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Sequential Adaptation of Vero Cell Lines in Serum Free Medium for Fixed Rabies Virus Propagation

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Abstract

Background: Vero cells are derived from kidney of the African green monkey *(Cercopithecus aethiops)* and proliferated in artificial growth medium. They are used as substrate for rabies virus propagation to produce cell culture based vaccines; however serum supplemented medium to proliferate Vero cells are very expensive and source of different contaminant agents that affect the quality and affordability products.

Objectives: The aim of this research was to adapt Vero cell lines in serum free medium through sequential serum reduction for propagation of Pasteur virus (PV) and Evelyn Rokitnicki Abelseth (ERA) rabies viruses.

Methods: Vero cells were proliferated in various serum concentration supplemented medium by gradual reducing from 10% to 0% serum concentration. Viable cells were counted and sub- cultured to passage seven in each serum concentrations. PV and ERA rabies were used to infect the cells proliferated in each serum concentration based on their multiplicity of infection and Virus titers of both viruses were calculated and expressed as tissue culture infectivity dose fifty($TCID_{50}$).

Results: The maximum viable cells density of Vero cells at each serum supplemented medium (0%, 1%, 2.5%, 5%, 7.5% and 10%) were 2.85×10^6 cells/ml, 2.75×10^6 cells/ml, 2.75×10^6 cells/ml, 2.70×10^6 cells/ml, 2.92×10^6 cells/ml and 2.75×10^6 cells/ml, respectively. The maximum virus titers were $10^{5.36}$ $TCID_{50}$/ml and $10^{5.61}$ $TCID_{50}$/ml for PV and ERA respectively in 0% serum concentration medium proliferated cells.

Conclusion: From the results of this study; it can be concluded that Vero cells adapted in serum free conditions were produce sufficient rabies virus titers propagation used for anti-rabies vaccine production.

Keywords: Cells density; Serum; Rabies; Titer; Vero cells

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Introduction

Cell culture is a process of growing the cells under controlled and aseptic conditions, generally outside of their natural environment. Vero cell lines were derived from African green monkey *(Cercopithecus aethiops)* kidney in Chiba University, Japan and sub-cultured several times before taken to culture collections [1]. They are continuous cell lines of mammalian origins proliferate on flasks, roller bottle and micro-carrier for different scale biopharmaceuticals production and biomedical researches [2]. The quality of initial Vero cells adhesion is affected by the amount of cells inoculated during subculture, constraints of surface area for anchorage of cells and accumulation of metabolic end products that inhibit or stimulate growth [3,4]. As Vero cells reach their confluence, they stop further proliferation and lift from culture surface or start to die; therefore, it is extremely important to monitor them and subculture as they form confluent monolayer. Vero cells were anchorage dependent cell lines that used mainly in virology and other applications including study of intracellular bacteria detection. For instance, Vero cells are used

as substrate for production of different viral rotavirus, measles virus, rubella virus, polioviruses and influenza viruses that gain great acceptance from regulatory authorities [5,6]. Vero cell lines are the candidate of choice for viral vaccine production, due to their: efficiency of primary virus isolation and replication to high titers [7]. Several vaccine manufactured by various pharmaceutical companies used Vero cells as main substrate due to its important characteristics for propagation of the desired virus on specified medium that allows proliferation of cells as well as extensive pathogens growth. Serum in culture medium supports the cells as additional nutrition, culture stimulating factors, protecting agents for both biological protection and prevention of mechanical damage. However, serum containing culture medium is becoming undesirable for production of vaccines [8]. There are various disadvantages of serum supplementation in culture media; such as; inconsistency of product and high protein content that hinders product purification [9]. The vaccine produced from serum free proliferated Vero lines were very important in terms of safety [10]. The production of cell culture based anti-rabies vaccine from Vero cells proliferated in serum supplemented medium has been conducted previously in our laboratory without considering those side effect and costs of serum in culture medium. Therefore, this work is aimed to gradual adaptation of Vero cell lines in serum free conditions for propagation of Pasteur virus (PV) and Evelyn Rokitnicki Abelseth (ERA) rabies viruses used for human and animals' vaccines production by avoiding mentioned problems of serum in culture medium.

Materials and Methods

Study setting and design

The study was carried out in Ethiopian Public Health Institute (EPHI), vaccine and diagnostic production directorate; cell culture based anti-rabies vaccine production laboratory involves laboratory based experimental study with quantitative and descriptive methods.

Cell lines and virus strains

Vero cells originally from American Type culture collection of vaccines such as rabies virus, (Vero ATCC CCL-81) used in this study was provided by National veterinary institute (NVI) Bishoftu, Ethiopia. Rabies virus strains such as, Pasteur virus (PV) and Evelyn Rokitnicki Abelseth (ERA) donated by Center for Disease Control and Prevention, Atlanta (CDC), stored in vaccine and diagnostic production laboratory.

Culture medium

medium used in this study was Minimal Essential Medium Eagle (MEME) (Sigma Life science, Batch # 021M8316) powder, dissolved in pure distilled water following manufacturer's instruction with Fetal bovine serum (FBS) (Sigma Life science, F7524, Lot: BCBR0718V) and the mixture was agitated to homogenize. The medium was sterilized by filtering with microbiological filter with pore size of 0.22 µm. For cells adaptation the medium was prepared by reducing the serum content expressed in percent as; 10% FBS, 7.5% FBS, 5% FBS, 2.5% FBS, 1% and 0% FBS in separately [11].

Cells reviving and passaging

Vero cells were taken from cryopreserved in the medium containing 20% fetal bovine Serum and 10% Dimethyl Sulfoxide (DMSO) (Chemicals Udyo- 121001(India), Batch no.44880LR), defrosted in water bath and transferred to 75 cm^2 T-flask and allowed to revive in MEME supplemented with 10% fetal bovine serum. Cells reviving and passaging were carried out after cells reached confluent stage; the flasks contained anchored cells were washed twice by pipetting 10 mL of Phosphate Buffer Saline (PBS) (Sigma Life science, Lot # SLBR3488V) and rinsed gently according to the protocol described by Ammerman et al. [12]. To trypsinize the cells; 2 mL of 0.05% trypsin-EDTA (Ethylene diamine Tetra acetic Acid) [Gibco, 0.05% Trypsin-EDTA, 1X, 25300] was dropped in (5% - 10%) serum grown cells, 2 mL of 3:1 diluted 0.05% trypsin-EDTA in (2.5% and 1%) serum grown cells and 2 mL of 1:1 diluted 0.05% trypsin-EDTA in 0% of serum grown cells, allowed to cover the bottom of the flask gently to ensure trypsin contact with all cells on the surface. The cells were incubated at 37°C for 4 minutes. The cells proliferated in 0% serum were centrifuged and re-suspended in fresh medium to dislodged the cells were gently tapped to the hands to facilitate the trypsin penetration and enhance detachment.

Cells counting

Cells were counted in a hemocytometer (Germany) after staining with 0.4% of trypan blue (Sigma, T8154, Lot 34K2375) for identification of viable and dead cells. The cells counting was carried out by adding 50 µL cell sample, 50 µL trypan blue and 400 µL PBS together in Eppendorf tube and mixed gently. Then 10 µL of sample was loaded into hemocytometer slide. Then cells counting were done by using inverted phase contrast microscope (Fisher Scientific).

Cells adaptation

cells adaptation was started with 1.50×10^6 cells and continued sequentially in all serum concentration levels. Cells were sub-cultured every 72 and 96 hours for cells proliferated in 2.5% - 10% FBS supplemented medium. The cells grown in 0% and 1% FBS supplemented medium were sub cultured every 120 and 144 hours incubation. Throughout the proliferation; the cells were sub cultured for seven passages in each serum concentration to check the consistency of growth and evaluate the cells growth and viability [13].

Cells infection and virus titration

Cells infection was carried out with Virus multiplicity of infection 0.01and 0.001 for PV and ERA respectively. The desired volume of respective viruses were inoculated in 50 mL test tube which contain fresh harvested Vero cells suspension, incubated at 37°C with 5% CO_2 for 30 minutes and agitated properly. Then transferred to T- flasks contained respective serum supplemented

culture medium and incubated for 72 hours at 37°C with 5% CO_2 for cells proliferated in 2.5% - 10% serum contained medium; whereas those cells grown in 0% and 1% serum contained medium were incubated for 96 hours at 37°C with 5% CO_2 for PV virus production. For ERA rabies virus production the cells were proliferated in 2.5% - 10% serum contained medium incubated for 96 hours at 37°C with 5% CO_2. Those cells grown in 0% and 1% serum concentration were incubated for 120 hours at 37°C with 5% CO_2 for ERA rabies virus production. Freeze thawing was proceeded to disrupt the cells and to remove the viruses into the supernatant; 1 mL sample was taken from each serum concentration virus cultures for virus titration test. To determine virus titer, the cells was adjusted at 5×10^5 cells and 50 µL suspension of Vero cells was distributed in each 96 wells of microtiter plates were infected with tenfold serially diluted virus samples. All dilutions of both viruses (PV and ERA) were distributed in four replicas on plates and incubated for 48 hours at 37°C with 5% CO_2 and the test was carried out three times at each serum concentration grown cell [14].

Then spent medium was discarded from the plates and cells were fixed with 50 µL per well of 80% cold acetone (Sigma-Aldrich, Lot # STBF7534V) [15], incubated at room temperature for 30 minutes. The plates were washed with 100 µL per well of phosphate buffered saline (PBS) twice and then 100 µL per well of Fluorescein Isothiocyanate (FITC) anti- rabies monoclonal globulin (Fujibio Diagnostics. Inc., Malvern, PA 19355) was added and incubated for 1 hour at 37°C with 5% CO_2 [16]. The microtiter plates were washed with PBS twice and dried at room temperature. The plates were observed under fluorescence microscope (Carl Zeiss micro Imaging Gmbh - Germany) and virus titer was expressed as tissue culture infectivity doses fifty ($TCID_{50}$) as calculated by Spearman- Kärber method.

Data analysis methods

Data were analyzed by using SPSS statistical software version 20 and expressed in tables and figures. One-way analysis of variance was used to test the titers of both viruses and viable cells density proliferated in each serum concentration. To determine statistical difference, $P < 0.05$ was regarded as statistically significant.

Results

Adapted Vero cell lines through serum reduction

Vero cells were proliferated through gradual serum reduction and the relative preferable incubation times at which the cells reach confluent stage as shown in **Figure 1**; to yield the maximum viable cells in each serum supplemented culture medium. As incubation time extended the cells were grow to its peak viable cells density and begin to detach off from the growth surface that decrease their viability. The morphology of Vero cells grown in all serum supplemented culture medium was similar throughout the passages. The culture aggregates formation at confluence stage was not observed **(Figure 2)** in each stages of serum supplement and the viable cells counted were higher than the initial inoculants in all passages that make the sequential serum reduction to proliferate the Vero cells important technique. The result indicated that viable cells yield through adaptation in all serum supplements were no significant difference in maximum viable cells density (p-vale=0.43). Therefore; reducing the serum in culture medium didn't affect the adapted viable cells density in each serum level that is important technique to grow Vero cells used as substrate for different rabies virus strains propagation. Through sequential serum reduction in culture medium the incubation time required the cells to reach confluent stage was extended in each stage. The serum free (0% serum) supplemented medium resulted maximum viable cells density on sixth passage at 144 hours incubation. This indicated that the Vero cells proliferate in serum free medium need longer time and serum supplemented grown cells to reach their confluence stages.

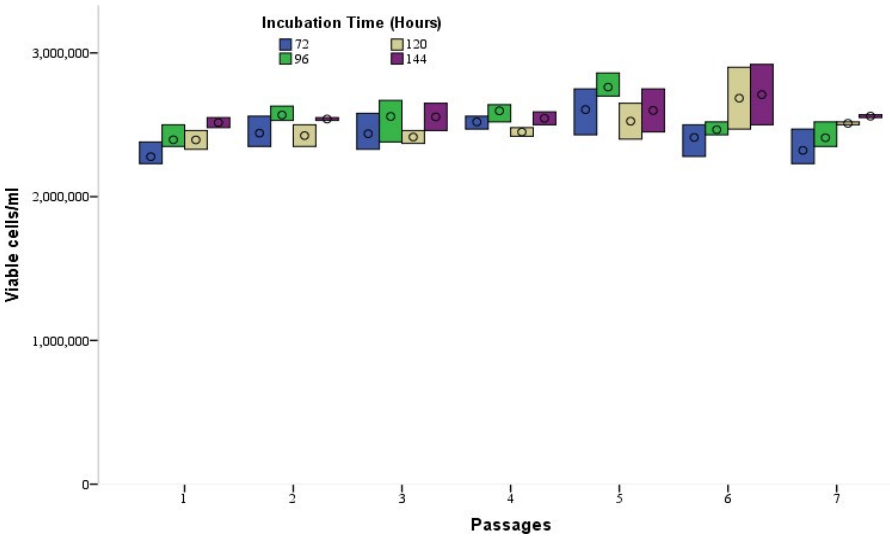

Figure 1 Viable Vero cells counted in different serum concentration supplemented medium.

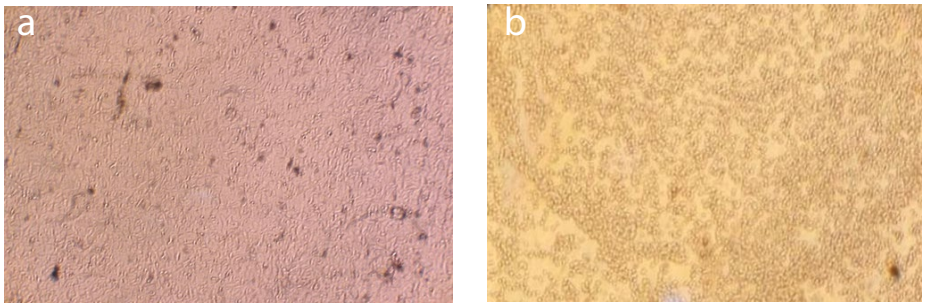

Figure 2 Microscopy image of Vero cell lines. (a)-Confluent Vero cells lines (b)-Trysinized Vero cell lines.

Table 1 Titers of PV virus propagated in Vero cells grown in different serum concentration supplemented medium.

Virus	Medium	Serum conc. (%)	Infected cells mL^{-} x10^{6}	Incubation hours	Maximum Virus titer ($TCID_{50}$)mL^{-}
PV	MEME	10	2.42	72	$10^{5.11}$
		7.5	2.6	72	$10^{4.61}$
		5	2.33	72	$10^{4.46}$
		2.5	2.67	72	$10^{4.61}$
		1	2.56	96	$10^{5.11}$
		0	2.35	96	$10^{5.36}$

Description: MEME- Minimal essential medium eagle, mL-milliliter, PV- Pasteur virus, MOI- Multiplicity of infection, TCID-Tissue culture infectivity dose.

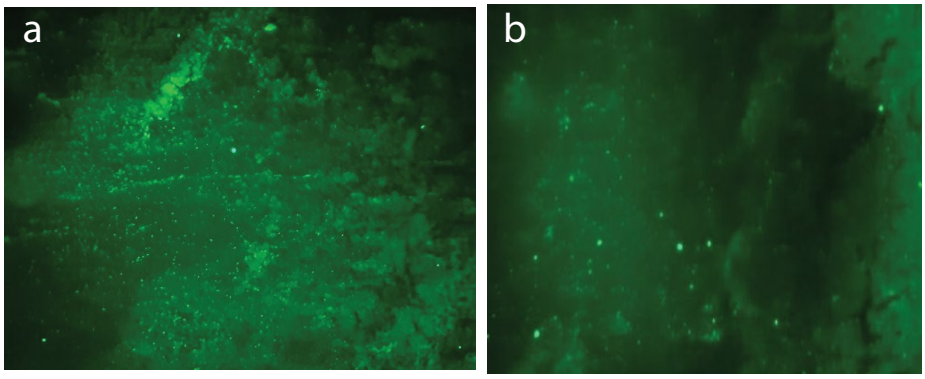

Figure 3 Image of florescent microscope (a) - Positive (b) – Negative.

Rabies Virus titer, Titer of PV virus: Titration of PV rabies strains were carried out with various serum concentrations supplemented medium propagated Vero cell line as shown in **Table 1**. The maximum virus titer obtained in this study was $10^{5.36}$ $TCID_{50}$ mL^{-} from the cells proliferated in 0% serum supplemented medium by careful focusing each wells as indicated **(Figure 3)** and recorded (positive and negative) wells to avoid considering none specific particles in the fields. Cells number and passage from which the cells used for titration don't affect virus titer because it's adjusted based on multiplicity of infection after 96 hours incubation. The result indicated that the cells proliferated in serum free medium were sensitive and the PV rabies prefer to propagate at high titer and the cells used for infection in this study after they were grown in respective serum concentration at any passage. However; virus titer obtained in this study was no significant difference (p-value=0.34) with the virus propagated in both serums supplemented and serum free medium grown cells.

Titer of ERA virus: the multiplicity of infection used was 0.001 and the maximum ERA virus titer obtained was $10^{5.61}TCID_{50}$ mL^{-} after 120 hours incubation that was better than serum supplemented culture medium. This result indicated that serum reduction in culture medium don't affect the ERA rabies virus titer. Therefore; Vero cells proliferated in medium without serum is important to produce ERA rabies virus titer that was greater than those proliferated in serum supplemented grown cells. As shown in **Table 2**; the incubation time extended in reduced serum concentration grown cells propagated; virus titer was also greater as compared to serum supplemented proliferated cells. The result showed that the virus titer obtained in reduced serum supplemented and serum free medium propagated ERA rabies was no significance difference ($p>0.05$) that indicates the virus production in serum free condition avoid the side effects and costs for vaccine productions.

Table 2 Titers of ERA Rabies virus propagated in Vero cells grown in different serum concentration supplemented medium.

Virus	Medium	Serum conc. (%)	Infected cells mL^{-} x10^{6}	Incubation hours	Maximum Virus titer ($TCID_{50}$)mL^{-}
ERA	MEME	10	2.55	96	$10^{5.11}$
		7.5	2.5	96	$10^{4.36}$
		5	2.51	96	$10^{4.61}$
		2.5	2.42	96	$10^{5.11}$
		1	2.56	120	$10^{4.86}$
		0	2.55	120	$10^{5.61}$

Description: MEME- Minimal essential medium eagle, ERA-Evenly Roktincki Abelseth, mL-Milliliter, MOI - Multiplicity of infection, TCID -Tissue culture infectivity dose, %-Percent.

Discussion

Growth medium those contained animal derived product components like serum in culture medium hinders the biological products used them as substrate for cell line proliferation. Serum is source of various contaminant agents; it also holds major cost of all culture medium components and collection of serum require loss of life of many fetal bovine that contradict the animal welfare [17]. To reduce the risk associated with the use of biological reagents of animal origin, regulatory authorities strongly encourage the development of industrial-scale processes that are free of animal and human derived components. Currently most vaccine industry use serum to proliferate Vero cell lines for rabies virus propagation to produce vaccine for animal and human use [18,19]. Reduction of serum in the culture medium doesn't affect the cells density that showed normal growth in number; cells morphology and enhance rabies virus titers of propagation. Therefore; the result obtained in current study is preferable in terms of maximum viable cells harvested used to proliferate the rabies virus. The incubation time and higher viable cells may be due to the greater initial inoculants and availability of surface for cell growth. The study done by Butler et al. [9]; grown Vero cells in serum free medium in micro- carrier based and reported maximum cells density 2.7×10^{6} cells mL^{-} that is in line with current study. The growth condition of current study was T-flask that was not advanced for Vero cells proliferation, but the viable cells counted were slightly greater than those grown in micro-carriers bioreactor. This indicated that growing of the cells adapted in this serum concentration in advanced growth mode may be resulted in higher viable cells. Virus titer of PV rabies from the cells proliferated in serum free medium obtained in this study was the greater as compared to serum supplemented grown cells propagated cells; that is similar with the investigation carried out by Frazattigallina et al. [20]. Others investigators [21]; grow Vero cells in VP-SFM 1% serum supplemented medium and obtained higher PV virus titer $10^{4.23}$ FFD per 0.05 mL^{-} after 6 days incubation time that was the longer incubation time considered to obtain high virus titer as compared to current study. The result obtained in current study showed that the higher virus titer record in serum free adapted cells which met the goal of harvesting sufficient virus for various biological products. Therefore; the result of this study used to propagate the PV rabies virus that is essential for human vaccine production with limited cost and great quality. The study carried out by Perrin et al. [22], harvest PV after the 120 hours incubation time in serum free medium was $10^{8}TCID_{50}$ mL^{-} in bioreactors for the production of experimental rabies vaccines. In serum free medium proliferated cells virus titer of ERA rabies virus was greater; this indicated that ERA rabies strains virus infectivity is enhanced as the cells are grown in serum free medium. Previous study obtain maximum titer of ERA strain rabies viruses was $10^{7.25}$ $TCID_{50}$ mL^{-} after 96 hours incubation in serum supplemented medium grown cells propagated virus [23]. The variation between our results and data reported may be difference between virus titers, which is probably due to the cells growth and virus infection technique; they used roller bottle to cells proliferation and virus infection. The results from the present study seeks further authenticate that adaptation of Vero cells through serum reduction and virus propagation is an important method for anti-rabies vaccines production.

Conclusion

Proliferation of Vero cell lines through reduction of serum in growth medium showed normal cells morphology and viability in all stages. The virus titers in each serum concentration medium grown Vero cells were little variation as compared to serum supplemented cells. Both rabies virus strains (ERA and PV) showed the maximum virus titer in 0% serum supplemented medium grown Vero cells, this indicated that the virus infectivity of those virus strains were sensitive to Vero cells proliferated in serum free medium to attain maximum titer. Therefore, Gradual reduction of serum concentration in growth medium for Vero cell lines proliferation didn't affect the cells density and virus titer yielded. The incubation times considered in this study at each stages of serum concentration to grow the cells, to maximum cells density were 96, 120 and 144 hours for serum concentrations of 10% to 2.5%, 1% and 0% respectively. Therefore, the time required for cells to reach the confluence stage was extended as serum content in the medium decreased to harvest Vero cells and rabies virus production. Generally it is concluded that through gradual adaptation the viable counted Vero cells in serum free medium and rabies virus strains propagated were sufficient for vaccine production for human as well as animals use.

References

1 Yasumura Y, Kawakita Y (1963) A line of cells derived from African green monkey kidney. Nippon Rinsho 12: 1209-1210

2 Souza MCO, Freire MS, Castilho LR (2005) Influence of culture conditions on Vero cell propagation on non-porous microcarriers. Braz Arch Biol Technol 48: 71-77.

3 Yokomizo AY, Antoniazzi MM, Galdino PL, Azambuja NJ, Jorge SA, et al. (2004) Rabies Virus Production in high vero cell density cultures on macroporous micro- carriers. Biotechnol Bioeng 5: 506-515.

4 Nahapetian AT, Thomas JN, Thilly GW (1986) Optimization of environment for high density vero cell culture: effect of dissolved oxygen and nutrient supply on cell growth and changes in metabolites. J Cell Sci 6: 65-103.

5 Barrett PN, Mundt W, Kistner O, Howard K (2009) Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines. Expert Rev Vaccines 5: 607- 618.

6 Brunner D, Frank J, Appl H, Schöffl H, Pfaller W (2010) Serum-free Cell Culture: The Serum free Media Interactive. ALTEX 27: 10.

7 Montomoli E, Khadang B, Piccirella S, Trombetta C, Mennitto E (2012) Cell culture derived influenza vaccines from vero cells: A new horizon for vaccine production. Expert Rev Vaccine 5: 587.

8 WHO (2013) Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. TECH REP SER 978.

9 Butler M, Burgener A, Patrick M, Berry M, Coombs K (2000) Application of a serum free medium for the growth of vero cells and the production of reovirus. Biotechnol Prog 5: 854– 858.

10 Merten O (2002) Development of serum-free media for cell growth and production of viruses or viral vaccines safety issues of animal products used in serum free media. Dev Biol 111: 233–257.

11 Hassandezadeh M, Zavareh A, Shokrgozar M, Ramezani A, Fayaz A (2011) high vero cells density and Rabies virus prolifration on Fibracel Disks versus Cytodex-1 in spinner flask. Pak J Biol Sci 14: 441-448.

12 Ammerman NC, Sexton BM, Azad AF (2008) Growth and maintenance of vero cell lines. Curr Protoc Microbiol 4: 4-12

13 Rourou S, Ark A, Vendel T, Kallel H (2007) A Microcarrier cell culture process for propagation of rabies virus in vero cells grown in a stirred bioreactor under fully animal component free conditions. Vaccine 25: 3879-3889.

14 Paldurai A, Singh RP, Gupta PK, Sharma B, Pandey KD (2014) Growth Kinetics of Rabies Virus in BHK-21 Cells Using Fluorescent Activated Cell Sorter (FACS) Analysis and a Monoclonal Antibody Based Cell-ELISA. J Immunol Vaccine Technol 1: 103.

15 Trablesi K, Samia R, Houssem L, Sammy M, Hella K (2005) Comparison of various culture modes for production of rabies virus by vero cells grown on micro carriers in 2-l bioreactor. Enzyme Microb Tech 36: 514-519.

16 Fayaz A, Zavarei A, Howaizi N, Eslami N (1997) Production of Rabies Vaccine Using BHK-21 with Roller Bottle Cell Culture Technique. Iran Biomed J 1: 35-38.

17 More S, Bicout D, Botne A, Butterfield A (2017) Animal welfare aspects in respect of the slaughter or killing of pregnant livestock animals (cattle, pigs, sheep, goats, horses). EFSA J 5: 47-82.

18 Merten OW, Kallel H, Manuguerra J (1999) The new medium MDSS2N, free of any animal protein supports cell growth and production of various viruschenes. Cyto technology 30: 191-201

19 Chen A, Swan P, Dietzsch L, Kong Y (2011) Serum-free microcarrier based production of replication deficient Influenza vaccine candidate virus lacking NS1 using Vero cells. BMC Biotechnol 11: 81.

20 Frazattigallina MN, Mour MR, Paoli LR, Higashi AS (2004) Vero-cell rabies vaccine produced using serum free medium. Vaccine 23: 511-517.

21 Frazzati-Gallina NM, Rosana LP, Regina MM, Soraia AC, Carlos SA (2001) higher production of rabies virus in serum-free medium cell cultures on microcarriers. J Biotech 92: 67-72.

22 Perrin P, Madhusudana S, GontierJallet C, Petres S, Tordo N (1995) An experimental rabies vaccine produced with a new BHK-21 suspension cell culture process: Use of serum-free medium and perfusion-reactor system. Vaccine 13: 1244-1250.

23 Birhanu H, Abebe M, Newayesilassie B, Sisay K, Kelbessa U (2013) Production of cell culture based anti-rabies vaccine in Ethiopia. Proc Vaccinol 2: 2-7.