

## Evaluation of Multiplicity of Infection (MOI) and Harvesting Time on the Production of CVS-11 Strain of Rabies Virus in BSR Cell Line

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### ABSTRACT

**Introduction:** Rabies is a zoonotic fatal viral disease caused by the rabies virus of the genus *Lyssavirus*, and the family *Rhabdoviridae*. Challenge virus standard (CVS-11) strain of rabies virus is a key element in rabies reference laboratories, as many gold-standard tests depend on a suitable titer of this strain for interpretation of results. The present study investigated the optimal CVS-11 production in BSR cells (a clone of BHK-21). **Methods:** We analyzed the kinetic growth of BSR cells in a T-flask and inoculated BSR cells with different MOI of CVS-11 strain of rabies virus, and harvested the produced virus at different time points. **Results:** Our data showed that BSR cells had a doubling time of around 24-30 h, and at least 95% of cells kept their viability three days after culture. The virus reached the highest titer when the cells were infected at an MOI of 0.1 in DMEM medium, equal to  $1.5 \times 10^7$  fluorescent focus units (FFU)/ml. Time-course analysis of CVS-11 titer showed that the highest titer was achieved around 72 h post-infection. All tests were performed in triplicate. **Conclusion:** Since producing the virus in mammalian cell culture is an expensive and complicated method, optimizing the virus production process may be an excellent strategy to lower the cost, save the laboratory resources and maximize productivity.

### INTRODUCTION

Rabies is a zoonotic fatal viral disease caused by the rabies virus of the genus *Lyssavirus*, and the family *Rhabdoviridae* [1, 2]. The virus is enveloped and bullet-shaped, measuring about 75×180 nm, with a single-stranded negative-sense RNA genome encoding five structural proteins [3, 4]. Annually, at least 60,000 people are estimated to die from rabies [5]. The disease remains endemic [6] and a health problem in developing countries where dogs, especially stray dogs, serve as the main reservoir [7-9]. The mass vaccination of dogs is the appropriate approach and new global strategy plan recommended by the World Health Organization (WHO) to control human rabies by 2030 [10-12]. However, unfortunately, there are no organized and comprehensive rabies control programs at the national level in countries where rabies is endemic [12, 13].

Given the importance of controlling rabies, it is essential to have national reference centers to diagnose and monitor the disease in rabies endemic countries. With timely diagnosis and exploring the hot spot of disease, and reporting to the Center for Disease Control and Prevention, Ministry of Health and Medical Education (Iran), these centers have an outstanding

indirect role in controlling the disease by helping the health authorities in the planning of infection-control strategies. [14]. Other crucial tasks of these centers are performing specialized tests like evaluating rabies vaccine potency and challenge experiments. Performing rabies vaccine potency tests employ infectious viruses, which necessitates special containment facilities and biosafety concerns assessment. So these activities usually are not possible in other laboratories.

The challenge virus standard (CVS-11) strain of rabies is considered a laboratory standard in rabies reference centers. CVS-11 is a well-characterized and widely used strain of rabies virus [15] initially obtained by adaptation in mice from the original Pasteur rabies virus isolated in 1882 by Louis Pasteur [16]. CVS-11 is approved for rabies vaccine manufacture and used as a challenge virus in the rapid fluorescent focus inhibition test (RFFIT) to measure rabies neutralizing antibodies in sera of vaccinated persons [15].

The CVS-11 virus is used as a challenge virus in the RFFIT test to measure rabies neutralizing antibodies in vaccinated individuals' sera [17-19]. This test is the only gold standard serological assay recommended by WHO

for this purpose [20]. The titer of CVS-11 used as a challenge virus in this test is critical in obtaining satisfactory results. Therefore, developing an optimized production protocol is crucial for producing the CVS-11 virus with high and sustainable titer. On the other hand, each produced batch of virus needs downstream experiments like titration and calibration for use in the RFFIT, making it more expensive to produce [21]. For these reasons, optimizing the virus production process in lab-scale in quantities required for the performing of RFFIT assays and other virological studies is essential and necessary.

Furthermore, with increasing numbers of commercial growth media and FBS in the markets that differ in their quality that certainly affect the result of work and yield of the virus, it has become imperative to optimize virus production with given reagents for maximum virus yield.

In this study, to produce optimal titer of CVS-11 virus, we investigated the kinetic growth of BSR cells (a clone from BHK-21) and the effects of the multiplicity of infection (MOI) and harvesting time as affecting factors in the production of virus. Such efforts are essential for reference laboratories to lower virus production cost and maximize productivity.

## MATERIALS AND METHODS

The Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% (V/V) heat-inactivated fetal bovine serum (FBS; Gibco, USA), 100 IU/ml penicillin, and 100ug/ml Streptomycin (Sigma) was used as the serum-containing medium. The adherent BSR cell (a clone from BHK-21 cells) was used for the experiments. The cells were incubated at 37°C with 5% humidity and used as monolayers for virus inoculation. The cells were passaged twice per week in T-flasks.

**Virus strain.** The CVS-11 strain of rabies virus (passage 23), adapted to grow in cell culture, was used throughout the study and kept at -80 °C.

**Cell growth assay.** The cell growth kinetic of BSR cells was determined four days in four separate T-25 flasks with seeding of  $5 \times 10^5$  cells in each flask according to the standard protocol for cell culture recommended by WHO (World Health Organization. Laboratory techniques in rabies, volume 1. 5th ed. 2018; 2: 289). All experiments were performed at 37 °C, 5% CO<sub>2</sub>. Briefly, each flask content was rinsed with PBS and trypsinized, and the total number of viable cells was counted. Cell growth was followed for 96 h; samples were taken daily to determine cell concentration [22]. Viable BSR cells were determined by the trypan blue exclusion method. Briefly, 10 µL of 0.4% trypan blue stain was added to 10 µL of the BSR cells suspension, and then cells were counted using a Neubauer hemocytometer. [23].

**Virus amplification.** Monolayers of BSR cell lines grown in a T-75 flask were washed twice with PBS (pH 7.4) and trypsinized [24]. Cells were infected with CVS-11 in sterile tubes at MOIs of 0.01, 0.1, and 1. After incubation for 1 h at 37 °C, the tube contents were centrifuged at 500g for 10 min, and the inoculums were removed. The cells were mixed with fresh medium in T-75 flasks and incubated at a 35 °C incubator containing 5% CO<sub>2</sub> for various periods, depending on the experimental design. The culture supernatants were assayed for virus titration at 48, 72, and 96 h. All assays were performed in triplicate.

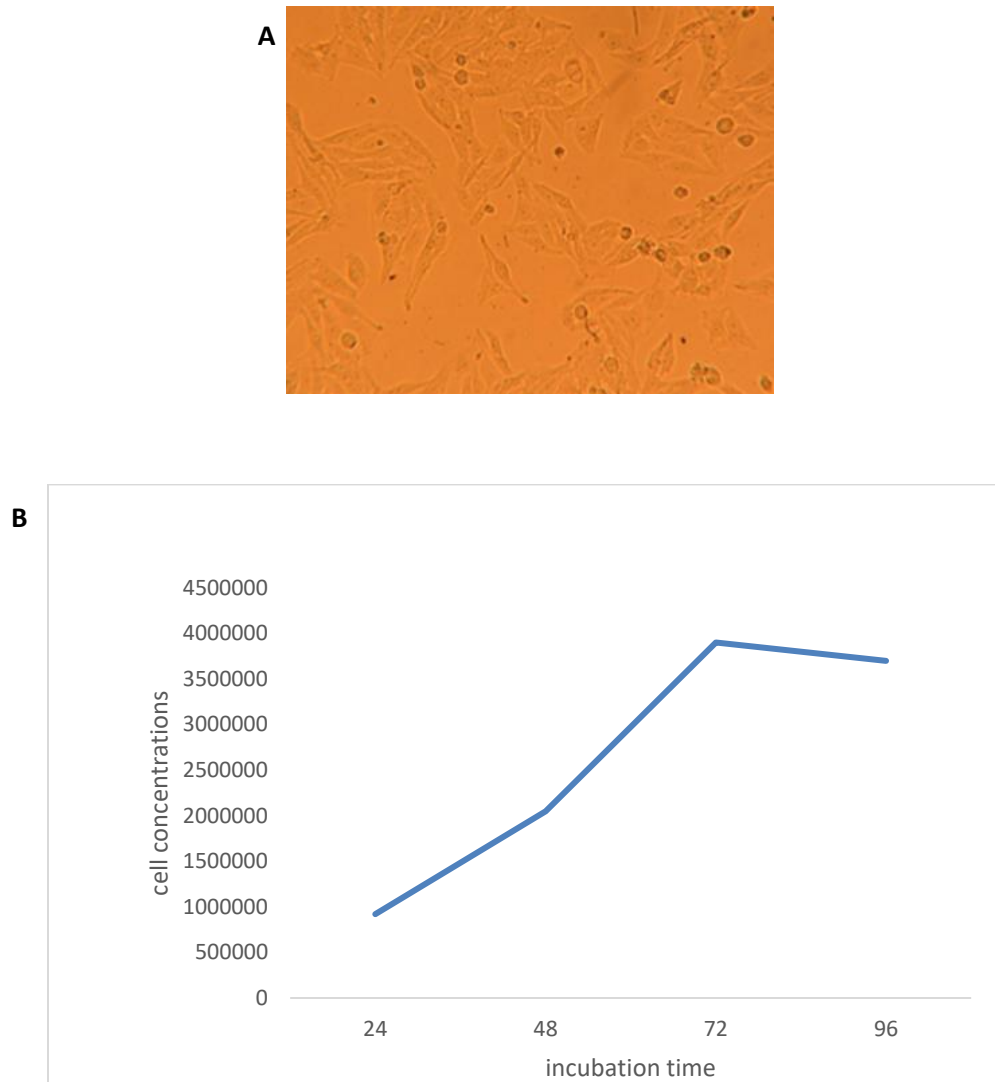
**Virus harvesting.** For harvesting the virus, the supernatant of T-75 flasks was collected under a biosafety hood. The debris was removed from the supernatant by centrifugation at 1000 g for 10 min at 4 °C. Following aliquoting, the harvested viruses were kept at -80 °C until used.

**Virus quantification by FFU method.** The CVS-11 virus was titrated using a modified focus forming unit (FFU) test described by others [25]. Briefly, after harvesting the rabies CVS-11 strain, 10-fold dilutions of the virus samples were prepared with culture medium in sterile tubes at 4 °C. Virus dilution was performed up to  $1 \times 10^{-7}$  dilution (100 µl of the virus was added to 900 µl of culture medium for sequential dilution). In each well of 8-well chamber slides containing 100 µl of the diluted virus,  $1 \times 10^5$  BSR cells were added. The chamber slides were incubated at 37 °C, 5% humidity, for 20-24 h.

**Immunofluorescence staining (IF).** Slide chambers were washed briefly with phosphate-buffered saline (PBS), fixed with 80% cold acetone at 4 °C for 30 min, and then stained with the rabies anti-nucleocapsid (FITC)-conjugated antibody (Bio-Rad), for 1 h at 37 °C. The slides were washed two times with PBS, 5 min each wash, and were examined by fluorescence microscopy. The highest dilution with fluorescent foci was defined as endpoints, and the numbers of fluorescent foci presented in the highest dilution were recorded, and virus titers were expressed in FFU/ml.

## RESULTS

**Growth kinetics of the BSR cells.** Adherent BSR cell (Fig. 1A) was used to investigate the cell growth kinetic for determining the best time for cell infection by CVS-11 strain. Growth kinetic assays of BSR cells were performed for 96 h, using the BSR cells in T-25 flasks. The growth curve of the BSR cells indicated a doubling time of around 24-30 h (Fig. 1B). The complete monolayer was observed after 72 h. There was a decline in viable cells after 72 h, so the best time for infecting cells was between 24 to 72 h when the cells were at the logarithmic phase. At least 95% of the total population of cells had their viability until the third day of the cell passage.



**Fig. 1.** BSR cell grown on T-flask using DMEM and incubated at 37 °C. A) Morphology of the BSR cells, B) growth curve of BSR cell line.

**Effect of MOI on CVS-11 production in adherent cell culture.** The influence of the number of CVS-11 used for cell infection (Multiplicity of infection, MOI) on virus production was investigated by infecting BSR cells at different MOIs, 0.01, 0.1, and 1. Our results indicated the maximum titers achieved in BSR cells when MOI increased from 0.01 to 0.1 (Fig. 2). At the MOI= 0.1, the maximal virus titer was obtained, and it reached  $1.5 \times 10^7$  FFU/ml.

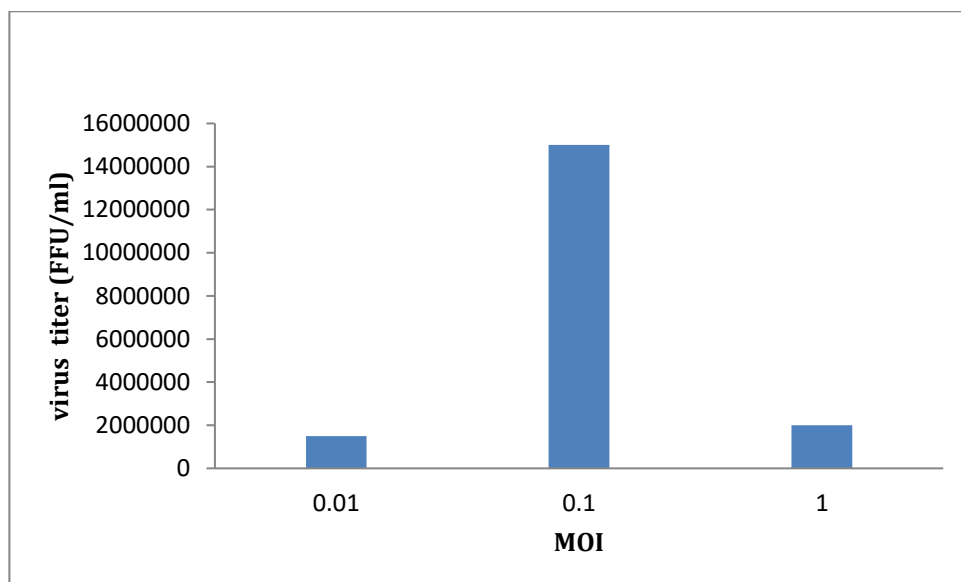
**Time-course analysis of CVS-11 production in BSR cell.** For determining the best time for virus harvesting, we harvested the produced virus at different times. Time-course analysis of CVS-11 titers showed that the highest titer was achieved at 72 h post-infection.

**Immunofluorescence staining of BSR cells.** Staining of BSR cells with rabies anti- nucleocapsid FITC-

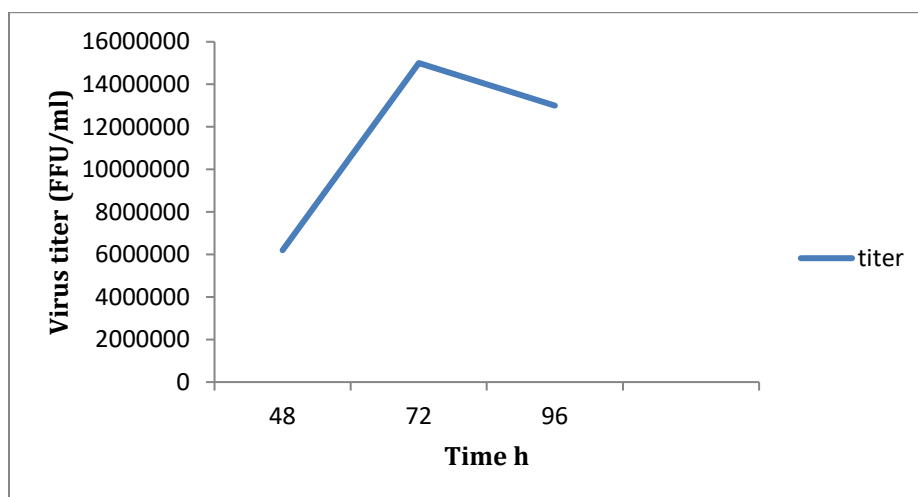
conjugated, infected cells with CVS-11 exhibited brilliant, green-apple fluorescence particles under an immunofluorescence microscope representing rabies virus antigens (Fig. 4).

## DISCUSSION

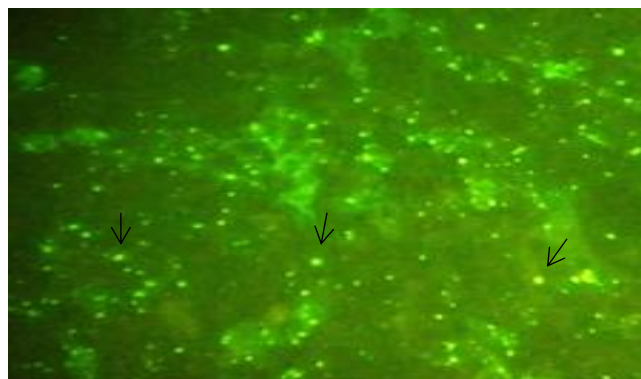
Nowadays, cell culture systems play a significant role in the research and production of viruses. Many approved viral vaccines were produced by amplifying virus in cell culture systems, and many viral antigens in diagnostic kits were developed by producing viral antigens as recombinant in cell culture systems [26, 27]. On the other hand, many aspects of viral life cycles have been understood by *in-vitro* research in cell culture systems [28, 29].



**Fig. 2.** Growth kinetics of cell-adapted virus using different MOI. Growth kinetics of BSR cell adapted CVS11 rabies virus using 1, 0.1, and 0.01 MOI. Note that 0.1 MOI of the virus is the most suitable for optimum virus titer (about  $1.5 \times 10^7$ ), at around 72 h post-infection.



**Fig. 3.** Time-course analysis of CVS-11 using MOI of 0.1. The highest titer was achieved at 72 h post-infection and then decreased gradually.



**Fig. 4.** BSR cells infected with CVS strain of rabies virus and stained by a fluorescent antibody test. Note bright apple-green fluorescent foci in 80–90% of cells.

Baby hamster kidney (BHK-21) cell line is commonly used in research and the industries with biopharmaceutical products [30]. This cell line was first established by Stoker and Macpherson in 1961 [29]. The advantages of BHK cell lines like their robust cell growth, easy maintenance, high cell densities, susceptibility for various viruses, and their ability to grow in suspension culture systems enable this cell line to be a suitable candidate for laboratory and large scale applications like manufacturing rabies vaccines [15, 31].

Most studies on the production and optimization of different rabies virus strains have focused on suspension culture systems at large scales for vaccine production [32-34]. On the other hand, in available protocols for CVS-11 production on laboratory scales, there is no clear indication of which MOI of CVS-11 is suitable for BHK cell and the exact time of virus harvesting [24]. Furthermore, commercial reagents quality like FBS can affect the results and virus yield [35]. So, the establishment and normalization of virus production with given reagents in each laboratory are mandatory. Moreover, in reference laboratories, practical procedures for planning and selecting internal quality control (IQC) are essential to maintain assays reproducibility [24, 36]. In this respect, we designed this study to find an optimal condition of CVS-11 virus production in adherent cell culture systems for lab-scale.

In this study, we observed that infection of BSR with MOI of 0.1 resulted in a suitable titer of CVS-11 virus, but infections with MOI 0.01 or 1 could not yield a suitable titer. In the recent WHO recommendation for CVS-11 production [37], there is no detail about the optimal MOI and virus harvesting time in the BHK-21 cell line, and it has been indicated that they should be found experimentally. In the OIE recommendation for CVS-11 production, the MOI ranges from 0.1 to 0.5, so each laboratory requires achieving an optimal MOI. Our finding was in agreement with other previously published data [15, 38], in which MOI of 0.1 resulted in a high titer of virus. Patel *et al.* (2015) reported that maximum virus titer obtained by infection of cells with 0.1 MOI, but inoculation with MOI of 0.01 or 1, resulted in low titer of the virus [15]. Although they used mice passaged of CVS-11 strain of rabies virus (passage-2) after adaptation (10-passage) in BHK-21 cells, while we used cell-cultured adapted CVS-11 strain of rabies virus (passage-23), but the optimal MOI was similar. This similarity suggests that CVS-11 can quickly adapt to the BHK-21 cell line. A similar observation was reported for the rabies virus PV strain using the BHK-21 cell line [38]. It is evident that using a high MOI will result in simultaneous infection of all the cells, affecting the cell growth rate, and only one virus replication cycle of the virus will occur. As cytopathic cell effects occur more rapidly, a lower virus titer is produced. Furthermore, using high MOI is not suitable from an economic standpoint and results in decreased viral laboratory

stocks and increased laboratory costs. On the other hand, using very low MOI will cause minimum infection of cells, slow down virus kinetic growth, and delay virus harvesting in proper time [39]. We also found that the optimum time of harvest at 35 °C was ~72 h post-infection. Within three days, virus titers increased significantly to  $1.5 \times 10^7$  FFU/ml. As before mentioned, in the WHO protocol for CVS-11 production [37], no apparent optimum time for virus harvesting is available, and it should be determined experimentally. In our study, the optimum harvesting time was not similar to others reporting an optimum harvesting time of 48 h post-infection [15, 38]. The variation might be due to the difference in temperature for virus propagation, as they practiced virus propagation at 37 °C. Also, depending on other parameters like the virus strain, adaptation level, and MOI, optimum harvest time for rabies virus ranges from 2 to 7 days [40-42].

The present work optimized two critical parameters, i. e., MOI and harvesting time in the BSR cell line, to produce a suitable CVS-11 virus titer for using in the Rabies Reference Laboratory of Pasteur Institute of Iran as a challenge virus. Finally, optimizing processes like virus production on a laboratory scale can save laboratories resources and lower the cost by maximizing productivity.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

## REFERENCES

1. Singh R, Singh KP, Cherian S, Saminathan M, Kapoor S, Manjunatha Reddy G, et al. Rabies—epidemiology, pathogenesis, public health concerns and advances in diagnosis and control: a comprehensive review. *Vet Q.* 2017; 37 (1): 212-51.
2. Williamson JG. Rabies : symptoms, treatment and prevention. New york: Nova Science Publishers, Inc.; 2010.
3. Leung AK, Davies HD, Hon K-LE. Rabies: epidemiology, pathogenesis, and prophylaxis. *Adv Ther.* 2007; 24 (6): 1340-7.
4. Riedel C, Vasishtan D, Pražák V, Ghanem A, Conzelmann K-K, Rümenapf T. Cryo EM structure of the rabies virus ribonucleoprotein complex. *Sci Rep.* 2019; 9 (1): 1-6.
5. Petersen BW, Rupprecht CE. Human rabies epidemiology and diagnosis. In: Thacker S, ed. Non-

flavivirus encephalitis. Rijeka, Croatia: InTech. 2011; 247–78.

6. Meeting WECBS, Organization WH. WHO Expert Committee on Biological Standardization: Sixtieth Report: World Health Organization; 2013.

7. Nadin-Davis S, Simani S, Armstrong J, Fayaz A, Wandeler A. Molecular and antigenic characterization of rabies viruses from Iran identifies variants with distinct epidemiological origins. *Epidemiol Infect.* 2003; 131 (1): 777-90.

8. Rhodes CJ, Atkinson RPD, Anderson RM, Macdonald DW. Rabies in Zimbabwe: reservoir dogs and the implications for disease control. *Philos Trans R Soc Lond B Biol Sci.* 1998; 353 (1371): 999-1010.

9. Yahiaoui F, Kardjadj M, Laidoudi Y, Medkour H, Ben-Mahdi MH. The epidemiology of dog rabies in Algeria: Retrospective national study of dog rabies cases, determination of vaccination coverage and immune response evaluation of three commercial used vaccines. *Prev Vet Med.* 2018; 158: 65-70.

10. Gibson AD, Mazeri S, Lohr F, Mayer D, Burdon Bailey JL, Wallace RM, et al. One million dog vaccinations recorded on mHealth innovation used to direct teams in numerous rabies control campaigns. *PLoS One.* 2018; 13 (7): e0200942.

11. Minghui R, Stone M, Semedo MH, Nel L. New global strategic plan to eliminate dog-mediated rabies by 2030. *Lancet Glob Health.* 2018; 6 (8): e828-e9.

12. WHO. WHO expert consultation on rabies: third report: World Health Organization; 2018.

13. Nel LH. Discrepancies in data reporting for rabies, Africa. *Emerg Infect Diseases.* 2013; 19 (4): 529-33.

14. Velasco-Villa A, Escobar LE, Sanchez A, Shi M, Streicker DG, Gallardo-Romero NF, et al. Successful strategies implemented towards the elimination of canine rabies in the Western Hemisphere. *Antiviral Res.* 2017; 143: 1-12.

15. Patel AC, Upmanyu V, Ramasamy S, Gupta PK, Singh R, Singh RP. Molecular and immunogenic characterization of BHK-21 cell line adapted CVS-11 strain of rabies virus and future prospect in vaccination strategy. *Virusdisease.* 2015; 26 (4): 288-96.

16. Wiktor T, Koprowski H. Antigenic variants of rabies virus. *J Exp Med.* 1980; 152 (1): 99-112.

17. Yager ML, Moore SM. The rapid fluorescent focus inhibition test. *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention, Volume 2:* Elsevier; 2015; 199-215.

18. Krämer B, Schildger H, Behrendorf-Nicol H, Hanschmann K, Duchow K. The rapid fluorescent focus inhibition test is a suitable method for batch potency

testing of inactivated rabies vaccines. *Biologicals.* 2009; 37 (2): 119-26.

19. Qin S, Volokhov D, Rodionova E, Wirblich C, Schnell MJ, Chizhikov V, et al. A new recombinant rabies virus expressing a green fluorescent protein: A novel and fast approach to quantify virus neutralizing antibodies. *Biologicals.* 2019; 59: 56-61.

20. Timiryasova TM, Hodge SA, Zheng L, Singer A, Vincent D, Rahman M, et al. Preparation and qualification of internal rabies reference standards for use in the rabies rapid fluorescent focus inhibition test. *Sci Rep.* 2020; 10 (1): 1-9.

21. Meza DK, Broos A, Becker DJ, Behdenna A, Willett BJ, Viana M, et al. Predicting the presence and titre of rabies virus-neutralizing antibodies from low-volume serum samples in low-containment facilities. *Transbound Emerg Dis.* 2020.

22. Kallel H, Jouini A, Majoul S, Rourou SJJ. Evaluation of various serum and animal protein free media for the production of a veterinary rabies vaccine in BHK-21 cells. *J Biotechnol.* 2002; 95 (3): 195-204.

23. Kang W, Huang H, Cai M, Li Y, Hou W, Yun F, et al. On-site cell concentration and viability detections using smartphone based field-portable cell counter. *Anal Chim Acta.* 2019; 1077: 216-24.

24. World Health Organization. Laboratory techniques in rabies, volume 1. 5th ed. 2018; 2: 289.

25. Smith JS, Yager PA, Baer GM. A rapid reproducible test for determining rabies neutralizing antibody. *Bull World Health Organ.* 1973; 48 (5): 535-41.

26. Bordignon J, Zanetti CR. Rabies Diagnosis: Demonstration of Viral Antigens by Flow Cytometry. *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention:* Elsevier; 2014; p 43-51.

27. Wunner W, Clark H. Regeneration of DI particles of virulent and attenuated rabies virus: genome characterization and lack of correlation with virulence phenotype. *J Gen Virol.* 1980; 51 (Pt 1): 69-81.

28. Leland DS, Ginocchio CC. Role of cell culture for virus detection in the age of technology. *Clin Microbiol Rev.* 2007; 20 (1): 49-78.

29. Hernandez R, Brown DT. Growth and Maintenance of Baby Hamster Kidney (BHK) Cells. *Curr Protoc in Microbiol.* 2010; 17 (1): A.4H.1-A.4H.7.

30. Sartori R, Leme J, Caricati CP, Tonso A, Núñez EGF. Model comparison to describe BHK-21 cell growth and metabolism in stirred tank bioreactors operated in batch mode. *Braz J Chem Eng.* 2018; 35 (2): 441-58.

31. Pay T, Boge A, Menard F, Radlett P. Production of rabies vaccine by an industrial scale BHK 21 suspension cell culture process. *Dev Biol Stand.* 1985; 60: 171-4.

32. Gallo-Ramírez LE, Nikolay A, Genzel Y, Reichl U. Bioreactor concepts for cell culture-based viral vaccine production. *Expert Rev Vaccines*. 2015; 14 (9): 1181-95.
33. Perrin P, Madhusudana S, Gontier-Jallet C, Petres S, Tordo N, Merten O-W. An experimental rabies vaccine produced with a new BHK-21 suspension cell culture process: use of serum-free medium and perfusion-reactor system. *Vaccine*. 1995; 13 (13): 1244-50.
34. Frazzati-Gallina NM, Paoli RL, Mourão-Fuches RM, Jorge SA, Pereira CA. Higher production of rabies virus in serum-free medium cell cultures on microcarriers. *J. Biotechnol*. 2001; 92 (1): 67-72.
35. Alfano R, Pennybaker A, Halfmann P, Huang CYH. Formulation and production of a blood-free and chemically defined virus production media for VERO cells. *Biotechnol Bioeng*. 2020; 117 (11): 3277-85.
36. Westgard JO. Internal quality control: planning and implementation strategies. *Ann Clin Biochem*. 2003; 40 (6): 593-611.
37. World Health Organization. Laboratory techniques in rabies. 1. 2018.
38. Paldurai A, Singh R, Gupta P, Sharma B, Pandey K. 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*. 2014; 1 (1): 103.
39. Chapman W, Ramshaw I, Crick J. Inactivated rabies vaccine produced from the Flury LEP strain of virus grown in BHK-21 suspension cells. *Appl Microbiol*. 1973; 26 (6): 858-62.
40. Mengesha A, Hurisa B, Tesfaye T, Lemma H, Niguse D, Wold G, et al. Adaptation of local rabies virus isolates to high growth titer and determination of pathogenicity to develop canine vaccine in Ethiopia. *J Vaccines Vaccin*. 2014; 5 (245): 2.
41. Guo C, Wang C, Luo S, Zhu S, Li H, Liu Y, et al. The adaptation of a CTN-1 rabies virus strain to high-titered growth in chick embryo cells for vaccine development. *Virol J*. 2014; 11 (1): 1-8.
42. Hurisa B, Mengesha A, Newayesilassie B, Kerga S, Kebede G, Bankovisky D, et al. Production of cell culture based anti-rabies vaccine in Ethiopia. *Procedia Vaccinol*. 2013; 7: 2-7.

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