic scale. Statistics were performed using two statistical Mann-Whitney U-test and Wilcoxon W-test for determine differences between Vero and Suspension cell line for viral replication. All statistical analyses were performed using

SPSS Version 22 and P values <0.05 were considered significant.

**III. RESULTS**

F9 cells, a suspension cell line with a very high capacity for population doubling, were seeded in flask after retrieving from the freezer and thawing the ampoule.

At least, three sequential passages from the cells has considerable importance after thawing, as the potential increase in cell number, growth rate, uniformity of the cells and quality improvement of culture.

By dilution cloning 4 cell clones were selected that they exhibited more preferences (e.g. high viability, cluster arrangement and low doubling time) than others (data not shown).

For assessed sensitivity of the cell clones to the virus, three sequential culture of virus on the cell clones were performed. The results presented in Table I, show that F9C6 cell clone provided higher titer from PPRV (6.7 Log<sub>10</sub> TCID<sub>50</sub>/ml) in the less time (4 days). In other words, F9C6 cell colon has more efficiency in replication of PPRV.

TABLE I. SENSITIVITY EVALUATION OF 4 CELL CLONES TO PPR VIRUS DURING THREE SEQUENTIAL PASSAGES

Cell colons	F9C6	F9D8	F9E2	F9G4
1 <sup>st</sup> Passage	6 days	7 days	7 days	7 days
2 <sup>nd</sup> Passage	5 days	6 days	6 days	6 days
3 <sup>rd</sup> Passage	5 days	6 days	6 days	6 days
Viability	10%	17%	16%	14%
Final titer (CCID <sub>50</sub> /ml)	10 <sup>6.4</sup>	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>6</sup>

TABLE II. GROWTH CHARACTERIZATION OF VERO AND F9 CELL LINE

Responses	Vero (flask)	F9C6 (flask)	F9C6 (cell spin)
Maximum Cell no (cell/ml)	6.8×10 <sup>5</sup>	1.7*10 <sup>6</sup>	3.42*10 <sup>6</sup>
doubling time (td), hour	32.2	18.6	14.3
Avg. Specific growth rate (μ)	0.021	0.037	0.049

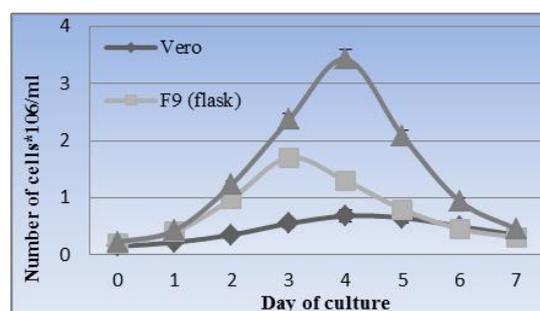


Figure 1. Growth curves of Vero and F9 cell lines.

According to Table II, that shows the growth curves of Vero cell and Suspension cell line in static and stirred

culture, the growth rate of F9 cells (suspension) is rather than Vero cells (monolayer). The peak of F9 cell concentration was  $(1.7 \pm 0.1) \times 10^6$  cell/ml in flask and  $(3.42 \pm 0.2) \times 10^6$  cell/ml in cell spin and the ratio of maximum number of F9 cells to Vero cells was 2.5-3 in the flask and 5 in the cell spin, nearly. The doubling time of F9 cell was calculated in several timescales of logarithmic phase, repeatedly and was  $18.6 \pm 0.3$ (h) in flask and  $14.3 \pm 0.2$ (h) in cell spin and the values of specific growth rate were significant, too (Fig. 1).

To evaluate efficiency of Suspension cell line in PPRV replication and compared it with Vero cell line, PPRV was inoculated to both cells in the same conditions during 3 sequential passages. The maximum PPRV titer obtained for each cell line is presented in Fig. 2. The virus was able to replicate and the titer increased by at least three orders of magnitude for Vero and F9 cells. In the case of F9 cells, the visible CPE was observed on 48 h post infection and the maximum volumetric PPRV productivity occurred after 4 days that was  $6.5 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$  but the CPE in the Vero cells was begun after 72h and the maximum titer of PPRV was  $5.3 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$  after 6 days then a delay of 48h was observed for Vero cells, where the maximum titer was achieved. Since the F9 cells is a suspension cell and more susceptible to PPRV, provides higher titer of virus in the less time. Statistical analysis for determine effect of different cell substrates (Vero and Suspension cell line) in viral replication using two statistical Mann-Whitney U-test and Wilcoxon W-test (+0.037;  $p < 0.05$ ) was showed increasing the titer of PPR virus propagated on Suspension cell line.

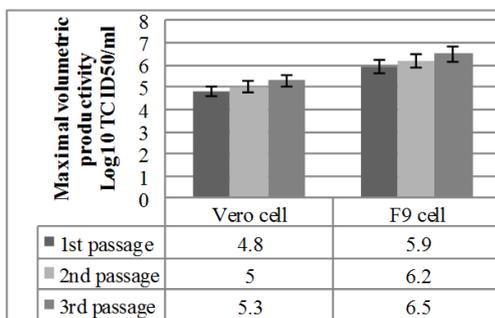


Figure 2. Titer comparison of PPRV on Vero and F9 cell lines

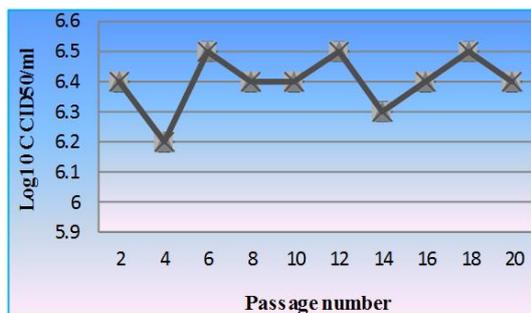


Figure 3. Sensitivity stability of suspension cell line to PPRV

The titer of virus yields propagated on consecutively 20 passage of Suspension cell line presented in Fig. 3. The end point values of virus propagated on first passage

of F9 cell was  $6.4 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$  and it was remained constant nearly in further passage that showed sensitivity of suspension cell line to PPRV has not change.

To assess the effect of the MOI in PPRV productivity using static conditions, F9 cells were infected with MOIs of 0.1, 0.05, 0.02, 0.01 and 0.001. The results were showed that PPRV productivity was slightly higher when a MOI of 0.05 ( $6.6 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$ ) was used.

The lower productivity was obtained from MOI of 0.001 ( $5.8 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$ ), but no significant difference was observed.

In order to select a suitable medium for F9 cell growth and PPRV production, different conditions of culture, including two available media (DMEM and Stoker), two serum (FBS and CS with different values) and different initial cell concentrations where tested for their ability to produce PPRV. The F9 cells were cultured in mentioned conditions and were infected with a MOI of 0.05. Cell culture in DMEM medium containing 5% serum showed to be able to slightly increase the PPRV volumetric productivity ( $6.5 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$ ) comparing to the PPRV productivity obtained using the other conditions. The type of serum no significant effect in titer of PPRV but the values of serum is important in the increase the PPRV productivity. The efficacy of DMEM and Stoker in cell growth and virus production were similar but the pH of Stoker medium becomes acidic sooner and need to pH setting. The use of Stoker medium resulted in 0.2 log decrease in the PPRV productivity.

Also, to assess the effect of the initial cell density and the time of virus inoculation in PPRV productivity using static conditions, F9 cells were seeded at different concentration and virus were added as 24h post-inoculation and co-inoculation. The results were showed that PPRV productivity was slightly higher when an initial cell concentration of  $0.8 \times 10^6$  cell/ml was used and cell and virus culture as co-cultivation was performed ( $6.7 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$ ). At MOI of 0.5 the cells stopped growing and the cell concentration started to decrease and CPE in cells started at about 24h post-infection.

Briefly, in the static conditions, the maximum viral titer were obtained when the F9 cells were seeded at  $0.8 \times 10^6$  cell/ml with the DMEM medium containing 5% serum and were infected with MOI of 0.05 as co-cultivation.

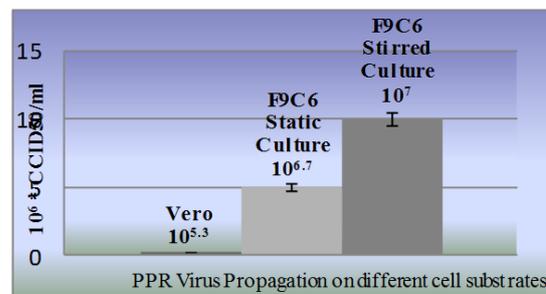


Figure 4. PPRV propagation comparison in different situation

To evaluate the feasibility to produce PPRV in stirred tank bioreactors F9 cells were grown in cell spin with DMEM and Stoker supplemented with 5% CS and

infected with MOI of 0.05. The maximum cell concentration was achieved for cell grown in cell spin ( $3.42 \times 10^6$  cell/ml) and because the cell concentration increased in the stirred culture as expected the titer of virus increased, too (Fig. 4).

The maximum volumetric PPRV titers are shown in Fig. 4. The maximum viral yields were obtained 5 days post infection from stirred culture of PPRV on suspension cell line ( $7 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$ ).

#### IV. DISCUSSION

PPR is an economically significant disease of small ruminates with an increasing global incidence [23]-[25]. At present, mass vaccination in endemic areas has been the best measure to protect sheep and goats from PPR [26]. Taking advantage of the close antigenic relationship between RPV and PPRV, tissue culture rinderpest vaccines had been used for a long time to protect small ruminants against PPR until the PPRV isolate Nigeria 75/1 was successfully attenuated by multiple passages in Vero cells [27]. Vero cell is a monolayer and adherent cell and require a surface for growth and differentiation [20]. Given the importance of controlling PPR outbreaks and limitations of scalable Vero cell culture, the aim of this work was to evaluate an alternative condition for production of a PPR attenuated vaccine instead of the currently available PPR vaccine produced in Vero cells. A new suspension cell line derived from lymph nodes of cow was showed high sensitivity to PPR virus because lymphoid cells are a major target of morbilliviruses. It is a suspension cell line that can be grown to a higher density than adherent conditions would allow.

In this study, we evaluated the sensitivity of a new cell substrate to PPR virus. It is a lymphoid cell line with a very high capacity for population doubling derived from lymph nodes of cow. This new cell line is highly efficient for PPRV propagation and production of PPR vaccine. At the first, the single cell cloning of suspension cell line was performed by dilution cloning method. It is the technique that is used most widely, based on the observation that cell diluted below a certain density form discrete colonies. Cell cloning isolate pure cell and help to reduce the heterogeneity of a cell culture and in fact a cell colony was derived from one cell [20]. To evaluate the ability of different suspension cell clones to produce PPRV, the cell clones were infected with PPRV during Three sequential passages and final yields were titrated. Finally F9C6 clone was selected as the most sensitive cell clone that it provides more efficiency in the replication of the virus. Suspension cell line is a globular and suspended cell with a smooth membrane and cumulative arrangement similar to cluster of grapes. For drawing growth curve of suspension cell line, the results of daily cell counts were plotted on a log-linear scale. Cell-growth curve can be useful in evaluating the growth characteristics of a cell line. From a growth curve, the lag time, population doubling time and saturation density can be determined [28]. The growth curves of cells showed the density and growth rate is higher and doubling time is shorter in the culture of F9C6 cell than Vero cell culture

and greater differences can be seen in stirred culture condition. Comparison of propagated

PPR virus on Suspension and Vero cell line showed that the Suspension cell line provides higher titer of virus in the less time. The titer of virus yields propagated on consecutively frequent passages of Suspension cell line showed sensitivity stability of suspension cell line to PPRV. The end point values of virus propagated on first passage of F9 cell was  $6.4 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$  and it was remained constant nearly in further passage. Finally, PPR virus culture conditions on Suspension cell line were optimized. To evaluation of the MOI effect in PPRV productivity using static conditions, F9 cells were infected with different MOIs. Also, in order to select a suitable medium for F9 cell growth and PPRV production, different conditions of culture and different initial cell concentrations were tested. In the static conditions, the maximum viral titer were obtained when the F9 cells were seeded at  $0.8 \times 10^6$  cell/ml with the DMEM medium containing 5% serum and were infected with a MOI of 0.05 as co-cultivation.

To evaluate the feasibility to produce PPRV in stirred tank bioreactors F9 cells were grown in CELLSPIN. It is a spinning system comprising stirring units for the gentle cultivation of cells and the preparation of cell products in spinning vessels. It is designed to provide a high surface to volume ratio ensuring improved oxygenation over standard designs. The maximum cell concentration was achieved for cell grown in CELLSPIN ( $3.42 \times 10^6$  cell/ml) and because of higher cell concentration in the stirred culture, as expected the titer of virus increased, too. The maximum viral yields were obtained 5 days post infection ( $7 \text{ Log}_{10} \text{ TCID}_{50}/\text{ml}$ ).

Therefore, we evaluated the sensitivity of a new suspension cell line to vaccinal strain of PPRV that can be used as cell substrate for Virus propagation in vaccine production. Because F9 lymphoid cell line is suspension prefer than Vero cell line in PPR vaccine production process so high virus titer can be achieved by this cell line.

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