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RABIES VIRUS IN McCOY CELL LINE.
Part I — Cytophatic effect and replication*

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ABSTRACT: The cytopathic effect caused by rabies virus is not easy to detect, although the McCoy cell line showed high sensitivity to this virus.

The titre increased at each new passage and the ERA strain reached titre higher than $10^{8.0}$ LD_{50}/ 0,03 ml after eight successive passages only.

The kinetics of infection showed that 48 hours after inoculation, the cell reaches the peak of infecton and the celular proteins synthesis is inhibited.

The data are reproducible suggesting that this cell line could be used for diagnostic purposes and for virus mass production.

DESCRIPTORS: *McCoy cell line. Cytopathic Effect (CPE). Rabies virus. ERA strain. Viral mass production.*

INTRODUCTION

Rabies virus propagations using the replication technique in cell systems (other than from nervous origin) is not established with success: the evidence of cytopathic effect (CPE) and its association with virus replication is also unclear according to Kissling, Atanasiu *et alii*, Wiktor *et alii*.[2,15,22]

In 1972, Wiktor and Clark[23] studied the chronic infection of rabies virus in mammalian and reptile cell cultures and no CPE was observed. These authors concluded that interferon produced was responsible for the persistent infection in these cells hiding the occurrence of CPE.

With the aim of producing anti-rabies vaccine for human use Atanasiu *et alii*, Fernandes *et alii*, and Montagnon *et alii* [3,8,19] used several kinds of cell lines and after about 20 - 30 passages of the virus in each cell line used, obtained primary culture of calf foetus, human diploid cell (WI - 38) and more recently also the cell line from green monkey (VERO). Although no cell alteration was observed, maybe the WI - 38 showed some signs of alternation, but not significant or typical of cytopathic effect.

Other autors, Kawai *et alii*; Kawai *et al.*[12,13], used a series of methods to obtain CPE in the BHK-21, clone 13 cell, such as: variation of incubator temperature, irradiation of cells, interference with DI particles, etc. In 1985, Honda *et alii*[10] used cells named Sym-1, clone 04, of human neuroblastom infected with rabies virus HEP (Flury strain), and proved that the virus replication produces large amounts of interferon maintaining the persistent infection. With the use of anti-interferon serum, the replication was stimulated with the presence of CPE.

Persistent infection with rabdovirus has been studied in great detail and over long periods by Holland *et alii*[11]. The presence of DI particles has been observed in vesicular stomatites and rabies virus by Villarreal *et al.* and by Youngner *et al.*[20,24]

During the revision of specialized literature it was verified that there was no systematic evidence of persistent presence of CPE as a result of replication of rabies virus.

In 1982 and 1987 Nogueira[17,18] showed the sensitivity of the McCoy cell line to rabies virus, with definitive and constant presence of CPE when rabies virus was

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inoculated. Besides, it was referred the adaptation of the rabies virus in these cells to be easy.

This paper has the aim to show the replication of rabies virus in McCoy cell line, the persistent CPE presence and the maintenance of the immunochemistry characteristic of the virus in the subsequent passages. This finding can be potentially a method to be used in the rapid diagnosis of infection caused by this virus. Titration of the antigen and serum neutralization reactions can be performed with this cell line.

MATERIALS AND METHODS

Cell Culture

The cell culture used was the McCoy cell line obtained from American Type Culture Collection (ATCC) catalogued as # 1696.[1]

For the assay with rabies virus it was cultivated in Eagle's medium supplied by 2% of foetal calf serum (FCS).

Virus

The ERA strain (vaccine) from Biovet Laboratory was used. The titre was $10^{4.5}$ LD_{50}/0.03 ml obtained from mice brains. The end point was calculated by the Reed-Muench method.[16]

The infection mock medium and the infected cells were harvested, and the samples were used for titration and part of them was frozen to -20°C.

Mice

Swiss-Webster mice were used for titration purposes.

Kinetics of Virus Replication

Mult-well plates, Limbro-Flow, with 24 wells were used. Briefly reported, cell monolayers were split in concentration of $4.2 \times 10^{5.0}$ cells/ml. Twenty-four hours before, the cells grew in the growth medium (Eagle's medium + 5% FCS).

Afterwards, the medium was removed, the cells washed with Buffer Balanced Saline (BBS) and the virus from the 6th passage, which titre, $10^{6.2}$ LD_{50}/0.03 ml, was added to each well.The adsorption was performed during 30 minutes at 33°C temperature in the incubator and each well completed with 1.5 ml by maintenance medium (Eagle's medium + 2.0% FCS) and again incubated at 33°C temperature. At intervals of 24, 48, 72 and 96 hours, the cells were removed, stained for immunofluorescence antibody reaction (FA), and observed in epifluorescence microscope (IM-35 Zeiss) objetive 40. Photos were taken and the film used was the Ilford 400 ASA, automatic exhibition.

Kinetics of Inhibition of Protein Cell's Synthesis

A radioactive label (Leucine labelled with Sulphur) Leu-^{35}S was used for incorporation in cells' synthesis.

Following that, $4.2 \times 10^{5.0}$ cells/ml were put in each of the multi-well plates (24 wells) 24 hours before. The next day the cells number was $1.3 \times 10^{6.0}$ cells/ml. The M.O.I. was 0.5. Radiation pulses were given during 30 minutes in intervals of 24, 48, 73 and 96 hours. Each sample was harvested after the pulses, respectively, and was worked for cpm determination in beta-Beckman Scintillation Apparatus. The process was: a) removing the sample, b) adding litic buffer, c) filtering in HA membrane through vacuum for macromolecules retation, d) fixing with Trichloro acetic acid (TCA), e) collecting the membrane in the scintillation vials and f) adding 3.0 ml of scintillation liquid (POP-POPOP), as described previously by Wagner *et al.*[21]

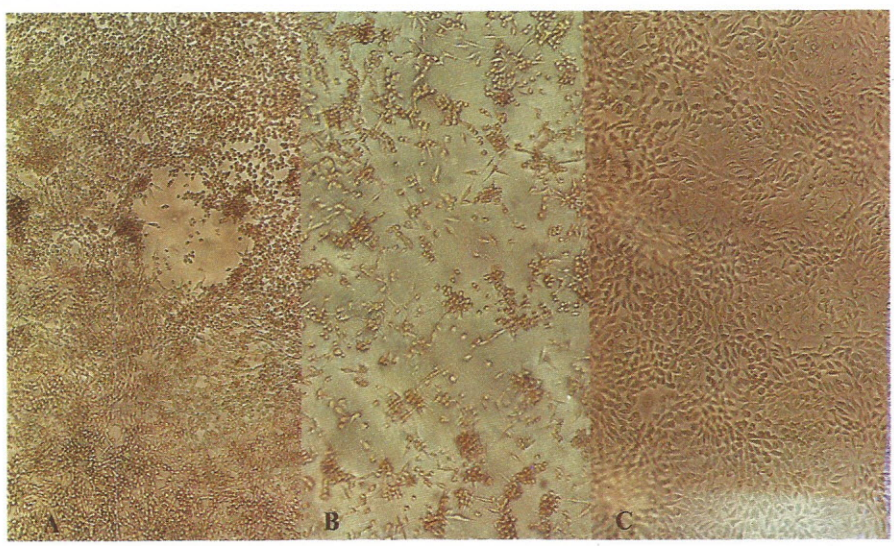

FIGURE 1:
Cytopathic Effect in McCoy Cell Observed by Photomicrography

System: A and B-McCoy cell infected; C - Control (optic microscopy; objective 6.3 , IM 35-Zeiss microscope). The cytopathic effect begins in individualized cells that suffer morfological alterations sequentially. At first the cells become like fibroblasts (oblong cells), then they detach from the monolayer making a litlle hole, the virus is released and infects the cells around (fig. 1A). After the total cellular lise the cells detach from the monolayer and become round cells (fig. 1B). The McCoy cell line is an uniform monolayer with polyhedral cells and epithelioid aspect (fig. 1C).

Electron Microscopy

The brains of paralytic mice were fixed in 2% of glutaraldehyde 0.1M phosphate buffer, pH=7.2 for two hours at room temperature and washed twice in phosphate buffer. Then they were kept overnight in uranyl acetate at 4°C and washed in distilled water once. The brains were post-fixed in osmic acid 1%, stained with uranyl acetate and treated with Polylite T.200 and 1200 overnight before polymerization. Sections were prepared using Poteblum MT 1 microtome, collected on 300 mesh grids and stained again with uranyl acetate and Reinold's stain solution. The sections were studied in a Phillips EM 400 T electron microscope.

RESULTS

The titres were calculated according to the method of Reed-Muench[16], starting with one vaccine (ERA strain), which titre, $10^{4.5}$ LD_{50}/0.03 ml, was imoculated intra-cerebrally in 5-to-9-day-old baby mice.

This strain of virus inoculated in McCoy cell line didn't increase its titre at first and second successive passages. Already in the third passage the titre increased significantly and improved in the 7th passage, which titre was $10^{8.0}$ LD_{50}/0.03 ml, also obtained in mice brains (table 2, figure 2).

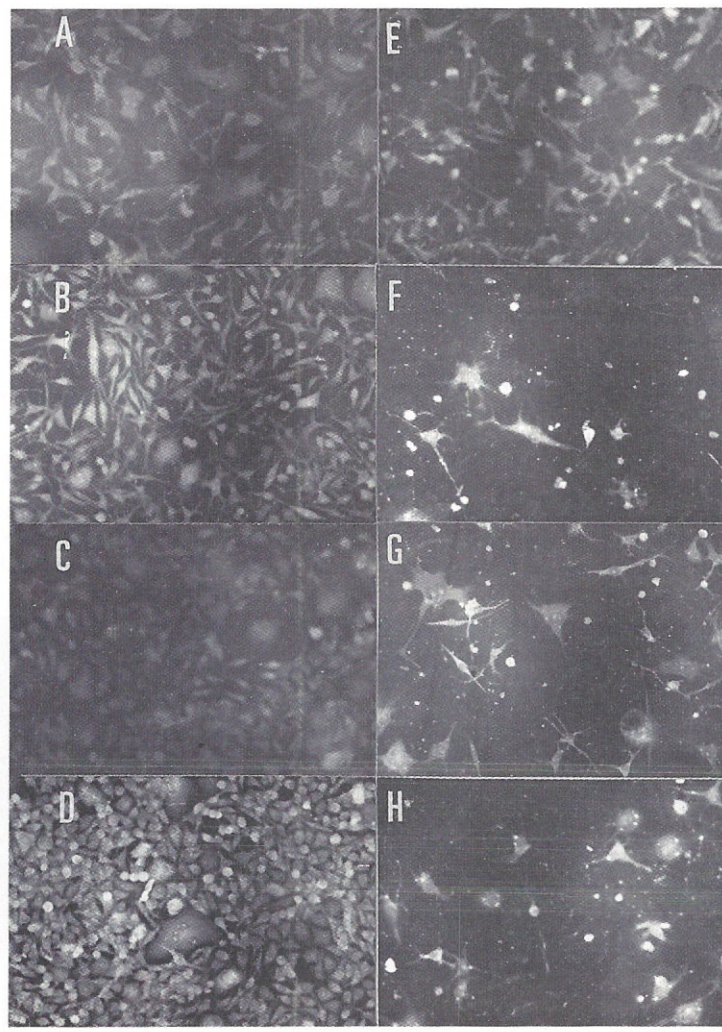

FIGURE 2
Kinectics of Viral Infection

Seventh passage of rabies virus, ERA strain, in McCoy cell line stained with direct immunofluorescent reaction (FA). A, B, C and D are CONTROL CELLS with 24, 48, 72 and 98 hours. A - uncompleted monolayer; B - semi confluent cells; E, F, G and H - INFECTED CELLS with rabies virus with 24, 48, 72 and 98 hours (titre = $10^{8.0}$ LD_{50}/0.03 ml)

Table I shows morphologic alterations. The cytopathic effect (CPE) with one to four plus (+ to ++++) was observed in these cells until the 7th day of observation, after the inoculation of ERA strain of rabies virus, and until the 6th subsequent passage.

It was observed that the CPE began in the first 48 hours (second day) after virus inoculation and became more evident (++) in the first passage, only on the 5th day of observation and increased on the 6th and 7th days. In the 3rd passage it was verified that CPE was evident (++) on the 4th day after inoculation. In the 5th and 6th subsequent passages the CPE was evident on the second day after virus inoculation. Cultures showed CPE (++) already improved to 3 or 4 plus, with about 75% to 100% of the cells alterated. The altered morphology by CPE was observed on the 3rd and 4th days of observation. It is important to note that in all these passages the controls of normal cells didn't show any alteration of CPE or toxic degeneration, as it can be observed in figure 1C.

The cytopathic effect is described in figures 1, 2 and 4.

According to obtained evidences it can be assumed that the McCoy cellular system shows high sensivity to the replication of rabies virus. It can also be assumed that the virus replication in these cells is followed by morphologic alteration, which is easily observed in optic microscopy.

The first kinetics results show the immunofluorescence antibody reaction (FA) as can be seen in figure 2 and the respective controls, with 24, 48, 72 and 96 hours after virus inoculation. In figure 2, A, B, C and D are control cells and E, F, G and H are infected ones. Cellular alteration was clear and could easily be seen. The samples showed improved CPE. The titre of each sample harvest in these intervals is shown in figure 3, where data was plotted showing the increase of the virus titre 96 hours after virus inoculation.

It was observed that in the first 12 hours after the virus inoculation a decrease of the size of McCoy cells occurs when compared to the peak of infection (in 48 hours after virus inoculation). It was observed that the cells' size seemed to increase when compared to the respective control, as can be confirmed in figure 1, suggesting that the cellular permeability increases until the cellular lise.

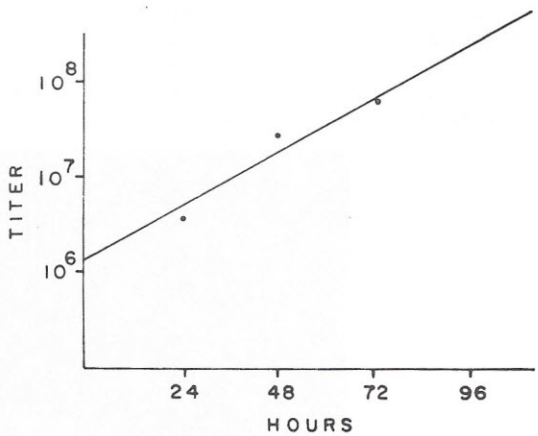

FIGURE 3
Plot of the titre obtained (log of dilution) from the each point of the kinetics.

As observed in figure 3, the titre of the virus replication in this passage was $10^{8.0}$ LD_{50}/0.03 ml. Figure 4 shows a photomicrography (optic microscopy) of an immunofluorescence reaction of the McCoy cells with the rabies virus (ERA strain) and the respective control cells.

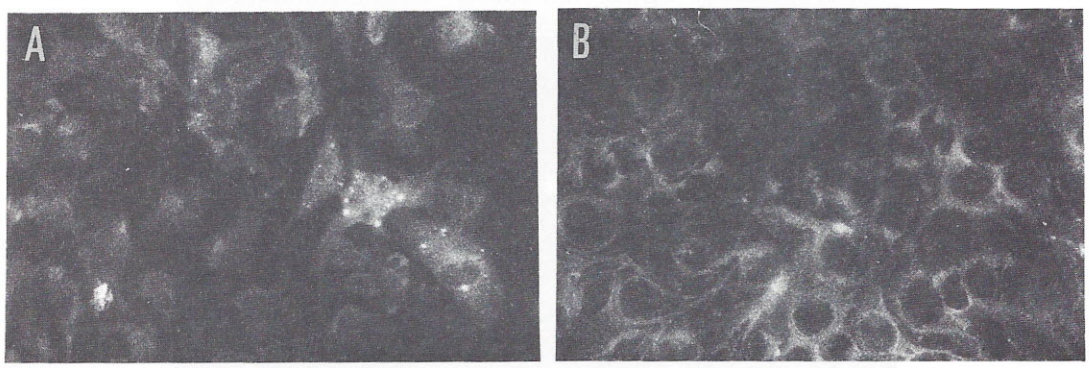

FIGURE 4
Photomicrography of McCoy Cells Infected with Rabies Virus.

ERA strain (A) and control cell (B), stained by immunofluorescence reaction, with imersion oil and objective 100x. The infected cell develops a star-like aspect (fig. 4A) and seems to be large than the control cells, not infected (fig. 4B).

The second kinetics results show the peak of inhibition of protein cells' synthesis (% of control) that occurs 48 hours after virus inoculation, as showns in figure 5.

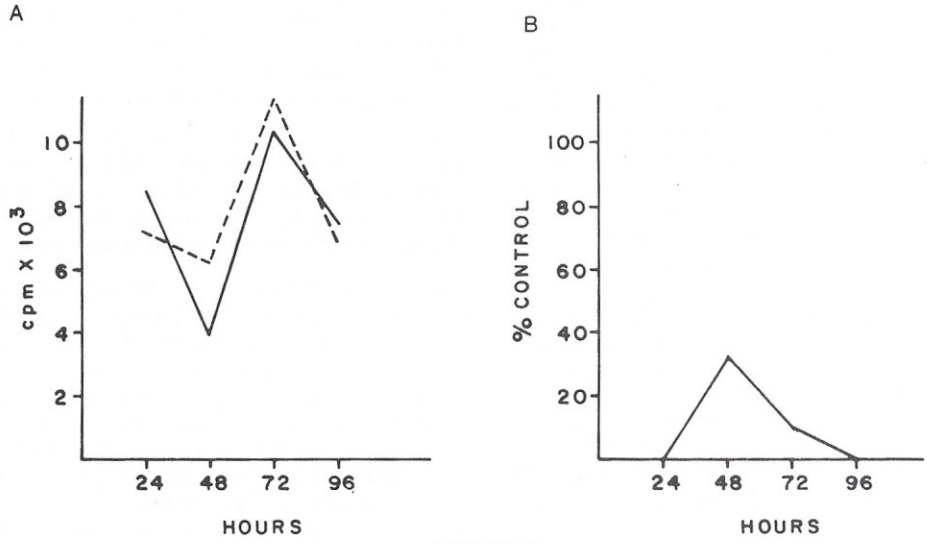

FIGURE 5
Kinectics of Inhibition of Protein cell's Syntesis.

Using radioactive label (Leu-^{35}S). A - cpm counting (_ _ _) infected cells and (—) control cells. B - The % of control, showing the inhibition of protein of cellular synthesis.

Figure 1 shows three photomicrographies in optic microscopy of McCoy cells infected with rabies virus, ERA strain (1A and 1B) and the control (1C), Observe the clear CPE in the cells caused by rabies virus.

The 7th passage of the ERA virus in McCoy cells was inoculated again, in mice brains, when the animals showed signs of infection (paralythic symptoms). The brains were harvested and included in glutaraldehyde 2% and processed for electron microscopy. In figure 6 it is possible to observe infected neurons by numerous particles with typical rabies virus morphology (Rabdovirus).

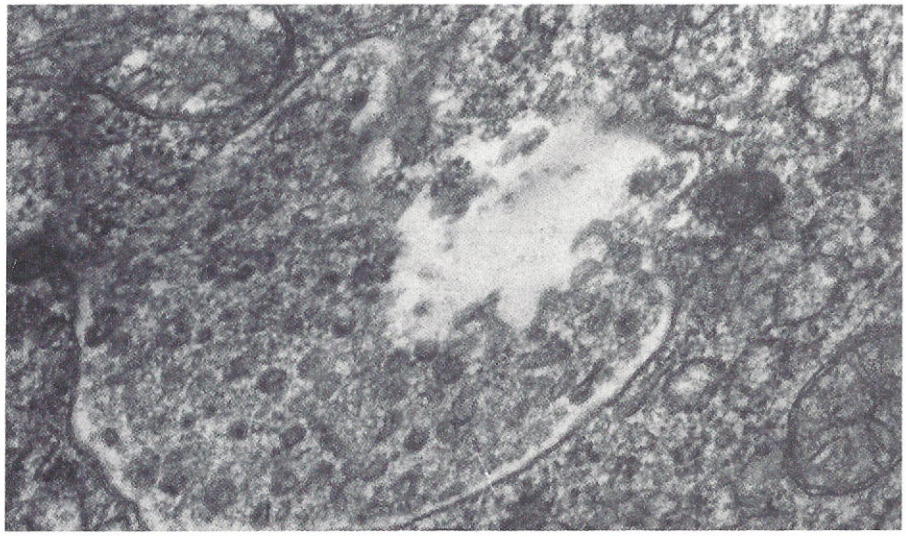

FIGURE 6

Electron Micrography of a Section from an Infected Brain Mice Inoculated with the 7th Passage of Rabies Virus on the McCoy Cell Line.

DISCUSSION

There is no doubt about the affirmation and demonstration that the continuous McCoy cell line, when inoculated with rabies virus "vaccine" or "street virus" shows cytopathic effects in normal conditions of tissue culture, not described until the present moment, in the specialized literature. Although many works have showed the persistent or chronic infection [9,10], the occurence of CPE in cells, such as in WI-38 was only obtained around the 17th seried passages.[22] On the other hand, the results of Kawai *et al.* [14] in 1982, where it was assumed that CPE was obtained, cannot be compared with the results presented here because those authors used artificial and different conditions, such as the elevation of temperature incubation of the used system from 34-36°C to 39-40°C. Yet, it was mentioned that a decrease of CPE could be obtained when defective interferent particles virus (DI) were inoculated in the used culture. Wiktor *et alii*[22] preferred to atribute the absence of CPE to the production of endogen interferon. It is interesting to note that Fernandes, 1959[6,7], working with McCoy cell line with bluetongue virus, assumed that these cells were very sensitive. When irradiated by ultra violet radiation (UV), the cell became a giant cell during the infection. This doesn't occur with human amnion cells, which are more sensitive to the bluetongue virus in normal conditions, yet less sensitive than McCoy cells after UV radiation.

In this work there is no interest so as to confirm these matters because the infection by rabies virus causes visible and constant CPE in this cellular system, as well as in primary isolation from cephaloraquidian liquid (CRL) of a suspected patient.

The results proved that the McCoy cell, initially after the inoculation suffered a decrease in its size, which later increased during the development of the infection. This fact was observed and described in relation to the mass production of poliovirus by Dunnenbacke *et al.*[5] The observation of the increase of McCoy cell size may be related to high titre production and higher sensitivity to rabies virus in this cells

Atanasiu *et al*[4] made studies with three different kinds of cell lines: VERO, HAK, BHK-21. They obtained good results in virus replication as well as good titres ans showed that established cell lines could be an efficient method to obtain purified rabies virus.

Due to the high sensivity of this cellular system with easy observation of CPE and the easy obtainment of end point, it can be used in titration of rabies virus, as a substitute for the classic inoculation in mice brains. Although our experience with isolation of rabies virus from biological material has been only from human CRL, it can be suggested that these cells could be an alternative process for inoculation and rapid diagnosis of this infection. So, to perform the neutralization proof in these cells using hiperimmune serum of human use produced by Instituto Vital Brazil to identify the isolated virus, the possibility to perform reactions of titration or neutralization antibody assay against this infection, either in humans or animals, is enlarged.

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TABLE 1
Viral Titre

Passages of ERA strain in McCoy cells line	Titre in LD_{50}/0.30 ml mice brain inoculation
ORIGINAL VACCINE*	$10^{4.5}$
1st passage	$10^{4.6}$
2nd passage	n.d.**
3rd passage	$10^{5.17}$
4th passage	n.d.**
5th passage	$10^{6.0}$
6th passage	$10^{6.21}$
7th passage	$10^{8.0}$

* BIO-RABDO-VET vaccine of veterinarian use (ERA strain in pig kidney cells).

** Not determined

TABLE 2
Cytopathic Effect Observed in McCoy Cell Line

Passages of ERA strain in McCoy cell line	Days after incubation						
	1st	2nd	3rd	4th	5th	6th	7th
1st passage	-	+	+	+	+	+++	++++
2nd passage	-	+	+	+	+	+++	++++
3rd passage	-	+	+	+	+	++++	
4th passage	-	+	+	+	+	+++	
5th passage	-	+	+	+++	++++		
6th passage	-	+	+++	+++	++++		

System: successive passages of rabies virus, ERA strain, in McCoy Cell line.

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RESUMO: O efeito citopático provocado pelo vírus da raiva não é de fácil detecção, embora a linhagem celular McCoy tenha mostrado elevada sensibilidade a esse vírus. O título aumentou a cada passagem, a cepa ERA alcançou titulação superior a $10^{8,0}$ DL 50%/ 0,03 ml após oito passagens sucessivas. A cinética de infecção mostrou que, 48 horas após a inoculação, as células atingem o pico de infecção e a síntese de proteínas celulares é inibida. Os dados são reproduzíveis, sugerindo que essa linhagem possa ser empregada para fins de diagnóstico e para produção de massa viral.

DESCRITORES: *Linhagem celular McCoy. Efeito citopático (ECP). Vírus rábico. Cepa ERA. Produção de massa viral.*

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