

LISTADO DE ARTE PREVIO CITADO – NO PATENTES

- ATCC Product Sheet Vero (Vero (ATCC® CCL-81™))
- Neuza M. Frazatti-Gallina, Regina M. Mourão-Fuches, Rosana L. Paoli, Maria L.N. Silva, Cosue Miyakib, Elizabeth J.G. Valentini, Isaias Raw, Hisako G. Higashi. Vaccine 23 (2004) 511–517. *Vero-cell rabies vaccine produced using serum-free medium* [Frazatti et al. "Frazatti" (Vaccine, 2004, 23:511-517)]



Product Sheet

Vero (ATCC® CCL-81™)

Please read this FIRST



Storage Temp.
liquid nitrogen
vapor phase



Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: Vero (ATCC® CCL-81™)

American Type Culture Collection
PO Box 1549
Manassas, VA 20108 USA
www.atcc.org

800.638.6597 or 703.365.2700
Fax: 703.365.2750
Email: Tech@atcc.org

Or contact your local distributor

Description

Organism: *Cercopithecus aethiops*

Tissue: kidney

Disease: normal

Age: adult

Morphology: epithelial

Growth Properties: adherent

Cytogenetic Analysis: This is a cell line with the hypodiploid chromosome count. The modal chromosome number was 58 occurring in 66% of cells. In most cells, over 50% of the chromosomes in each cell complement belonged to structurally altered marker chromosomes. Normal A3, A4, B4, and B5 were absent; B2, B3 and B7 were occasionally paired; and B9, C1 and C5 were mostly paired. The rate of cells with higher ploidies was 1.7%. Other chromosomes were mostly present in single copy.

Batch-Specific Information

Refer to the Certificate of Analysis for batch-specific test results.

SAFETY PRECAUTION

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

Unpacking & Storage Instructions

1. Check all containers for leakage or breakage.
2. Remove the frozen cells from the dry ice packaging and immediately place the cells at a temperature below -130°C, preferably in liquid nitrogen vapor, until ready for use.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Transfer the vial contents to a centrifuge tube containing 9.0 mL complete culture medium and spin at approximately 125 x g for 5 to 7 minutes. Discard supernatant.
4. Resuspend the cell pellet with the recommended complete medium and dispense into a 25 cm² culture flask. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).
5. Incubate the culture at 37°C in a suitable incubator. A 5% CO₂ in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).
2. **If the cells are still attached**, aseptically remove all but 5 to 10 mL of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO₂ in air atmosphere until they are ready to be subcultured.
3. **If the cells are not attached**, aseptically remove the entire contents of the flask and centrifuge at



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125 x g for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 mL of this medium and add to 25 cm² flask. Incubate at 37°C in a 5% CO₂ in air atmosphere until cells are ready to be subcultured.



Subculturing Procedure

Volumes are given for a 75 cm² flask. Increase or decrease the amount of dissociation medium needed proportionally for culture vessels of other sizes. Corning® T-75 flasks (catalog #430641) are recommended for subculturing this product.

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:6 is recommended

Medium Renewal: 2 to 3 times per week



Cryopreservation Medium

Complete culture medium described above supplemented with 5% (v/v) DMSO.

Cell culture tested DMSO is available as ATCC® Catalog No. 4-X.



References

References and other information relating to this product are available online at www.atcc.org.



Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

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Vero-cell rabies vaccine produced using serum-free medium

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Abstract

A new rabies vaccine was developed from Vero cells adhered to microcarriers, cultivated in a bioreactor in serum-free medium and infected with the PV/VERO-Paris rabies virus strain. The viral suspensions were concentrated by tangential filtration, purified by chromatography and inactivated with β -propiolactone. In immunogenicity studies performed in mice immunized with three doses of the new vaccine (seven batches) and the commercial Verorab and HDCV, mean titers of neutralizing antibodies of 10.3–34.6, 6.54 and 9.36 IU/ml were found, respectively. The vaccine presented stability during 14 months at 2–8 °C, 30 days at 37 °C and 8 h at 45 °C. The use of serum-free medium facilitated the downstream process leading to residual cellular DNA values <22.8 pg per dose of vaccine in all produced batches. The effective immunogenicity induced in mice by this vaccine, the degree of purity of the product, the high antigen yield and the reduction of the cost of the product due to the virus production and purification processes, makes this technology very important for countries where rabies presents a great public health problem.

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Keywords: Rabies vaccine; Vero cell; Serum-free medium

1. Introduction

Rabies is a worldwide fatal disease and represents a severe public health problem in developing countries. Domestic animals, mainly dogs and cats, or wild animals (bats, monkeys and foxes) are important virus reservoirs and transmit this disease to human and animals of great economical interest, such as cattles, horses and others [1].

The World Health Organization notified that in 1998, in the whole world 32,451 human deaths due to rabies occurred and 7,576,323 people received post-exposure treatment [2]. In Brazil, from 1988 to 1997 there were 452 reported human rabies deaths, with a yearly mean of 342,830 people exposed to rabies suspect animals. The dog was the main transmitter

of the disease (72.1%), followed by the bat (11.1%), cat (4.6%), fox (2%), monkey (1.5%), other animals (1.6%) and in 7.1% the transmitting species was not identified [3]. For post-exposure treatment and immunization of the professionals at risk (pre-exposure scheme) in this country approximately 2,500,000 vaccine doses are used per year, Fuenzalida & Palacios type [4] and cell culture vaccines.

The Butantan Institute started the production of human rabies vaccine in 1957 with the Semple-type [5]. The production of the vaccine in suckling mouse brain (Fuenzalida & Palacios type) was introduced in 1964 and the process was modified in 1987, with the introduction of a filtration to reduce the brain tissue present in the product [6]. This vaccine, although being immunogenic, may cause severe adverse neurological reactions, due to antibodies produced against myelin or other substances of the animal nervous tissue present in the product [7].

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Since the appearance of the first rabies vaccine with Pasteur in 1885, many others were developed using different substrates for virus cultivation (animal brains; avian embryos; primary mammalian cells, human diploid cell or Vero cells), the antigen type and the route of administration [8–18].

Among the vaccines for human use produced in cell cultures, recognized by the WHO, there is the Purified Vero Rabies Vaccine (Verorab) produced in Vero cells infected with rabies virus. These cells were established in Japan from the kidney of a normal adult African green monkey (*Cercopithecus aethiops*) [19]. A cell bank was established for the production of human vaccines in 1979 [10,20]. Extensive research was carried out to evaluate the oncogenic potential of the continuous lines. Vero cells showed to be free of oncogenic properties, not presenting risks to man when used as substrate in the production of vaccines [21–24]. The use of these cells allows also the large-scale production of vaccines using microcarriers and cultures in bioreactors [10,18].

Culture media, such as modified Eagle MEM, Leibovitz and others are used for the cultivation of Vero cells adhered to microcarriers in the production of viral suspensions. Supplementation of these media with fetal bovine serum, human or bovine albumin is necessary for the cellular growth in the bioreactor.

However, the use of serum in the cultures presents some disadvantages, such as the potential to induce hypersensitivity [25,26], the variability of the serum batches and the possible presence of contaminants (bacteria, fungi, mycoplasma, bovine viruses, etc.) and the high cost of a good quality serum [27]. There are some culture media formulated without any human or animal derived components, designed to support the serum-free growth of several cell lines of interest in virus production and biotechnology [28–34].

Data obtained with serum-free VP-SFM medium (GIBCO) showed that its use increases the cellular density and rabies virus replication, decreases the possibility of contamination due to serum and reduces the time required for a production cycle in the bioreactor, thus minimizing the costs of the product [34].

In this paper, we describe a methodology for the production of human rabies vaccine, prepared with rabies virus suspension obtained from infected Vero cells cultivated in a serum-free medium in the bioreactor.

2. Materials and methods

2.1. Cell lines

Cell banks (seed and working) used were made from Vero cells originated from passage 124 of the American Type Cell Collection (VERO-ATCC-CCL81), tested according to WHO requirements for cell lines [35]: the absence of fungi, bacteria, mycoplasmas [36] and other adventitious agents, determination of the karyotype and lack of tumorigenicity [37]. The banks were stored at -180°C .

2.2. Rabies virus

The seed and working banks were prepared with rabies virus suspensions obtained from Vero cells, cultivated in T-flasks (350 cm^2) and infected with fixed PV Pasteur virus (PV/RV/VERO Stock I-Pasteur, Paris). These banks were submitted to control tests such as viral identity, titration in BHK-21 cells [38], the absence of mycoplasma, fungi, bacteria and other adventitious agents [39].

2.3. Culture medium

Serum-free VP-SFM medium (GIBCO—Cat. no. 1168—Invitrogen Corporation) with 4 mM L-glutamine was used for the production of rabies virus in the bioreactor.

2.4. Cell culture system and viral infection

Cell cultures performed initially in 350 cm^2 T-flasks were inoculated into a bioreactor (Celligen-New Brunswick) of 3.7 l working volume, at a proportion of 16 cells per microcarrier (Cy todex 3-Pharmacia) with the initial seeding of $2.5 \times 10^5\text{ cell ml}^{-1}$. After 4 days of cellular cultivation in VP-SFM medium, the cells were infected with PV rabies virus (multiplicity of infection = 0.08).

The harvests of the culture supernatant were carried out 3 days after the virus inoculation and four times at intervals of 24 h. During the whole process, culture conditions were maintain at 60 rpm, pH = 7.15 and 5% of dissolved oxygen. Only the temperature varied from 36.5°C in the cellular growth phase of culture to 34°C after virus inoculation [34].

2.5. Concentration, purification and inactivation

Viral suspensions harvested from the bioreactor after clarification in membranes ($0.45\text{ }\mu\text{m}$) and concentration by tangential filtration (Pellicon-Millipore System, 10 kDa cassette) were purified by ion exchange chromatography (DEAE–Cellulose resin, Sigma) and inactivated with β -propiolactone (1:4000). Samples were taken during the process for quality control tests: checking for the absence of adventitious agents, antigen titration in BHK21 cells, identity, verifying of viral inactivation, potency, protein concentration, detection of residual cellular DNA and SDS–PAGE to determine purity.

2.6. Vaccine preparation

The purified harvests were diluted in phosphate buffered saline solution to present a potency of 8.0 IU (International Units) per dose. After the dilution, 1% of human albumin (Octapharma) and thimerosal (1:10,000) were added. The vaccine batches were packed in boxes with 10 vials of one dose. Samples of these vials were used for potency, adventitious agents, safety, stability and immunogenicity tests.

2.7. Detection of residual cellular DNA

The slot/blot hybridization technique was used to verify the amount of residual Vero cellular DNA. This method is sensitive to detect <15 pg for standard curves using fluorescent probe prepared by random prime labeling and Gene Images CDP-Star detection module (Amersham Life Science RPN-3510) [40–42].

2.8. Potency of the vaccine

The potency of the vaccine was tested according to the NIH (National Institutes of Health) test [43]. Two groups of NIH mice were vaccinated twice, 7 days apart, with dilutions of a reference vaccine (BR-006—Brazilian Institute of Quality Control) and the Vero cell vaccine. Seven days after the last vaccination the animals were intracerebrally challenged with CVS (Challenge Virus Strain) (20 LD₅₀ per 0.03 ml). The mice were observed for 14 days. The ED₅₀ (50% efficient dose) were calculated by the Spearman Karber method and the results expressed in IU per dose.

2.9. Immunogenicity test

Groups of 10 mice were vaccinated intraperitoneally with three doses of 0.25 ml of Verorab (PO881-2) and HDCV (PO537-4), both from Pasteur Mérieux Connaught, and the rabies vaccine in serum-free medium, on Days 0, 7 and 28 (human pre-exposure scheme). Blood samples were taken on Days 7, 21 and 56 to evaluate the level of neutralizing antibodies by the RFFIT test (Rapid Fluorescent Focus Inhibition Test) [38]. Titers higher than or equal to 0.5 IU/ml were considered satisfactory. The animals were challenged intracerebrally with 20 LD₅₀/0.03 ml of CVS.

2.10. Stability tests

Vaccine vials were incubated at temperatures of 2–8, 37 and 45 °C and samples were taken for potency tests at different time intervals: at 2–8 °C after 06–12–14–18 months of incubation; at 37 and 45 °C, on Days 05–10–30 and 2–4–8–12 h, respectively.

3. Results

3.1. Vero cell working bank

A Vero cell working bank, at passage 146, free of adventitious agents, fungi, bacteria and mycoplasmas was utilized for obtention of rabies virus in the bioreactor. Nude athymic mice inoculated with the cells of this bank did not present lung tumors or metastases after 21 days of observation. Cells of the bank and proceeding from the bioreactor presented a distribution frequency of the chromosomes of $2n = 58$.

3.2. Rabies virus working bank

The working bank was formed by a suspension of fixed virus (PV) adapted in Vero cells (Pasteur Institute, Paris) obtained after three passages at Butantan Institute. This bank, with an infective viral titer of $10^{4.98}$ fluorescent focus-forming dose (FFD₅₀/0.05 ml) presented satisfactory results in the control tests.

3.3. Virus production in the bioreactor

Seven batches of virus suspensions using serum-free VP-SFM medium were produced in the bioreactor (16 l per cycle) with a mean virus titer of $10^{4.0}$ FFD₅₀/0.05 ml.

3.4. Vaccine batch preparation

Seven rabies vaccine batches were prepared with concentrated viral suspensions that presented a mean viral titer of $10^{4.8}$ FFD₅₀/0.05 ml. This titer remained constant after purification in DEAE–Cellulose (Sigma). After dilution, the mean viral titer was $10^{3.7}$ FFD₅₀/0.05 ml. These batches presented satisfactory results in quality control tests required by the WHO for vaccines for human use. The values obtained in the potency, viral titration and residual cellular DNA tests are shown in Table 1. The degree of purity of the batches was demonstrated in SDS–PAGE (12.5% polyacrylamide gel) and the Verorab vaccine was used as control (Fig. 1).

3.5. Immunogenicity

All mice immunized with three vaccine doses produced in serum-free medium presented satisfactory neutralizing antibody titers (Table 2) and survived when challenged with CVS virus after the last dose of vaccine.

Neutralizing antibody titers obtained in mice immunized with serum-free rabies vaccine, when compared with HDCV and Verorab, after the administration of the first dose were

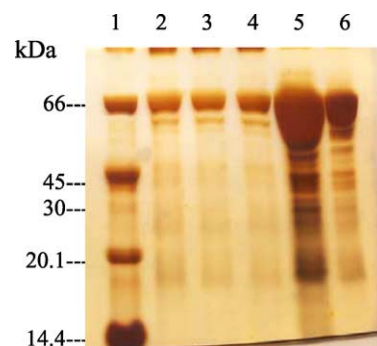


Fig. 1. SDS–PAGE of rabies vaccine produced in Vero cells. (1) Protein markers; (2, 3 and 4) rabies vaccine in serum free medium, batches 7, 8 and 9, respectively; (5) human serum albumin; (6) Verorab PO881-2 (Pasteur Mérieux Connaught).

Table 1

Results of potency tests, viral titer and residual cellular DNA values of rabies vaccine batches produced in Vero cells cultivated in serum-free medium

Tests	Vaccine batches							Geometric mean
	01	02	03	04	05	06	07	
Potency (IU per dose)	11.0	13.6	9.4	7.8	10.4	5.8	7.1	8.98 ± 2.45
Viral titer (FFD ₅₀ /0.05 ml)	10 ^{4.60}	10 ^{4.50}	10 ^{4.10}	10 ^{3.76}	10 ^{4.20}	10 ^{3.20}	10 ^{3.20}	10 ^{3.9±0.57}
Residual DNA (pg per dose)	<15	<15	<15	<15	<15	22.76	22.78	

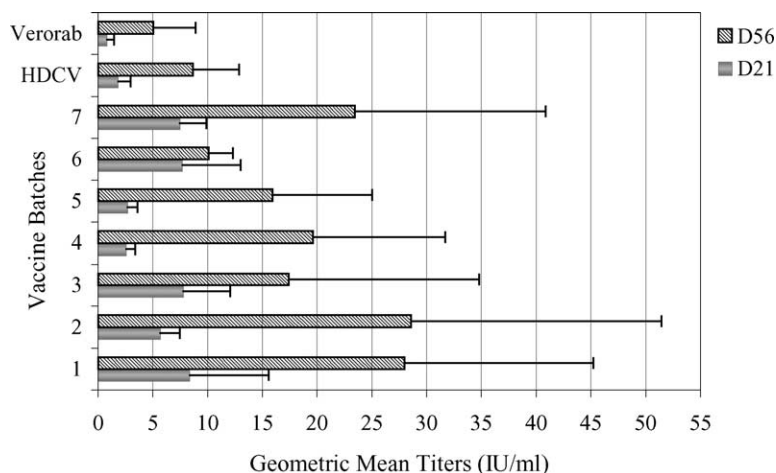


Fig. 2. Responses of neutralizing antibodies (IU/ml) in groups of mice (seven per group) immunized (i.p.) with three doses (D₀, D₇ and D₂₈) of 0.5 ml of the batches produced in serum-free medium, HDCV and Verorab vaccines. Blood samples were taken on Days 21 and 56 after the beginning of immunization to evaluate the second and third vaccine doses, respectively. Geometric means of antibodies were calculated, using the individual values obtained for each animal.

similar for all tested vaccines (data not shown). The values found for the second and third doses are shown in Fig. 2.

3.6. Stability

During 14 months of storage at a temperature of 2–8 °C all tested batches showed a satisfactory potency (≥ 2.5 IU per dose). At 37 °C, this occurred until 30 days and at 45 °C only 01 batch presented a potency lower than the required after 12 h (Fig. 3).

4. Discussion

Large-scale production of rabies vaccine in cell culture, in different systems (cell suspensions or cells adhered to microcarriers cultivated in bioreactor or roller bottles) requires a culture medium supplemented with fetal bovine serum or

albumin (human or bovine) for cell growth. The use of these components presents some disadvantages, such as potential to induce hypersensitivity [25,26], serum batch variability, possibility of contaminants, such as fungi, bacteria, mycoplasmas, bovine viruses, agent responsible for bovine spongiform encephalopathy (BSE) and the Creutzfeld-Jacob disease in humans [44,45].

The development of serum-free culture media reduced these problems, facilitating the downstream process required for the obtention of the final purity [27]. There are different serum-free media for growth of several cell types (BHK-21, BSR, VERO, CHO, MDCK, and others). The MDSS₂ medium (Axcell Biotechnologies) is one of the most used in vaccine production [28,29,32].

The serum-free VP-SFM medium, according to the manufacturer, was developed for Vero, BHK-21 and CHO cell growth. The use of this medium for Vero cells adhered to microcarriers, infected with PV rabies virus and cultivated in a Celligen bioreactor (New Brunswick), showed to be very

Table 2

Titers of neutralizing antibodies (IU/ml) obtained in groups of seven mice immunized with rabies vaccine produced in serum-free medium

Blood taken	Days	Vaccine batches						
		01	02	03	04	05	06	07
First	7	0.21 (0.1–0.5)	0.27 (0.1–0.9)	0.22 (0.2–0.4)	0.43 (0.1–0.8)	0.63 (0.4–0.8)	0.12 (0.1–0.2)	0.14 (0.1–0.3)
Second	21	8.37 (4.7–24.6)	5.67 (3.5–7.6)	7.77 (4.0–14.4)	2.56 (1.6–4.1)	2.70 (1.1–3.8)	7.69 (3.5–17.1)	7.47 (4.9–11.3)
Third	56	27.97 (12.3–56.6)	28.58 (13.2–69.7)	17.41 (9.3–42.9)	19.62 (4.8–40.0)	15.93 (9.3–34.8)	10.10 (8.7–14.1)	23.44 (11.5–60.6)

Mean and range of the antibody titers obtained in vaccinated mice.

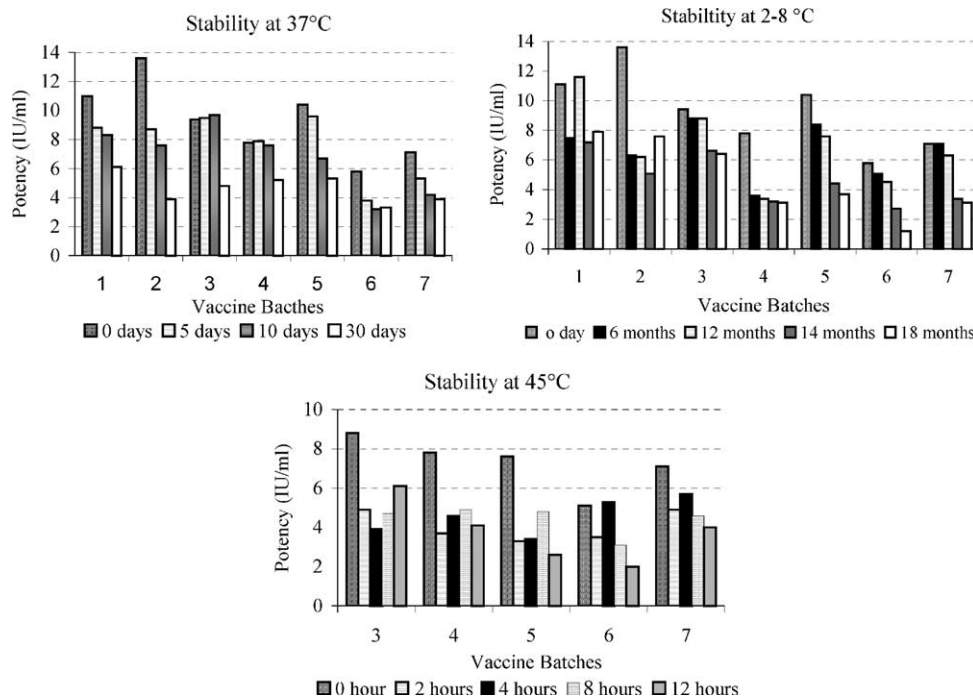


Fig. 3. Potency of the rabies vaccine batches produced in Vero cells cultivated in serum-free medium, and stored at different temperatures. The values in the figure are in international units of antigen per dose and indicate the potency of each vaccine batch stored at temperatures of 2–8, 37 and 45 °C at different time intervals. The tests for the determination of the potency were performed in NIH mice, and the reference vaccine was BR-006 (INCQS). These results correspond to the geometric mean of two tests performed with each sample.

efficient to increase the cell density and the viral multiplication [34]. Thus, this system demonstrated to be an excellent alternative for the obtention of viral suspensions for the preparation of rabies vaccine.

The potency of the vaccine batches prepared in VP-SFM medium showed to be much higher (5.8–13.6 IU per dose) than the minimum required for a human rabies vaccine (2.5 IU per dose) [39]. Correlation between the viral titers of suspensions utilized to prepare the batches and the results of potency was observed ($r = 0.87$) (Table 1).

In stability tests, all batches of rabies vaccine in serum-free medium maintained the minimum potency required at 37 °C after 30 days. The batches stored during 14 months at 2–8 °C presented potency ≥ 2.5 IU per dose. The same occurred after 12 h of storage at 45 °C. It is a very important factor in tropical countries such as Brazil with regions reaching approximately 41 °C. The increase in potency observed in some tests after a period of vaccine storage can be explained by the variability of NIH test [46].

Residual cellular DNA of the vaccines produced in Vero cells is one of the great concerns in the production of this immunobiological. The WHO recommends values ≤ 100 pg per dose. With the use of serum-free medium the downstream process was facilitated (one purification process by ion exchange chromatography) and the levels of residual cellular DNA obtained were lower than this value in all produced batches. Five of them presented values of 15 pg per dose, corresponding to the sensitivity

of the test used for detection and two others 22.8 pg per dose.

The immunogenicity test of the vaccine performed in mice with a pre-exposure scheme (D_0 , D_7 and D_{28}) presented excellent results when compared with the Verorab and HDCV vaccines. The statistical analysis demonstrated that there is correlation between the potency and the levels of neutralizing antibodies of the mice immunized ($r = 0.69$). However, independent of the potency value, all the animals immunized presented neutralizing antibodies titers higher than the minimum required after the second dose of vaccine.

The good results obtained with the rabies virus replication in the bioreactor and immunogenicity of this new vaccine produced in serum-free medium, permits the reduction of the costs of the process, in spite of the high price of serum-free media.

Thus, it is possible to obtain a high quality product with similar costs to the process that uses media with fetal bovine serum or bovine albumin for the production of rabies vaccine in Vero cells.

Acknowledgement

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References

- [1] Rupprecht CE, Hanlon CA, Hemachudha T. Rabies re-examined. *Lancet Infect Dis* 2002;2(6):327–43.
- [2] World Health Organization. World survey of rabies number 34 (for the year 1998). Geneva: WHO; 2000. http://www.who.int/emc/diseases/zoo/wsr98/HTML_version/wsr_98_index.html, accessed: August 2002.
- [3] Brazil Health Ministry. National Center of Epidemiology. Zoonosis and Poisonous Animals Control. Annual Report 1997. Brasília; 1998.
- [4] Díaz AM. Suckling-mouse brain vaccine. In: Meslin F-X, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*. Geneva: WHO; 1996. p. 243–50.
- [5] Seligmann Jr EB. Semple-type vaccine. In: Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*. Geneva: WHO; 1973. p. 192–8.
- [6] Frazatti-Gallina NM, Mourão-Fuches RM, Mendonça RMZ, Fang FLW, Valentini EJG. Stability study of rabies vaccine type Fuenzalida & Palacios after filtration process. *Arq Biol Technol* 1995;38(2):429–39.
- [7] Meslin F-X, Kaplan MM. General considerations in the production and use of brain-tissue and purified chicken-embryo rabies vaccine for human use. In: Meslin F-X, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*. Geneva: WHO; 1996. p. 223–33.
- [8] Wiktor TJ, Plotkin SA, Koprowski H. Development and clinical trials of the new human rabies vaccine of tissue culture (human diploid cell) origin. *Dev Biol Stand* 1978;40:3–9.
- [9] Burgoyne GH, Kajiya KD, Brown DW, Mitchel JR. Rhesus diploid rabies vaccine (adsorbed): a new rabies vaccine using FRhL-2 cells. *J Infect Dis* 1985;152:204–10.
- [10] Montagnon BJ, Fournier P, Vicent-Falquet JC. A new rabies vaccine for human use usage: preliminary report. In: Kurwert E, Mérieux C, Koprowski H, Bögel K, editors. *Rabies in the tropics*. Berlin: Springer-Verlag; 1985. p. 138–43.
- [11] Flamand A, Coulon P, Lafay F, Tuffereau R. Avirulent mutants of rabies virus and their use as live vaccine. *Trends Microbiol* 1993;1:317–20.
- [12] Mc Garvey PB, Hammond J, Dienelt MM, Hooper DC, Michaels FH, Fu ZF, Dietzschold B, Koprowski H. Expression of the rabies virus glycoprotein in transgenic tomatoes. *Biol Technol* 1995;13:1484–7.
- [13] Glück R. Purified duck-embryo vaccine for humans. In: Meslin F-X, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*. Geneva: WHO; 1996. p. 253–9.
- [14] Hanlon CA, Niezgoda M, Shankar V, Niu HS, Koprowski H, Rupprecht CE. A recombinant vaccinia-rabies virus in immunocompromised host: oral innocuity, progressive parenteral infection, and therapeutics. *Vaccine* 1997;15:140–8.
- [15] Pérez O, Paolazzi CC. Production methods for rabies vaccine. *J Ind Microbiol Biotechnol* 1997;18:340–7.
- [16] Kammer AR, Ertl HC. Rabies vaccines: from the past to the 21st century. *Hybrid Hybridom* 2002;21(2):123–7.
- [17] Morimoto K, McGettigan JP, Foley HD, Hooper DC, Dietzschold B, Schnell MJ. Genetic engineering of live rabies vaccines. *Vaccine* 2001;19(25–26):3543–51.
- [18] Mendonça RZ, Ioshimoto LM, Mendonça RMZ, De Franco M, Valentini EJG, Beçak W, Raw I, Pereira CA. Preparation of human rabies vaccine in VERO cell cultures using a microcarrier system. *Bra J Med Biol Res* 1993;26(12):1305–17.
- [19] American Type Culture Collection. Cell lines and Hybridomas. Vero Cell CCL-81. Available from <http://www.atcc.org/SearchCatalogs/CellBiologys.cfm>, accessed: July 2004.
- [20] Montagnon BJ, Fanjet B, Nicolas AJ. The large-scale cultivation of Vero cells in microcarrier culture for virus vaccine production: preliminary results for killed poliovirus vaccine. *Dev Biol Stand* 1981;47:55–64.
- [21] Furesz J, Fanok A, Contreras G, Becker B. Tumorigenicity testing of various cell line substrates for production of biologicals. *Dev Biol Stand* 1989;70:233–43.
- [22] Vincent-Falquet JC, Peyron L, Souvras M, Moulin JC, Tektoff J, Patet J. Qualification of working cell banks for the Vero cell line to produce licensed human vaccines. *Dev Biol Stand* 1989;70:153–6.
- [23] Horaud F. Absence of viral sequences in the WHO-Vero cell bank: a collaborative study. *Dev Biol Stand* 1992;76:43–6.
- [24] Montagnon BJ, Vincent-Falquet JC, Saluzzo JF. Experience with Vero cells at Pasteur Mérieux Connaught. *Dev Biol Stand* 1999;98:137–40.
- [25] Fishbein DB, Yenne KM, Dreesen DW, Teplis CF, Mehta N, Briggs DJ. Risk factors for systemic hypersensitivity reactions after booster vaccinations with human diploid cell rabies vaccine: a nationwide prospective study. *Vaccine* 1993;11(14):1390–4.
- [26] Swanson MC, Rosanoff E, Gurwith M, Deitch M, Schnurrenberger P, Reed CE. IgE and IgG antibodies to beta-propiolactone and human serum albumin associated with urticarial reactions to rabies vaccine. *J Infect Dis* 1987;155(5):909–13.
- [27] Froud SJ. The development, benefits and disadvantages of serum-free media. *Dev Biol Stand* 1999;99:157–66.
- [28] Merten OW, Kierulff JV, Castignolles N, Perrin P. Evaluation of the new serum-free medium (MDSS2) for the production of the different biologicals: use of various cell lines. *Cytotechnology* 1994;14(1):47–59.
- [29] Perrin P, Madhusudana S, Gontier-Jallet C, Petres S, Tordo N, Merten OW. 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;13:1244–50.
- [30] Price PJ, Evege EK. Serum-free medium without animal components for virus production. *Focus* 1997;19:67–9.
- [31] Voeten JT, Brands R, Palache AM, Van Scharrenburg GJ, Rimmelzwaam GF, Osterhaus A, Claas EC. Characterization of high-growth reassortant influenza A viruses generated in MDCK cells cultured in serum-free medium. *Vaccine* 1999;17(15–16):1942–50.
- [32] Merten OW, Manuguerra JC, Hannoun C, vanderWerf S. Production of influenza virus in serum-free mammalian cells cultures. *Dev Biol Stand* 1999;98:23–37.
- [33] Butler M, Burgener A, Patrick M, Berry M, Moffatt D, Huzel N, Barnabe N, Coombs K. Application of a serum-free medium for the growth of Vero cells and the production of reovirus. *Biotechnol Prog* 2000;16(5):854–8.
- [34] Frazatti-Gallina NM, Paoli RL, Mourão-Fuches RM, Jorge SAC, Pereira CA. Higher production of rabies virus in serum-free medium cell cultures on microcarriers. *J Biotechnol* 2001;92:67–72.
- [35] WHO. Requirements for continuous cell lines used for biological substances. *WHO Tech Rep Ser* 1987;745:99–115.
- [36] Miyaki C, Pral MM, Gallina NMF, Rizzo E. Mycoplasma as a contaminant of cell cultures maintained in laboratories of private and official institutions. *Rev Saúde Publ, S Paulo* 1989;23(1):39–44.
- [37] Assaf SLMR, Yokomizo AY, Serapicos EO, Frazatti-Gallina NM, Machado LD. Tumorigenicity testing of VERO cells. *Arq Biol Technol* 1997;40(3):596–600.
- [38] Smith JS, Yager PA, Baer GM. A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralizing antibody. In: Meslin F-X, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*. Geneva: World Health Organization; 1996. p. 181–9.
- [39] WHO. Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines, WHO Tech Rep Ser 198;760:167–89.
- [40] Yoneyama T, Zebai Q, Miyamura T. A sensitive method for the detection of residual cell DNA in a recombinant hepatitis B vaccine prepared from Chinese hamster ovary cells. *J Biol Stand* 1989;17:371–5.

- [41] Davis LG, Mark DD, Battey JF. Basics methods in molecular biology. New York: Elsevier Science; 1986.
- [42] Ausubel FM. Current protocols in molecular biology. Molecular biology. New York: Greene Publishing Associates and Wiley interscience; 1989.
- [43] Wilbur LA, Aubert MFA. The NIH test for potency. In: Meslin F-X, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies. Geneva: WHO; 1996. p. 360–8.
- [44] Brown P, Will RG, Bradley R, Asher DM, Detwiler L. Bovine spongiform encephalopathy and Creutzfeld-Jacob disease: background, evolution and current concerns. *Emerg Infect Dis* 2001;7(1):6–16.
- [45] Public health service recommendations for the use of vaccines manufactured with bovine-derived materials. The Center of Biologicals and Evaluation and Research (CBER) US Food and Droog. *MMWR Morb Mortal Wkly Rep* 2000;49(50):1137–8.
- [46] Barth R, Diderrich G, Weinmann E. NIH test, a problematic method for testing potency of inactivated rabies vaccine. *Vaccine* 1988;6:369–77.