

Pilot-Scale Production of Lyophilized Inactivated Rabies Vaccine Candidate in Vero Cells under Fully Animal Component-Free Conditions Using Microcarrier Technology and Laboratory Animal Trials

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ABSTRACT

The upstream process was carried out in an animal component-free medium on Cytodex 1 microcarriers. Recombinant trypsin is a non-animal derived protease used as an alternative to animal-derived trypsin. To inactivate recombinant trypsin, a soybean trypsin inhibitor (STI) should be added to the medium. A protocol was first tested in T-flasks and then passaged to 500 mL and 3 L spinner flasks. Cell detachment was completed in 10 - 12 min, and 0.4 g/L STI was added to a 3L spinner, and cells were transferred into a 30 L stirred tank bioreactor. On day 5, the cell density had reached its maximum (around 1.8×10^6 cells/mL). At an MOI of 0.3 with serum-free medium conditions, cell infection yielded a maximal rabies virus titer of 1.82×10^7 FFU/mL at 5 days. All cell culture conditions and virus growth kinetics in serum-free media were investigated. In conclusion, Vero cells were grown on Cytodex 1 with serum-free media and a high amount of rabies virus was obtained. A mouse challenge was used to determine the immune response to an inactivated rabies virus vaccine candidate. Also, we evaluated inactive rabies vaccine candidate safety, and immunogenicity in mice, sheep, horses, and cattle. We found that no horses, sheep, or cattle who were given vaccine IM at 3.2 IU/dose exhibited any clinical sign of disease and all developed high VNA titers (up to 10.03 IU/mL) by 3 - 4 WPI. After the accelerated stability studies, the lyophilized inactivated rabies vaccine candidate showed enough antigenic potency (2.6 IU/mL) in the mouse challenge test. Also, 18-month long-term stability studies showed enough immune response (1.93 IU/mL) on day 14. The activity of the vaccine candidate showed a good immune response and safety criteria that meet WHO requirements. This is the first pi-

lot-scale mammalian cell-based viral rabies vaccine production study in Türkiye that used microcarriers.

1. INTRODUCTION

Rabies virus, a rhabdovirus of the Lyssavirus genus, is a negative-strand virus containing a non-segmented, single-stranded, negative-sense genome RNA that encodes five proteins [1]. The rabies virus has a bullet-shaped morphology. The RNA is found to be associated with the nucleoprotein (N), forming the nucleocapsid core. The M1 protein as well as the transcriptase activity, presumably supplied by the L protein, is also associated with the nucleocapsid. M2 is associated with the lipid envelope, and a glycoprotein is found on the outer surface of the virion [2, 3].

Rabies is a viral disease that can affect all warm-blooded animals. Rabies remains endemic, mainly in developing countries [4]. An epidemiological study from 1991 [5] shows that the dog is the most important rabies reservoir (63% of countries are concerned about dog rabies) and that humans are most likely to be infected by dogs (93% of human cases in Africa; 72% in Asia). It has been reported that rodents may very rarely be infected with the rabies virus [2]. The rabies virus reaches the brain through peripheral nerves. The disease can only be diagnosed after the onset of symptoms. The first rabies vaccine was developed by Louis Pasteur and Émile Roux in 1885. Their first rabies vaccine was obtained from infected rabbits' brains. The cell culture-based rabies vaccine was established in 1967. Purified chicken embryonic cell-derived vaccines, human diploid cell-derived vaccines and purified Vero cell rabies vaccines are currently in use [2]. Today, Vero cells are considered a more suitable substrate to produce viral vaccines. The Vero cell line has been recommended by The World Health Organisation (WHO) to produce human vaccines. Rabies virus LP2061 which we used in this study is a Vero adapted strain and optimised to produce rabies vaccine in large-scale production. Adherent Vero cells can be used in different kinds of microcarriers for industrial production in cell culture bioreactors. Vero cells have been accepted for viral vaccine production [6] and are currently used in the manufacture of rabies [7], polio [8] and Japanese encephalitis [9] vaccines. Some cell additives and chemicals such as surfactants are used to increase host cell growth and virus titer [8, 10]. Vaccines that have at least 2 IU of antigenic value in each dose should be approved by the WHO committee [11, 12].

Media composition is one of the major safety concerns in vaccine production. In classical vaccine production processes, animal-derived compounds are widely used in the medium content, such as serum, trypsin, etc. Serum-containing media is generally used for the cell growth phase at a 10% ratio and sometimes also for the virus production phase at a 1% to 2% ratio. The animal serum is required for the growth of animal cells that are used as a substrate. The serum is a source of nutrients, growth factors, and proteolytic enzyme inhibitors for cell growth. Moreover, serum supports the adhesion and spread of cells on the surface and provides protection against mechanical damage and shear forces [10]. Despite all these benefits, animal-derived components such as serum and trypsin can be a source of contamination. Also, foetal bovine serum and newborn calf serum are expensive components in vaccine production and increase the impurities in the purification process. It has been previously reported by Merten *et al.* and Frazatti-Gallina *et al.* that a serum-free medium can be used in the production of the Rabies virus if the process is properly validated [13-16]. Trypsin is a necessary substance for passaging all cell substrates and presents a certain threat due to its origin in the animal pancreas (mainly porcine-derived). Trypsin, the main detaching agent, should be replaced by non-animal derived proteases or by recombinant trypsin produced by microorganisms. However, using the porcine-derived trypsin in the production process is not preferred in some religions because of the contamination and its residues.

This work describes a fully animal component-free process for rabies virus production in Vero cells grown on T-Flasks and Cytodex 1 (positive charge-DEAE-dextran) in a 500 mL, 3 L spinner flask, a basket-type 30 L bioreactor, a commercially available VP-SFM AGT, and, additionally, epithelial growth factor supplements. The critical parameters that affect cell attachment to the type of microcarriers, detach-

ment by recombinant trypsin and viral yield in VP-SFM and Glutamax I CTS supplement in spinner flasks and stirred bioreactors in batch mode, are reported.

Also, we evaluated Vero cell-derived lyophilized inactive rabies vaccine candidate safety, stability, and immunogenicity in sheep, horses, and cattle injected by the IM route.

2. METHODS

2.1. Cell Line

Vero cells at passage 131, provided by the Institute Pasteur de Tunis, (Tunis, Tunisia) and originally obtained from ATCC (CCL-81), were sub-cultivated and Vero cells at passage 141 used in this study. BHK-21 C13 cells at passage 77, provided by ATCC, USA for microtitration assay and CVS-11 strain production for challenge studies.

2.2. Rabies Virus Strain

Rabies virus Pastor strain LP-2061 at passage 22, provided by Institute Pasteur de Tunis, (Tunis, Tunisia) was conducted the following tests: sterility, mycoplasma, identity (by using the immunofluorescence technique). CVS-11 strain at passage 11, provided by Animal and Plant Health Agency, the UK for challenge studies.

2.3. Cell Adaptation to VP-SFM AGT and Preparation of Master Cell Bank (MCB)

One vial of Vero cells cryopreserved in MEM + 20% FBS + 10% DMSO, containing 20×10^6 cells was revitalized in serum-containing medium EMEM + 10% FBS according to standard protocol and let to attach for 3 - 4 h at 37°C, 5% CO₂ on T-175 Flasks' surface. Static cultures of cells adapted to VP-SFM were carried out in a seeding density of $6 - 8 \times 10^4$ cells/cm². The medium was then replaced by VP-SFM + Glutamax I CTS solution (Gibco, USA no: 30018), and the cells were incubated at 37°C, 5% CO₂ until the confluency [17]. VP-SFM must be supplemented with Glutamax I CTS solution (includes L-glutamine+ Epithelial growth factors) for cell growth and rabies virus production. Cells were expanded in VP-SFM over six passages to create a master cell bank. Cells were frozen in VP-SFM + 10% DMSO + 0.1% methylcellulose, cell density was equal to $5 - 8 \times 10^6$ cells/vial.

2.4. Preparation of Working Cell Banks (WCB)

Vero cells (ATCC CCL 81) are maintained for four passages in SFM for cell adaptation to this medium. The cells are amplified through subcultures in T-flasks using animal origin-free recombinant trypsin enzyme, and SFM. Vero cells from these flasks were used to prepare the Master Cell Bank (MCB). The cell suspension is distributed in cryogenic vials with 10×10^6 cells/vial and stored overnight at -80°C, and subsequently in liquid nitrogen. Using VP-SFM + 10% DMSO + 0.1% methylcellulose as a freezing medium for Vero cells was found efficient in growing performance after thawing. Vials from this bank are taken for quality control tests and the MCB is considered certified only after it passed such tests. As described by Rourou et al a working cell bank of adapted Vero cells was established [10].

2.5. Cell Dissociation

Cells were sub cultivated using recombinant trypsin (TrypLE Express, GIBCO, Denmark). Cell sub-cultivation was performed as described by the manufacturer Briefly the cells were washed twice with Ca⁺⁺ and Mg⁺⁺ free phosphate buffer saline (PBS, Sigma) including 0.01% EDTA, 5 - 6 mL of recombinant trypsin solution was added to a T-175 flask and cells were incubated for 3 - 5 min at 37°C. Dislodged cells were then centrifuged at 800 - 1000 rpm for 10 min. The supernatant was discarded and fresh VP-SFM was added to the falcons. Cells were counted by an automated cell counter (Vi-cell XR, Beckman Dickenson, UK).

Spinner flask and bioreactor cultures with microcarriers, the stirrer was stopped and let the microcarriers down, the medium was discarded and microcarriers were washed with PBS and PBS + EDTA solution twice. 5 - 6 mL of recombinant trypsin solution was added to 500 mL and 3L spinner flasks or 30 L bioreactor and after a contact time of 10 - 12 min at 37°C, cell detachment was completed and 0.4 g/L STI was added to spinner or bioreactor (**Figure 1(A)**, **Figure 1(B)**). Dislodged cells were then centrifuged and washed as mentioned above.

2.6. Cell Counting

Five millilitres of Vero cell culture were washed three times with PBS then treated in 5 mL of 0.1M citric acid (Sigma) containing 0.1% crystal violet (Sigma) and 0.1% Triton X-100 (USB, Cleveland, OH, USA) and incubated at least for 1 h at 37°C. The released nuclei were counted using a hemacytometer (HBG, Germany). Dissociated cells from microcarriers were counted by the Vi-Cell XR cell counting machine (Beckman Dickenson, UK).

2.7. Monitoring of Cell Infection

Aliquots of 5 mL of infected microcarriers culture were washed three times with PBS and then resuspended in a final volume of 2 mL. $2 \times 50 \mu\text{L}$ were put into a microscope slide and let dry at room temperature in a Class II B cabinet. The slide was then fixed with 80% acetone and stained with fluorescein-labelled specific nucleocapsid monoclonal antibodies DFA reagent kit (EMD Millipore, Canada, Cat. Ref No. 5100). Slides were inspected using a fluorescent microscope with 490 nm excitation and 515 nm emission peak filters. Rabies protein in infected cells will fluoresce bright apple green (**Figure 2(A)**, **Figure 2(B)**).

2.8. Preparation of the Virus Seed Banks

Vero cells cultivated in 225 cm² T-flasks were inoculated with Rabies LP-2061 strain with a multiplicity of infection (MOI) of 0.25 - 0.3, and the cultures were maintained in VP-SFM at 34°C - 35°C for 3 days. The viral culture supernatants were harvested in a class II cabinet on days 4 after infection. The Rabies virus

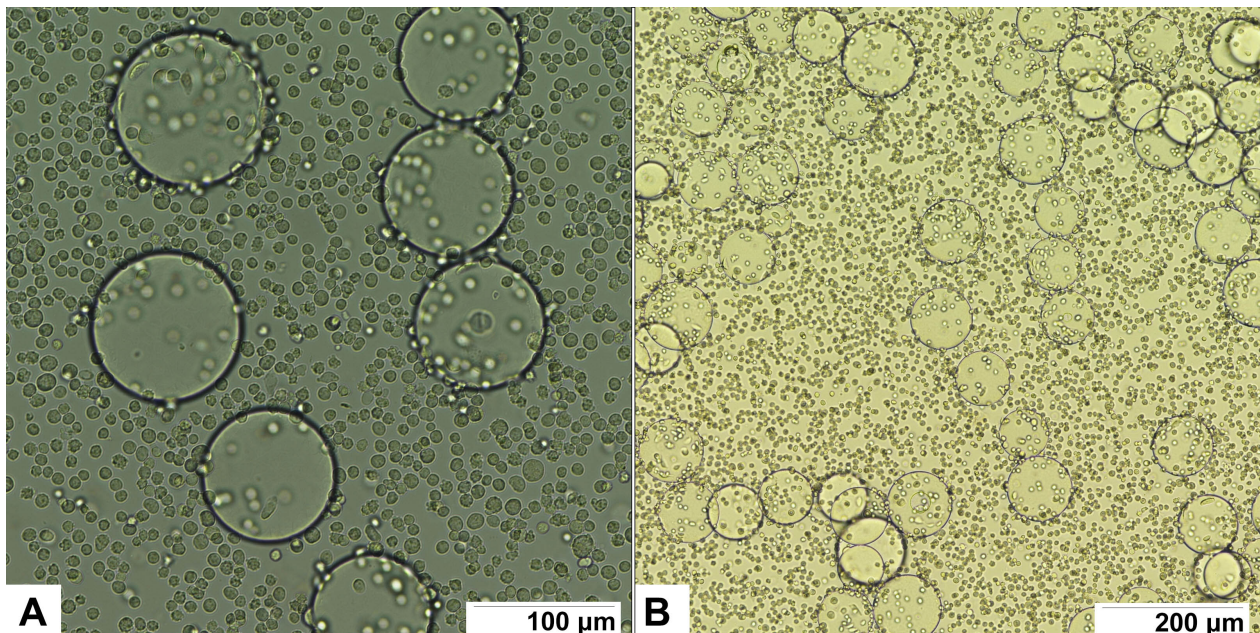


Figure 1. Cell dissociation from microcarriers at different magnifications, (A) 40× and (B) 10×, after the TrypLE express treatment.

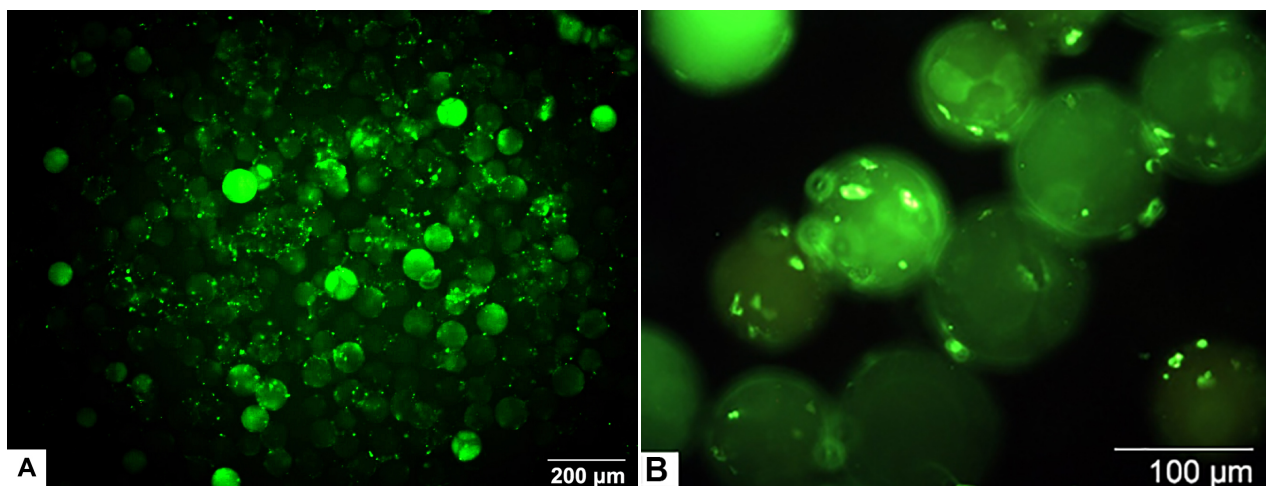


Figure 2. At 4 days post-infection, rabies virus-infected Vero cells on microcarriers at (A) 10× and (B) 40× magnification.

supernatants obtained in the harvests are clarified through a 0.45 to 0.6 μm membrane filter, and samples are taken for in-process tests. Then, the clarified Rabies virus harvests are stored at $-156^{\circ}\text{C} \pm 5^{\circ}\text{C}$ (in liquid nitrogen) for the viability of the live virus. After the determination of viral titer ($\geq 10^{5.0}$ FFD₅₀/mL), clarified harvests are used to prepare the Virus Master Seed lot.

The rabies virus Working Seed Lots were prepared from rabies virus Master Seed Lots obtained in a maximum of five virus passages. The harvests were clarified (0.45 to 0.6 μm filter), distributed in aliquots, and stored under $-156^{\circ}\text{C} \pm 5^{\circ}\text{C}$ as a mentioned by WHO. Samples of this bank were taken for quality control tests.

Advantageous agent analyses were carried out on 13 - 15 g weight of mice, suckling mice, and 350 - 450 g weight of guinea pigs according to European Pharmacopeia.

2.9. Production of Experimental Rabies Vaccine

Cytodex 1 microcarriers from Cytiva (Sweden) were used at 3 - 4 g/L throughout this study. Size ranges included 90 - 110 μm . They were prepared with PBS and sterilized according to the manufacturers' recommendations.

Cultures were carried out in 500 mL spinner flasks (Corning, USA) containing 500 mL of cultured cells in VP-SFM + Glutamax I CTS, at 37°C in a 5% CO_2 incubator as described by Trabelsi *et al.* [4]. The stirring speed was set up at 25 - 35 rpm with a slow speed magnetic stirrer (VWR, USA) in an incubator. The spinners were inoculated with $5 - 7 \times 10^5$ cells/mL. Samples were taken daily to observe cell confluency and growth parameters on microcarriers by an inverted microscope (Olympus, Japan). 3L spinner flasks (Corning, USA) containing 3000 mL of cultured cells, at 37°C in a 5% CO_2 incubator as mentioned above. The culture was started with 7×10^5 cells/mL and was maintained at 25 - 30 rpm stirring speed. Samples were taken daily to perform microscopic analysis and off-line analysis. pH set at 7.1 - 7.3 (regulated by the addition of NaHCO_3 at 88 g/L). 50% of the culture medium was replaced daily two days after cell inoculation. For the rabies virus production step, pH was maintained at 7.2 - 7.4, agitation rate at 30 rpm and temperature at $34 - 35^{\circ}\text{C}$. Once the temperature reached $34^{\circ}\text{C} - 35^{\circ}\text{C}$, cells were washed with PBS twice, added fresh VP-SFM at 30% volume of spinner flasks and then infected at an MOI of 0.3 with the Rabies virus LP-2061 strain. Two hours after cell infection, VP-SFM was completed to the final volume of spinner flasks. Samples were taken daily to determine the following parameters: cell count, microcarriers load, virus titer, and cell infection. Supernatants were harvested after 4 days of infection and then repeated every 48 - 62 hours for 5 additional harvests. Spinner flask culture was repeated three times.

30 L basket bioreactor (SARTORIUS, Biostat C) starting volume was equal to 30 L with VP-SFM +

Glutamax I CTS. The culture was seeded with $5 - 7 \times 10^5$ cells/mL from WCB, cultivated at $36.5^\circ\text{C} - 37^\circ\text{C}$ and was continuously agitated at 30 rpm until the cell's confluency was completed on the microcarriers (3 - 4 g/L). During the cell culture proliferation step, pH is set at 7.1 - 7.4 (regulated by injection of CO_2 or addition of NaHCO_3 at 88 g/L), pO_2 maintained at 45% - 75% air-saturation or pure oxygen when required, the temperature at 37°C and agitation rate at 30 - 50 rpm. Samples were taken daily to perform microscopic analysis and off-line analysis (Figures 3(A)-(D)). 50% of the culture medium was replaced daily two days after cell inoculation. Because the viability and growth parameters of cells could be affected by the shear environment, we used Pluronic F-68 10% (Gibco, USA) in the cell culture medium in a concentration range of 0.1% [18, 19]. The addition of shear protectants such as Pluronic F-68 to a serum-free medium is often needed to protect cells against shear forces [20, 21].

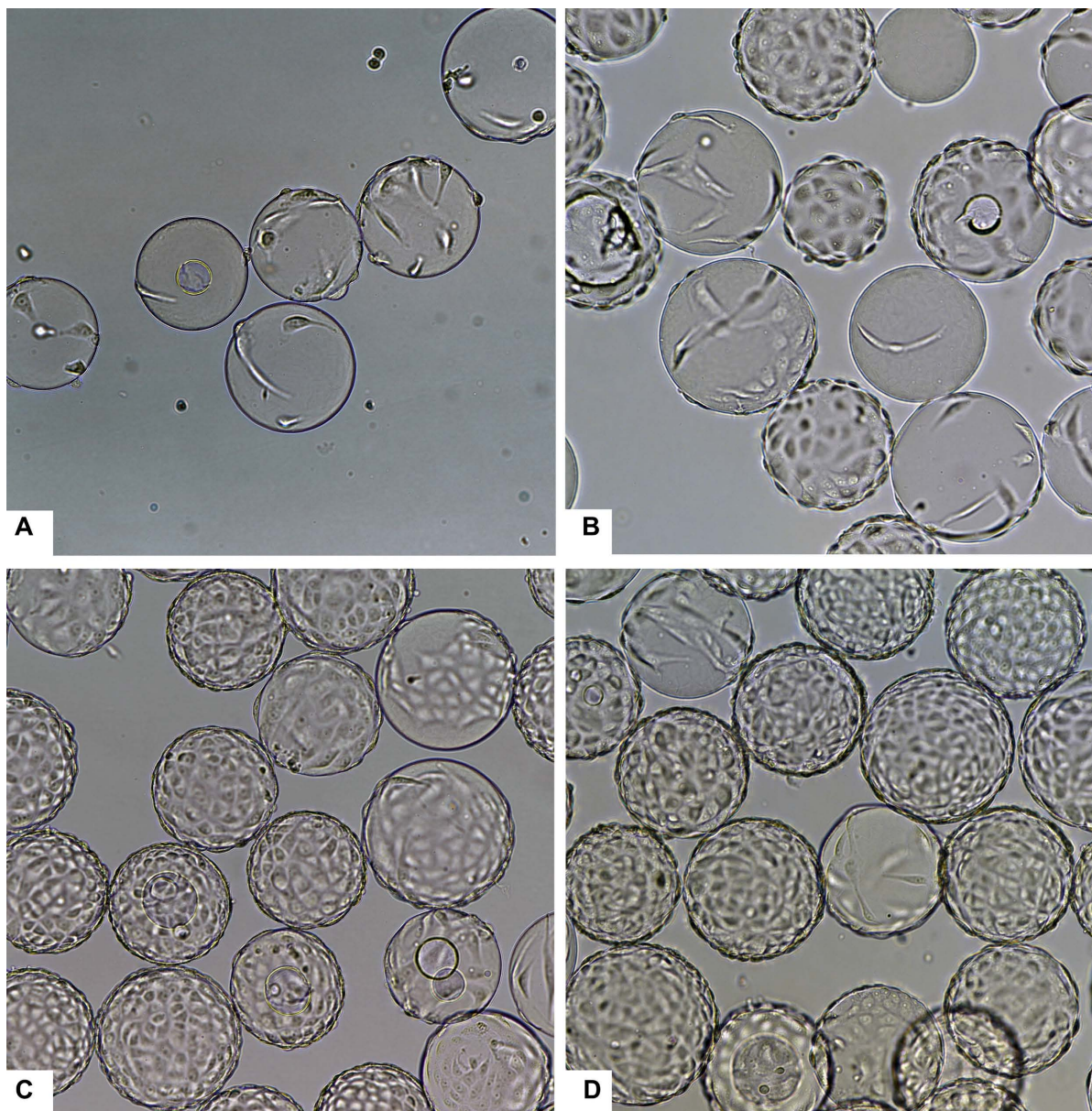


Figure 3. VERO CCL-81 cells grown in serum-free medium on Cytodex I (3 g/L) microcarriers in 30 L bioreactor. Cells were observed with an inverted light microscope without staining at 100× magnification: (A) 24 hours, (B) 48 hours, (C) 80 hours, and (D) 120 hours after seeding.

For the rabies virus production step, pH was maintained at 7.2 - 7.4, pO_2 at 30% - 40% air-saturation, agitation rate at 30 - 50 rpm and temperature at 34°C. Once the temperature reached 34°C, cells were washed with PBS twice, added fresh VP-SFM at 30% volume of bioreactor and then infected at an MOI of 0.3 with the Rabies virus LP-2061 strain. Two hours after cell infection, VP-SFM was completed to a final volume of 30 L basket bioreactor. Supernatants from this culture are harvested as mentioned above. Samples of the harvests are used for offline analysis.

The viral suspensions obtained in the harvests were clarified by low-speed centrifugation (15 min 3500 × g) and filtration (0.45 μm) and then harvests were concentrated 10 - 20-fold and buffer exchanged by Tangential flow filtration using Sartorius benchtop System (Sartorius Stedim, Germany) at a flow rate of 27 mL/min equipped with Slice 200 cassette Hydrosart (cut off 100 kDa) (Sartorius Stedim, Germany), transmembrane pressure (TMP) was around 2.7 bar. Concentrated bulk was inactivated by β-propiolactone (Acros, Germany) (final dilution 1/4000) at 2°C - 6°C for 18 - 24 hours [22]. The inactivated bulk was transferred to a sterile vessel and stored by slow speed stirring at 25°C for 2 h. After this period, samples are taken for sterility and effective virus inactivation tests [23].

Inactivated bulk was purified by AKTÄ Avanth system (GE Healthcare Life Sciences, Uppsala, Sweden) using XK 16 20 chromatography column. 450 cm/hr velocity was used. Capto Core 700 chromatography resin (GE Healthcare Life Sciences, Uppsala, Sweden) was equilibrated with 10 Column volume with 20 mM Tris, 150 mM NaCl. A fraction that includes purified virus was collected in the flow-through; the impurities were discarded using 30 Column volume with 20 mM Tris and 1 M NaCl [23] (Figure 4).

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UNICORN 6.3
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Result: KRP01204112020-R3
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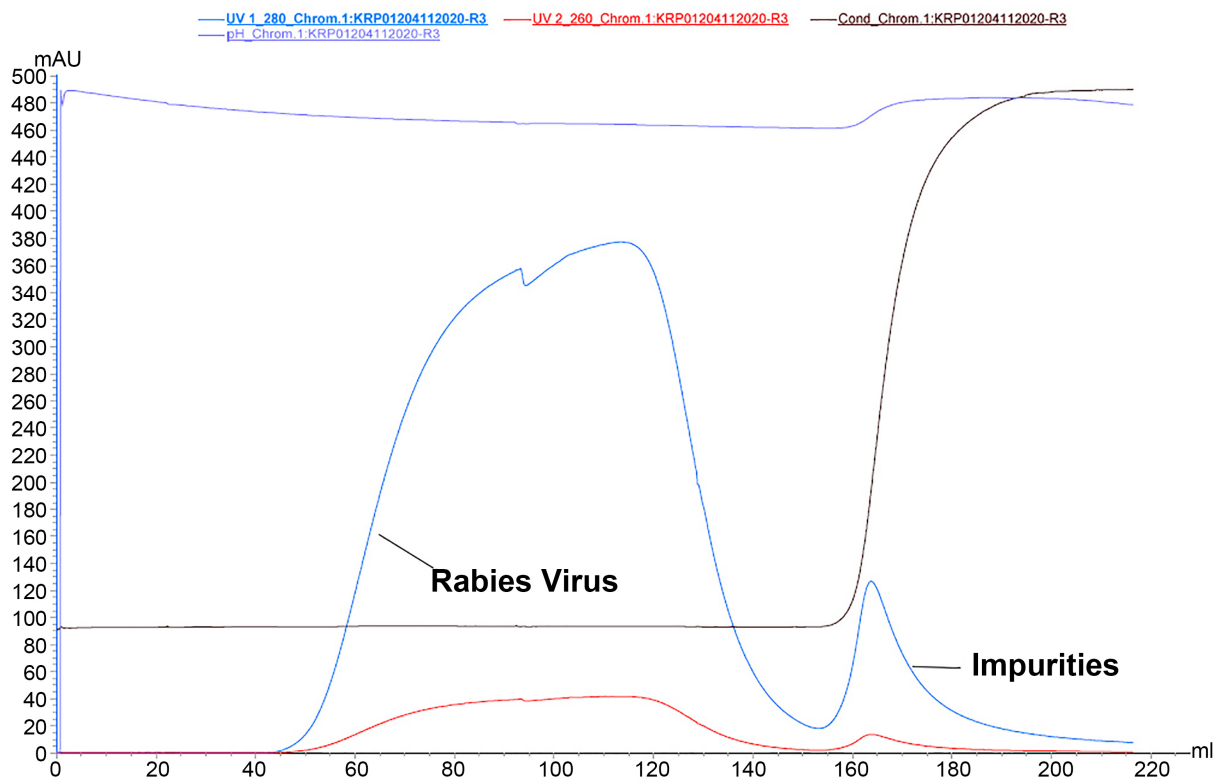


Figure 4. Purification by AKTÄ Avanth system. Rabies virus was collected in the flow through. The impurities were eluted using 30 CV 1 M NaCl and 20 mM Tris at pH 7.5.

The purified and inactivated Rabies virus concentrates obtained in a bioreactor cycle are mixed, filtered (0.45 + 0.22-µm capsule filter) in a 50-L Flexibody bag, and stored at 2°C - 8°C. Samples of this mixed concentrate are taken for the quality control tests mentioned in European Pharmacopeia.

The residual Vero host cell DNA was measured using a res DNA-SEQ quantitative VERO HOST CELL DNA kit (Applied Biosystems, Life Technology, Warrington, UK). The isolation of the Vero cell DNA was performed according to the manufacturer's recommendations. DNA Quantitation kit was used to measure residual Vero host cell DNA in the purified bulk using the Roche LightCycler 480 instrument.

Residual host cell protein levels were quantified using the Vero Host Cell Proteins kit (Cygnus Technologies, Southport, USA) according to manufacturer recommendations. The results were read at 450 nm wavelength in an ELISA reader with a GEN-5 software interface (BIOTEK Epoch Agilent, USA).

The bulk rabies vaccine was adjusted by adding 0.01 M Phosphate Buffered Saline solution, glycine (Merck, Germany), sucrose (Merck, Germany), and human serum albumin 1% (Sigma, USA) to obtain a potency of a minimum of 3.0 IU/dose. Samples were taken for necessary quality control tests according to European Pharmacopeia.

The formulated product is filled according to vaccine presentation: after the buffer exchange and the formulation the samples were immediately frozen at -80°C and lyophilized, and subsequently, the samples were analysed. 0.7-mL vials for use in the freeze-dried process. The protocol (Table 1) was established with some modifications related to lyophilization described by Severo *et al.* [24]. Mean residual moisture obtained was given in Table 2 for the freeze-dried vaccine and the results were found according to WHO standards [25].

2.10. Transmission Electron Microscope (TEM) Experiments

The samples were negatively stained using 2% uranyl acetate (UA). Inactivated samples were mounted on pyloform-covered copper grids, stained with 2% uranyl acetate (UA), washed two times with sterile pure water, and monitored in a TEM (Jeol, Tokyo, Japan) [1].

2.11. Glycoprotein Titration

Rabies glycoprotein content was determined by commercial rabies antigen ELISA kit (DRG-ELISA, Germany) using monoclonal antibodies as manufacturer recommendations. ELISA assay was defined by M. Chabaud-Riou *et al.* as can quantify the rabies viral G-protein with high specificity, linearity, accuracy, and precision [26]. In the 2011 Ames international workshop (USA), worldwide rabies experts agreed on the need to replace the NIH test with an enzyme-linked immunosorbent assay (ELISA) [27]. The amount of rabies glycoprotein was read at 450 nm wavelength in a microtiter plate reader with GEN-5 software interface (BIOTEK Epoch Agilent, USA).

Table 1. Description of lyophilization protocol.

Step	Temperature (°C)	Duration (h)
Freezing	-42 to -50	3 to 5
Primary drying	-50 to 5	48 to 96
Secondary drying	+20 to 25	3 to 6

Table 2. Residual moisture results for lyophilized cakes.

Excipient	S1	S2	S3	Mean Residual Moisture
Sucrose, albumin, and glycine	2.91	2.48	3.22	2.85% ± 0.30%

2.12. Rabies Virus Titration

Virus titres were determined using the Vero cell line (ATCC; CCL-81) in microplates. Cells (5×10^4) were distributed in each well and infected with 50 μL of log₁₀ dilutions of either virus samples or a PV-LP2061/VERO reference virus previously titrated by the plaque-forming technique. After 24 h, microplates were washed with PBS (phosphate buffer saline supplemented with Ca^{2+} and Mg^{2+}) and fixed with cold 80% acetone. Cells were stained with fluorescein-labelled anti-rabies-nucleocapsid immunoglobulins (EMD Millipore, Canada, Cat. Ref No. 5100). Results were expressed in fluorescent focusing units per mL (FFU/mL) by comparison of the sample assays with the reference.

2.13. Nucleic acid Extraction and First-Strand cDNA Synthesis

Total nucleic acids were extracted from clarified viral supernatants using the Mini Pathogen Nucleic Acid Kit (Qiagen, Germany) according to the manufacturer's instructions and collected in 50 - 100 μL elution volumes. The viral RNA was reverse transcribed depending on Moloney murine leukaemia virus reverse transcriptase (Thermo Scientific, USA) according to the manufacturer's recommendations. At first, reaction mixtures were incubated for 60 min at 42°C, and then the reaction was completed by heating the mixture at 95°C for 5 min. The mixtures were chilled on ice, immediately.

2.14. Sequencing

48-hour (R9.4) sequencing protocol was performed using MinION™ control software, MinKNOW™ version 0.46.1.9 (R9.4). Read data were obtained based on 1.2.2 rev 1.5 workflows and software Metrichor™ agent (version 0.16.37960).

After sequencing, the results obtained in fast5 format were converted to fastq format using guppy v4.4.1 software (base-calling and de-multiplexing). Barcode and adapter sequences were cleaned using Porechop v0.2.3 software, and universal primers and tags were also deleted by deleting 45 bases from both ends of the sequences. After clearing the sequences, reads less than 100 bp in length were filtered and excluded from the analysis.

Cleaned reads were analysed with a customized workflow using the Genius Prime 2021.0.3 platform. The sequences were mapped by comparing the Rabies lyssavirus-NC_001542.1 viral whole-genome in the NCBI database, and the consistency of the resulting contigs and the organisms they matched in the database were scanned by de novo alignment. In addition, the consensus sequences produced polymorphism results and statistical information of contigs are presented in the manuscript.

2.15. SDS-Page and Western Blotting

β -Propiolactone inactivated Rabies virus samples were separated using the 10% polyacrylamide gels described by Laemmli and visualized using the Coomassie Blue staining kit (BIO-RAD).

2.16. Quantitative Real-Time PCR

The qRT-PCR assay was performed using the 2x One-Step RT-qPCR Master Mix, (Techne, UK). The Rabies Techne qPCR test is a highly specific in vitro diagnostic quantitative real-time reverse transcriptase PCR (qRT-PCR) assay to quantify nucleic acid from the Rabies virus. The assay utilizes real-time technology targeting the 3' leader and nucleoprotein region. Each 20- μL reaction contained 10 μL Lyophilised OneStep 2x Master Mix (Techne, UK), 1 μL 5 $\mu\text{mol/L}$ probes, 20 $\mu\text{mol/L}$ forward and reverse primer mix, 4 μL nuclease-free water, and 5 μL nucleic acid extract. Amplification was carried out on a ROCHE LightCycler 480 Real-Time PCR instrument (ROCHE). Real-time PCR conditions consisted of 10 min at 55°C for reverse transcription, 2 min at 95°C for enzyme activation, and 40 cycles of 10 s at 95°C and 60 s at 60°C for a polymerase chain reaction. Specific rabies virus nucleotide copy number/ μL is calculated based on the standard curve by absolute quantification of Roche 480 original software.

2.17. Determination of LD₅₀ of Rabies Virus CVS Strain

Determination of the rabies virus LD₅₀ (MIT—mouse inoculation test) was carried out in 8 - 10 g Balb/c mice [28, 29]. Intracerebral inoculation of mice was performed with 6.1 LD₅₀/0.03 mL of the CVS-11 strain. Five different tenfold dilutions (10⁻³ to 10⁻⁷) were administered to five groups of five Balb/c mice each. The only mouse that died between the 5th and 14th days after the challenge was considered to have died from rabies. Monitoring of the animals continued for 21 days. Specific death (was verified by FAT detection of the rabies antigen on brain imprints in a fluorescent microscope and, then the LD₅₀ of the challenge virus strain was calculated according to Reed and Muench (1938) [30].

2.18. Potency (NIH) Tests

The total protective efficiency of lyophilised inactivated rabies virus vaccine candidate was determined according to the NIH test in 13 - 15 g weight Balb/c mice. Balb/c mice were immunized at days 0 and 7 by IP (intraperitoneal) route and then challenged by the intracerebral (IC) route using the Challenge Virus Standard strain (CVS-11 strain) a week after the second dose. An international reference vaccine was used to calculate potency expressed in International Units/mL (IU/mL) [1, 31]. The only mouse that died between the 5th and 14th days post-inoculation was considered to have died from rabies. Brain imprints of non-vaccinated animals and challenged at day 21 with CVS-11 strain of rabies virus showed green-apple cytoplasmic inclusions intracerebrally challenged with CVS-11 strain (Figure 5).

2.19. RFFIT (Rapid Fluorescent Focus Inhibition) Tests

RFFIT measures the immune response after vaccination and the strain of seed virus used for vaccine production and then rabies virus neutralizing antibodies (RVNA) values are reported. Blood samples were collected from farm animals at 0, 7 and 14 days after one dose of rabies vaccine administration. Serum samples must be heated at 56°C for 30 minutes before testing to inactive complement. Serial dilutions of test sera may be prepared in an eight-well tissue-culture chamber slide. Screening dilutions of 1/5 and 1/50 are sufficient for routine evaluation of vaccination efficacy. Add 100 FFD₅₀/0.1 mL of the challenge virus (CVS) to all serum dilutions in eight-well tissue-culture chamber slide and then incubate at 35°C in a humidified incubator with 0.5% CO₂ for 90 minutes. During the incubation period, resuspend BHK-21 cells in EMEM to add a final concentration of 1 × 10⁵ cells/0.2 mL and incubate at 35°C in a humidified incubator

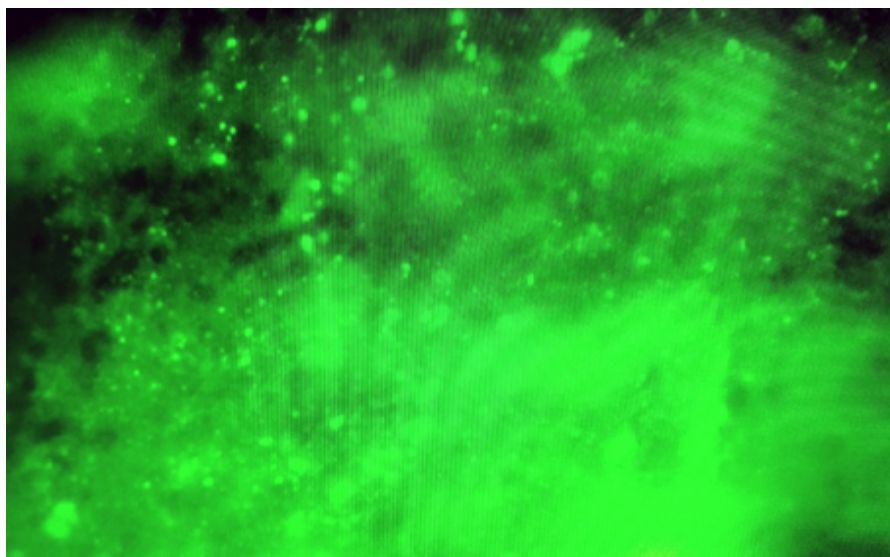


Figure 5. Direct specific fluorescent antibody-stained mouse brain tissue imprints in non-vaccinated animals with challenged CVS strain.

with 0.5% CO₂ for 24 hours. After 24 h, microplates were washed with PBS (phosphate buffer saline supplemented with Ca²⁺ and Mg²⁺) and fixed with cold acetone. Cells were stained with fluorescein-labelled anti-rabies-nucleocapsid immunoglobulins (EMD Millipore, Canada, Cat. Ref No. 5100). Observe the slides under a fluorescence microscope. The titre of antibody in the test serum (in IU/mL) can be obtained by comparison with the titre of the national reference standard.

3. RESULTS

3.1. Cells Growth Kinetics

Samples were taken daily to perform microscopic analysis and off-line analysis. The culture was seeded with 5×10^5 cells/mL and was continuously agitated at 30 - 50 rpm. On day 5, the cell density had reached around 1.8×10^6 cells/mL (Figure 6).

3.2. Transmission Electron Microscope (TEM) Results

TEM analysis verified that the rabies virus showed intact, bullet-shaped particles, approximately 200 nm in length after the final purification step (Figure 7). We have observed enveloped intact viruses on the negative (uranyl acetate) staining method in the TEM experiments.

3.3. Glycoprotein Titration of Final Vaccine Bulk

The commercial ELISA kit used in this study is based on monoclonal antibodies attaching specifically the native form of the G-protein of the Rabies virus. In the current study, for all batches that were found to comply with the NIH potency specification (2.5 IU/dose), consistent results were obtained with the ELISA. The potency of the final product was found 3.2 IU per single human dose by commercial ELISA. The batch at 3.2 ELISA IU/dose was tested 3 times by NIH, with titers varying between 3 and 3.3 IU/dose (Figure 8).

3.4. Rabies Virus Titration

Supernatants from infected cultures are harvested after 4 days of virus infection and then carried out

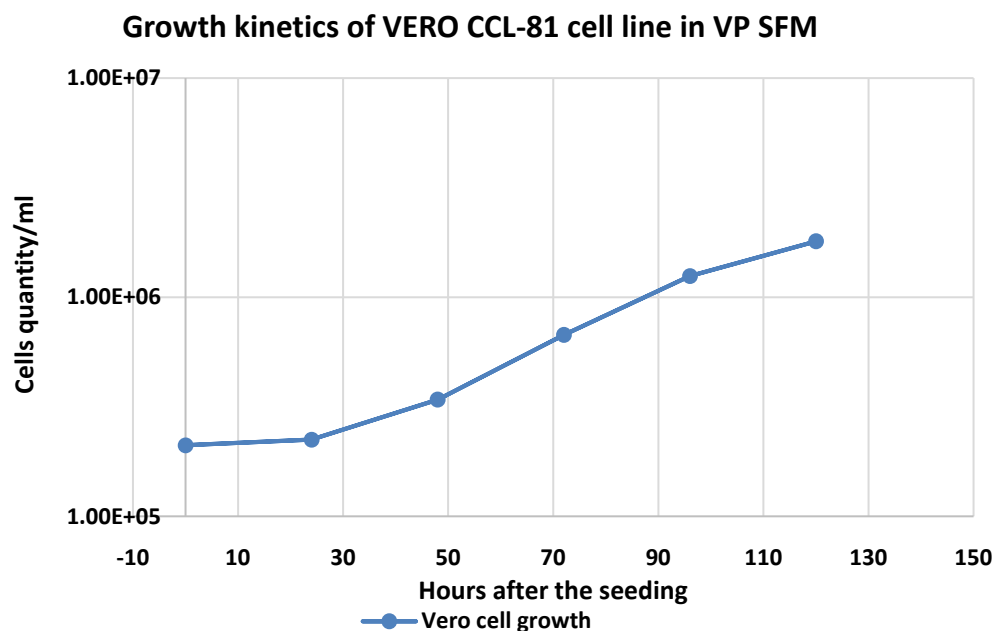


Figure 6. The curve of Vero CCL-81 cells growth on cytodex 1 in VP SFM + Glutamax I.

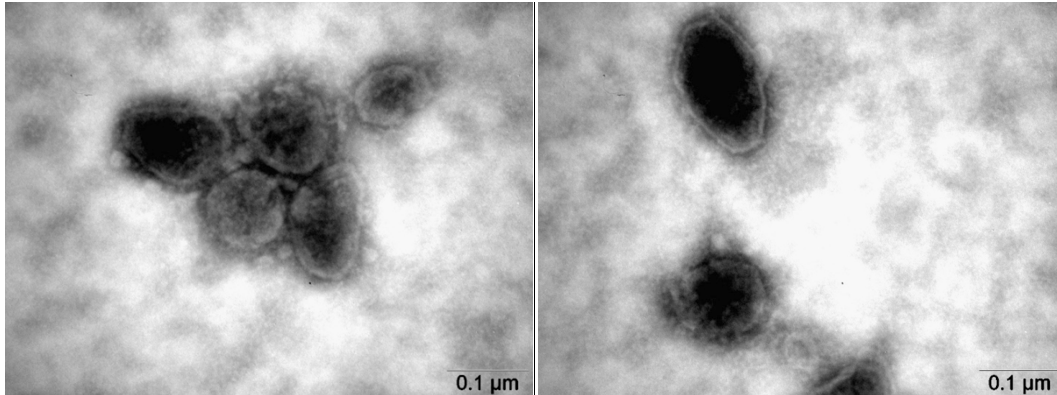


Figure 7. TEM images of bullet-shaped particles, approximately 200 nm in length after the final purification step.

EIA-2489 Glycoprotein of Rabies titre calculation

Analysed on:

Test	Sample	OD-value	END point Titre	RC	intersection	r ²
undiluted		2.148	1.8IU	-0.77979	3.477695652	0.951704
1:2 diluted		2.603	0.9IU			
1:4 diluted		3.128	0.45IU			
1:8 diluted		3.4	0.225IU			
1:16 diluted			IU			
1:32 diluted			IU			

Samples	Final Bulk	OD-value	END point Titre
9015-002		0.961	3.2

Fill in OD of positive curve
 Fill in sample values

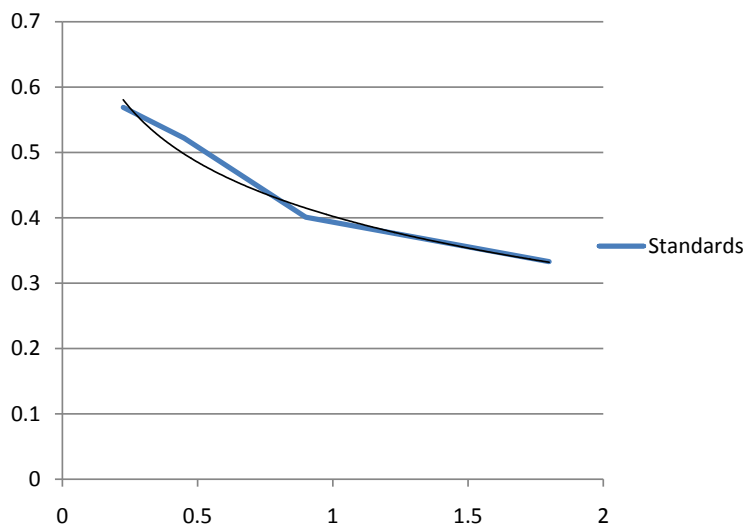


Figure 8. Quantification of rabies virus glycoprotein using rabies specific quantitative ELISA (DRG-EIA, Germany) as IU/dose.

viral titration as mentioned previously. Results were expressed in fluorescent focusing units per mL (FFU/mL). We showed that the optimum Rabies virus titer was equal to 1.82×10^7 FFU/mL on day 5 in serum-free conditions (Figures 9(A)-(E)).

However, the rabies virus growth kinetics in VP SFM medium through the 5 days was given below (Figure 10).

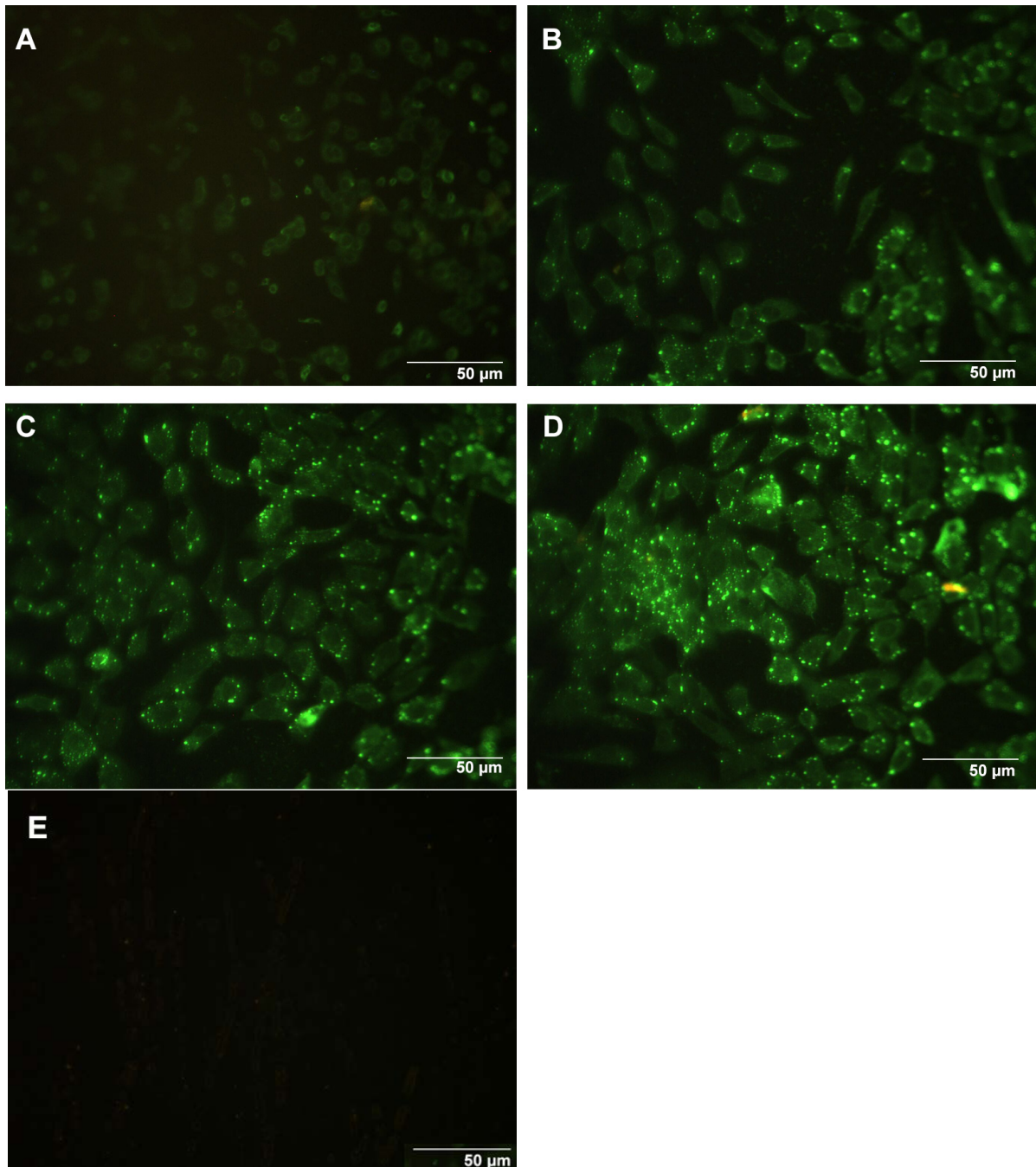


Figure 9. Quantification of rabies virus in VERO CCL-81 cell culture plates. Assays were performed on the harvested virus at 96 - 120 h post-infection, acetone fixed, and stained with rabies specific direct fluorescent antibody to visualize on a fluorescent microscope. (A), (B), (C), and (D) are ten-fold dilutions of the virus (10^{-7} to 10^{-4}). (E) is a negative control well.

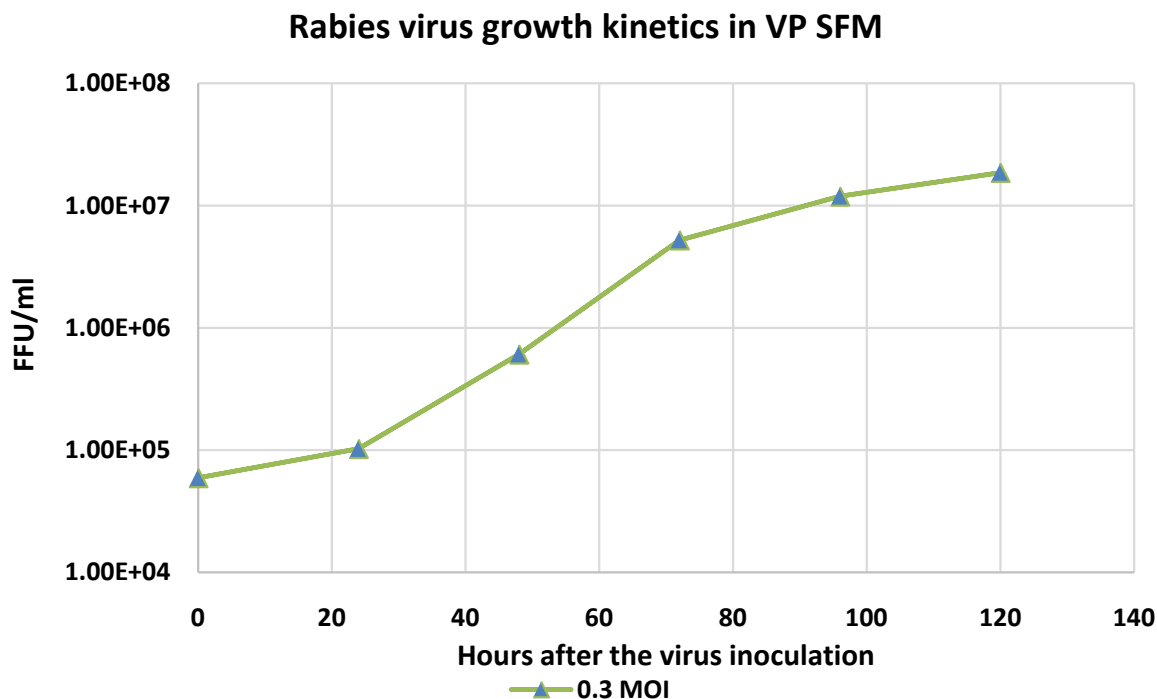


Figure 10. Rabies virus production on 3 g/L Cytodex 1 in VP SFM. Cells were grown in a 30-l bioreactor in a batch mode, and then the rabies virus was inoculated with 0.3 MOI 5 days after the start of the culture. Sampling was carried out daily.

3.5. Validation of Inactivation

No fluorescent signals were observed for three sub passages of inactivated virus bulk into the susceptible cell line. Inactivation was validated by passaging the inactivation agent (β -propiolactone) treated samples 3 generations ($n = 3$) without the appearance of fluorescence.

3.6. Vero cell DNA and Host Cell Protein Results

Residual cellular DNA quantification by q-PCR was also carried out and was equal to 8.6 ± 2 pg/dose in the final product. Concerning the concentrations of HCP in the final product, it was equal to 1180 ± 100 ng/mL.

3.7. Sequencing

Whole-genome sequence results of Kocak Rabies virus PV LP-2061 correlate to Rabies lyssavirus—NC_001542.1 isolate at a ratio of 98.379% in the NCBI database. Also, genetically variations were given below (Figure 11).

3.8. SDS-PAGE and Western blotting

The molecular weights of the full-length Glycoprotein-G and RNA-dependent RNA polymerase L are approximately 64 and 190 kDa, respectively (Figure 12).

3.9. Quantitative Real-Time PCR

Quantitative Rabies RT-PCR (Techne, UK) kit results are correlated with viral titer (FFU/mL) results. Rabies qRT-PCR is a fast and easy way to determine harvesting time in batch culture (Figure 13). We observed the viral load increase day to day by the qRT-PCR method.

Name	Sequence	Position	Polymorphism Type	Change	Maximum	Length	Type	Reference Nucleotide	Variant Nucleotide	Sequence (with extension)	Amino Acid Change	Coverage	Product	Protein Effect	Reference Frequency	Variant Frequency
A	C	3176	SNP (transversion)	C → A	3176	1	Polymorphism	C	A			33				
A	G	2763	SNP (transition)	G → A	2763	1	Polymorphism	G	A		V → I	15	M2 protein	Substitution	6.7%	93.3%
G	A	2934	SNP (transition)	A → G	2934	1	Polymorphism	A	G		N → D	26	M2 protein	Substitution	3.8%	92.3%
C	U	5155	SNP (transition)	T → C	5155	1	Polymorphism	T	C			38				92.1%
C	A	4067	SNP (transversion)	A → C	4067	1	Polymorphism	A	C			16	transmembrane glycoprotein G	None		87.5%
A	G	540	SNP (transition)	G → A	540	1	Polymorphism	G	A		S → N	16	nucleoprotein N	Substitution	6.3%	81.3%
A	C	11801	SNP (transversion)	C → A	11801	1	Polymorphism	C	A			12	L protein	Truncation	8.3%	75.0%
G		5078	Deletion (tandem repeat)	G ₄ → G ₃	5078	1	Polymorphism	G	G			45				71.1%
T	G	353	SNP (transversion)	G → T	353	1	Polymorphism	G	T		V → L	10	nucleoprotein N	Substitution		70.0%
C		5852	Deletion (tandem repeat)	C ₆ → C ₅	5852	1	Polymorphism	C	C			26	L protein	Frame Shift		89.2%
C		4553	Deletion (tandem repeat)	C ₅ → C ₄	4553	1	Polymorphism	C	C			64	transmembrane glycoprotein G	Frame Shift		68.8%
A		11598	Deletion (tandem repeat)	A ₄ → A ₃	11598	1	Polymorphism	A	A			16	L protein	Frame Shift		68.8%
G		4416	Deletion (tandem repeat)	G ₆ → G ₅	4416	1	Polymorphism	G	G			15	transmembrane glycoprotein G	Frame Shift		66.7%
C		11496	Deletion	-C	11496	1	Polymorphism	C				18	L protein	Frame Shift		66.7%
A	C	4569	SNP (transversion)	C → A	4569	1	Polymorphism	C	A		L → M	58	transmembrane glycoprotein G	Substitution	15.5%	65.5%

Figure 11. Genetic variations of nucleotides of rabies virus LP 2061 strain in whole-genome analysis.

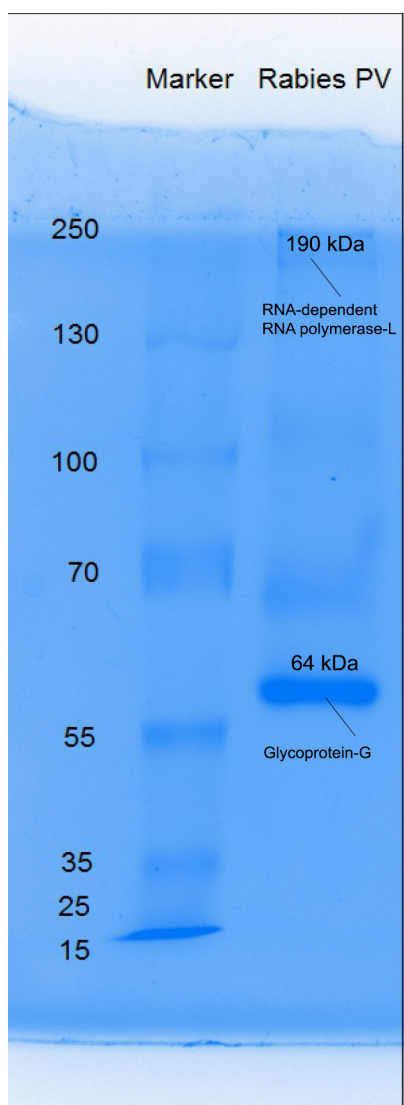


Figure 12. Rabies virus SDS gel electrophoresis with 250 kDa Marker.

Rabies Experiment Run Protocol One Step 26.07.18

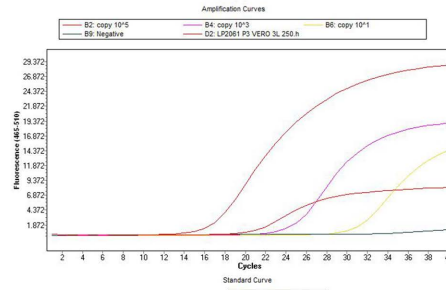
Experiment	
Creation Date	7/26/2018 9:02:19 AM
Last Modified Date	7/26/2018 11:30:21 AM
Operator	System Admin
Owner	System Admin
Start Time	7/26/2018 9:25:50 AM
End Time	7/26/2018 10:58:42 AM
Run State	Completed
Software Version	LCS480 1.5.1.62
Macro	Macro Owner
Macro Status	
Templates	Rabies Experiment Run Protocol One Step 23.05.17
Plate ID	06310977
Test ID	
Lot ID	
Color Comp ID	
Run Notes	

Programs							
Program Name Reverse Transcription							
Cycles	1	Analysis Mode None					
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
55	None	00:10:00	4.40		0	0	0
Program Name Enzyme Activation							
Cycles	1	Analysis Mode None					
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:02:00	4.40		0	0	0
Program Name PCR							
Cycles	40	Analysis Mode Quantification					
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:10	4.40		0	0	0
60	Single	00:01:00	2.20		0	0	0
Program Name Cooling							
Cycles	1	Analysis Mode None					
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
37	None	00:10:00	2.20		0	0	0

Abs Quant/Fit Points for rabies 26.07.18 (Abs Quant/Fit Points)

Settings			
Channel	465-510		
Color Compensation	Off		
Standard Curve	In-run		
Program	PCR		
First Cycle	1	Last Cycle	40
Background	2.6		
Noiseband Method	Auto	Noiseband	0.3171
STD Dev Multiplier	12.0000		
# of Fit Points	2	Threshold Method	Auto
Threshold	0.0470		

Results							
Inc	Pos	Name	Type	CP	Concentration	Standard	Status
<input checked="" type="checkbox"/>	B2	copy 10*5	Standard	8.42	2.09E5	2.00E5	
<input checked="" type="checkbox"/>	B4	copy 10*3	Standard	15.86	1.83E3	2.00E3	
<input checked="" type="checkbox"/>	B6	copy 10*1	Standard	22.88	2.09E1	2.00E1	
<input checked="" type="checkbox"/>	B9	Negative	Negative Control				
<input checked="" type="checkbox"/>	D2	LP2061 P3 VERO 3L 250.h	Unknown	8.94	1.50E5		



Error: 0.0557
Efficiency: 1.891
Slope: -3.614
Yintercept: 27.65

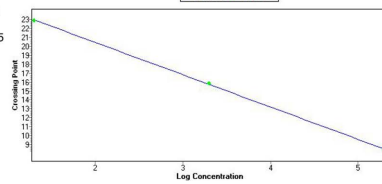


Figure 13. qRT-PCR results for rabies virus (LP-2061 strain) harvest samples cultivated in Vero CCL-81 cell line at 250 hours.

Calculation of virus LD50 in mice using the Spearman-Kärber method

Log10 virus dilutions	Mice	
	Dead	Alive
-1	10	0
-2	10	0
-3	10	0
-4	10	0
-5	10	0
-6	6	4
-7	0	10

World J Virol 2016 May 12; 5(2):85-86
Determination of 50% endpoint titer using a simple formula
Muthannan Andavar Ram akrishnan

X₀ = 5
d = 1
LD₅₀/m l = -6.1
10x LD₅₀/m l = 6.1

Spearman-Kärber method
 $\lg_{10} 50\% \text{ end point dilution} = -(\bar{x}_0 - d/2 + d \sum r_i/n_i)$
x₀ = lg10 of the reciprocal of the highest dilution (lowest concentration) at which all animals are positive;
d = lg10 of the dilution factor;
n_i = number of animals used in each individual dilution (after discounting accidental deaths);
r_i = number of positive animals (out of n_i).
 Summation is started at dilution x₀.

Figure 14. LD50 calculation of CVS rabies strain according to the Spearman-Kärber method.

3.10. Determination of LD₅₀ of Rabies Virus CVS Strain

6 Log 10 dilution of CVS strain was found to kill 50% of the animals in the experimental group (Figure 14).

3.11. Potency Tests

The rabies vaccine candidate batches used recombinant trypsin in the scale-up process and were cultivated in VP-SFM medium showed to be much higher potency (3 IU per dose) than the minimum required for a human rabies vaccine (2.5 IU per dose) (Figure 15).

3.12. Accelerated and Interim Long-Term Stability Results

During 18 months of long-term storage at a temperature of 2°C - 8°C and 6 months of accelerated stability experiments at 25°C in stability cabinet, all tested batches presented enough potency (≥ 2.5 IU per dose) according to WHO requirements in the NIH (mouse inoculation) test comparison with the national reference standard (Figure 16). Also, RFFIT assays showed enough immune response (≥ 0.5 IU per mL) and induced a 100% seroconversion on farm animals (sheep, cows, and horses) after the one dose vaccine administration in pilot experiments in long-term studies.

Fifty percent effective dose (ED 50): Spearman-Kärber method Date: 2021/11/24
Batch: 3

Log10 dilution	Dilution	Mice	Kocak Rabies		Std Vaccine	
			A live	Positive Ratio	A live	Positive Ratio
0.00	1	10	10	1	10	1
-0.70	0.2	10	9	0.9	9	0.9
-1.40	0.04	10	9	0.9	9	0.9
-2.10	0.008	10	7	0.7	2	0.2
-2.80	0.0016	10	0	0	0	0
dose= 0.5 ml			$\Sigma P = 3.5$		$\Sigma P = 3$	
Calculate m = $xk - (d)(EP - 0.5)$			-2.07		-1.725	

3 IU/ml 2.5 IU/ml standard

Figure 15. The calculation of potency of the vaccine batch 3 according to the Spearman-Karber method.

Fifty percent effective dose (ED 50): Spearman-Kärber method 6 months accelerated stability

Log10 dilution	Dilution	Mice	Kocak Rabies		Std Vaccine	
			A live	Positive Ratio	A live	Positive Ratio
0.00	1	10	10	1	10	1
-0.70	0.2	10	8	0.8	6	0.6
-1.40	0.04	10	5	0.5	3	0.3
-2.10	0.008	10	2	0.2	1	0.1
-2.80	0.0016	10	0	0	0	0
dose= 0.5 ml			$\Sigma P = 2.5$		$\Sigma P = 2$	
Calculate m = $xk - (d)(EP - 0.5)$			-1.38		-1.035	

2.66666667 IU/ml 2.5 IU/ml standard

Figure 16. The calculation of potency of the accelerated stability sample by NIH test after 6 months at 25°C according to the Spearman-Karber method.

Over an 18-month storage period, no trend was observed, and the stability data complied with the specifications.

4. DISCUSSION

Mammalian cell culture was established as a manufacturing technology for the preparation of viral vaccines in the late 1940s. Different animal-derived components are considered essential for either cell growth or as a cell detachment agent, but the use of these products has several disadvantages, such as the possibility of animal-based contaminants. The Serum-free media and animal origin-free trypsin-like enzyme used in the Vero-cell vaccine production significantly reduce the possibility of contaminant transmission to the final product, make the purification process easier, and increase Rabies virus production in the basket type bioreactor.

We found that the cell density had reached its maximum (around 1.8×10^6 cells/mL) on day 5. At an MOI of 0.3 with serum-free medium conditions, cell infection yielded a maximal rabies virus titer of 1.82×10^7 FFU/mL. All supporting activities such as cell banking, cell detachment, static culture, and stirred tank bioreactor culture conditions were investigated.

Bioreactor culture of Vero cells in serum-free media showed that cells could not grow unless the use of surface aeration for dissolved oxygen regulation; culture sparging was detrimental. Cultivating of Vero cells under serum-free conditions share stress induced by sparging. Therefore, the addition of shear protectants such as Pluronic F-68 to serum-free media is often needed to protect cells against shear forces.

We found that after a contact time of 10 - 12 min, cell detachment was completed, and 0.4 g/L soybean trypsin inhibitor (STI) was added in a 3 L spinner and cells were transferred into a 30 L stirred tank bioreactor. It appears that STI at 0.2 - 0.4 g/L inhibits the residual activity of recombinant trypsin without affecting Vero cell growth and attachment in monolayer and microcarrier culture. These findings are compiled from results previously reported by Rourou *et al.* [32].

When rabies vaccines are produced on an industrial scale from the Vero suspension cell culture process, an adult bovine serum is added to the medium for cell growth. We have recently shown that a serum-free medium (VP-SFM AGT and Glutamax I CTS) can be used to grow the Vero cell line. Vero cells grew as a monolayer in T-flasks and on microcarriers as adherent cultures. Both the cells and virus were adapted to VP-SFM conditions after the four passages. The use of VP-SFM + 10% DMSO + 0.1% methylcellulose as a freezing medium free of animal substances of Vero cells was found to be an efficient method for cell banking. These data were supported by Merten *et al.* [33] and Hesse *et al.* [34] who investigated BHK, CHO and MDCK cells cryopreservation in a serum-free freezing medium.

In this study, host cell impurities (host cell DNA and proteins) were found to be compatible with the impurity results previously reported by Trabelski *et al.* [23]. Residual cell DNA complies with the international requirements (≤ 10 ng/dose).

Satisfactory agreement was observed between the ELISA and the NIH test in the determination of the vaccine titer previously reported by Riou *et al.* [26]. Riou *et al.* reported that ELISA can be a good alternative to the NIH potency test and ELISA can detect slight changes in the antigen concentration, enabling the detection of batches that contain insufficient antigen amounts [26]. Gamoh *et al.* [35] also reported that there is a good agreement between the results of the potency test by ELISA and the results of the Glycoprotein potency test.

Also, we evaluated Vero cell-derived inactive rabies vaccine (PV LP 2061 strain) safety, and immunogenicity in sheep, horses and cattle inoculated via the IM route. We found that no horses, sheep, or cattle are given vaccine IM at 3.2 ELISA IU/dose and 3 NIH IU/dose exhibited any clinical sign of disease during the observational period and all developed high VNA titers (1.97 to 10.03 IU/mL) by 3 - 4 WPI. In the current study, the potency of a batch of the final product was 3.2 IU/0.5mL. Also, the lyophilized final product gave 1.93 IU/mL titers in sheep 18 months after the production. The inactivated lyophilised rabies vaccine candidate met the quality requirements of the WHO and provided enough protection in laboratory animal experiments.

In summary, the freeze-dried inactivated rabies vaccine candidate containing human serum albumin, glycine and disaccharide stabilizer at a validated ratio were suitable for long-term storage at 2°C - 8°C. Potency and safety were guaranteed by animal experiments.

In conclusion, in the present work, a complete animal components free process that enables to reach a high cell density of Vero cells grown on Cytodex 1 and a high amount of rabies virus was established in the pilot scale. All supporting activities such as cell banking, cell detachment, static culture, bioreactor culture conditions, purification, formulation, lyophilization and potency of the final product were investigated and found satisfied. This is the first pilot-scale mammalian cell-based viral rabies vaccine production study using microcarrier technology reported in Türkiye. All experiments were carried out using serum-free media and microcarrier technology without porcine-derived trypsin. Soon, it is planned to start clinical trials after obtaining the necessary ethics committee and ministry permissions.

DECLARATIONS

Funding

This work was supported by The Scientific and Technological Research Council of Türkiye (TUBITAK TEYDEB) (Cooperation Projects in the Field of Vaccine Development Grant-Project Number 3150337).

Ethics Declarations

The Animal Local Ethics Committee of Kocak Pharmaceuticals approved all animal experiments in this study (No. 2019-5, 2019-20, 2019-7).

Availability of Data and Material

The key information and data generated and/or analysed during this study were included in this article.

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Author Contribution Statements

E.A.O. directed the project. E.A.O and S.B. designed the project. E.A.O. and S.B. developed and formulated the vaccine candidate for Preclinical trials. E.A.O. and S.B. performed the experiments, and the analytic calculations and analysed the data. E.A.O. wrote the paper with input from all authors. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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