

Production of Rabies Vaccine Using BHK-21 with Roller Bottle Cell Culture Technique

Ahmad Fayaz, Alireza Zavarei*, Nader Howaizi, and Nasser Eslami

W.H.O. Collaborating Center for References and Research on Rabies, Pasteur Institute of Iran, Tehran 13164, Iran.

ABSTRACT

Compared to other methods of vaccine production, (e.g. culture flask and bioreactor system), the roller culture method for producing rabies vaccine is simpler, and the equipment easier to install and maintain, and also in the event of contamination of one roller, the entire batch need not be discarded. This system provides a much larger surface area than the culture flask, and also ensures a much higher surface area to volume ratio. Gas exchange is also made easier because the cells are alternately aerated and fed with medium every few minutes. Production of rabies vaccine using BHK-21 cultivated by roller bottle cell culture technique can be suitable for countries where rabies is an important health problem. For this project, the PV-PARIS/BTK-21 rabies virus strain was used. The vaccine thus obtained was tested on mice and a satisfactory immune response was observed, and also the potency of vaccine met the requirements of the World Health Organization (WHO).

Keywords: Rabies, Vaccine, Adjuvant, BHK-21 cell, Roller bottle

INTRODUCTION

Rabies is one of the most feared human diseases and is almost invariably fatal for animals. An epidemiological study by the World Health Organization in 1991 [1], shows that dogs are the main reservoir and vector of rabies virus to humans in the world wide, and our statistical study shows that in Iran the major animal group found to be infected with rabies are dogs. Humans that are affected by rabies virus must be treated with expensive vaccines.

Thus control of dog rabies through programs of animal vaccination and elimination of stray dogs can be a suitable and economical method for human prophylaxis, to reduce the incidence of human rabies. Cultured vaccines are free from many of the side effects of nerve tissue vaccines, store well and have good shelf life, and are highly antigenic [2] in this article the procedure for production of inactivated rabies vaccine, using BHK-21 cells in roller bottles for veterinary use is presented.

MATERIALS AND METHODS

Virus strain. The PV-PARIS/BHK strain is used for the preparation of veterinary vaccine. It is de-

rived from the PV-CEPANZO strain of fixed rabies, passaged in young rabbits (PV-PARIS, passage 11) and then serially passaged in BHK-21. For this project the fixed rabies virus strain used for infecting the BHK-21C13 cells was PVPARIS/BHK-21, obtained from Pasteur Institute of Paris [3].

Cell Lines and Medium. BHK-21C13 (ATCC: CLL10) [4], which was tested for absence of mycoplasma, was used for the culture of virus in roller bottles [5, 6]. Cells required for roller bottles were cultivated in T-flasks, and for cell counting Trypan blue was used. Minimum Essential Medium (MEM), containing 300 µg of glutamine and 30 µg of gentamicin supplemented with 10% fetal calf serum was used for cell culture. MEM maintenance medium, containing the above amount of gentamicin together with 0.3% bovine serum albumin, were used for vaccine preparation.

Production of rabies vaccine. The cells were multiplied by successive passage, initially in small static flasks, until the quantity of cells needed for producing a batch of vaccine in roller bottles were obtained [7]. The cell cultures were infected by adding the seed virus, with a titer of 7.5×10^6 focus forming unit per ml (FFU/ml) [2, 8], to the cell sus-

*Corresponding author. Tel.: 6403496; Fax: 6465132

pension, shaken for an hour at 34°C, and then transferred into culture bottles. The viral inoculum was added in a quantity calculated to obtain the optimum multiplicity of infection [9] (1 focus forming unit per 10 cells). The bottles were placed horizontally on rollers and rotated at a rate of 0.5 rpm at 37°C for 24 hours allowing infected cells to attach to the bottle walls, and then at 1 rpm for 48 hours. After attachment the infected cells to the walls, and at the beginning of viral replication, the growth medium was replaced by a maintenance medium without fetal calf serum and the cultures were incubated at 34°C and rotated at a rate of 1 rpm for 48 hours to obtain the maximum yield of virus in the maintenance medium. The supernatant containing the cultured virus from roller bottles, was harvested after 72 hours and was clarified by low-speed centrifugation for 10 minutes at 1000 ×g, to eliminate cell debris. This then was inactivated by β-propiolacton [10]. The final dilution of the β-propiolacton used, was 1/1000, because at this dilution full inactivity of rabies virus is obtained without reducing the antigenicity properties of rabies vaccine [11]. Furthermore at this dilution the residual cell DNA is sufficiently broken and inactivated [12]. Sodium merthiolate (0.01%) was used as preservative, and aluminium hydroxide (equivalent to 0.25 g of dry weight of aluminium per liter of vaccine) was used as adjuvant [2].

Rabies virus titration. Virus titer was determined using BHK-21 cells in 96 wells microplates. 5×10^4 cells were distributed in each well and infected with 50 µg of varying dilution of PV-PARIS /BHK virus. After 24 hours the microplates were washed with PBS (Phosphate buffer saline supplemented with Mg^{+2} , Ca^{+2}), fixated with cold 80°C acetone and then stained with fluorescein-labeled anti-rabies-nucleocapsid immunoglobulin. The results were studied with the method of focus forming unit per ml.

Glycoprotein titration. The transmembrane glycoprotein G mediates the attachment of the virus to the host cells and is of crucial importance, because it is also responsible for the induction and binding of virus neutralizing antibodies, as well as for the stimulation of T cells [13]. Rabies G protein is the major antigen capable of inducing rabies virus-neutralizing antibodies and conferring protection against intracerebral challenge. Furthermore, it can be titrated by the ELISA for testing the potency of rabies vaccines

[14]. Glycoprotein titration was determined by the ELISA immunocapture method using peroxidase-conjugated polyclonal antibodies.

Neutralization test. The titers of neutralizing antibodies developed in vaccinated mice serum, were tested by the rapid fluorescence focus inhibition test (RFFIT) [2]. The dilutions of heat-inactivated serum were incubated with a fixed amount of Challenge Virus Standard (CVS) strain of rabies virus, which was adapted to cell culture, for an hour at 37°C. Residual virus infectivity was then determined by cell culture, based on the foci of virus-infected cells were revealed by fluorescent antibody staining.

Rabies Vaccine Potency test. The potency of vaccine was determined according to the method of NIH test [2, 15]. The test measures the degree of protection conferred by inactivated rabies vaccines in immunized mice challenged with rabies virus. The test was conducted by vaccinating two groups of mice, 7 days apart, with dilution of a reference vaccine and the vaccine under test. 14 days after the Last vaccination, the immunized animals and a control group of mice were challenged with the Challenge Virus Standard (CVS) mouse-brain strain of fixed rabies virus (dilution 1:20,000 = 50 LD₅₀ per 0.03 ml). The mice were observed for 14 days and the median effective dose (DE₅₀) of the reference and test vaccines was determined, based on the number of survivors. The relative potency of the test vaccine was then calculated by comparing the DE₅₀ of the test vaccine with that of the reference vaccine.

RESULTS AND DISCUSSION

After clarification of supernatant containing the cultured virus, we have determined the titer of virus on the base of focus forming unit per ml (FFU/ml) method. The result of the virus titer before inactivation by β-propiolacton was 1.5×10^6 FFU/ml. After inactivation of virus we have titrated the glycoprotein for VSTI (non-concentrated inactivated virus). The result of the glycoprotein content determination by ELISA method using Antiglycoprotein polyclonal rabbit immunoglobulins (PAb-Gs) was 3.62 µg/ml.

The determination of the titers of neutralizing antibodies developed in vaccinated mice serum has done by RFFIT method. The results of the neutral-

Table 1. Results of neutralizing antibody titers following 14 days after the second vaccination of 20 mice by four different dilutions of test vaccine. For each dilution, 5 mice were injected, each with 0.5 ml vaccine intraperitoneally (I.P.) on days 0 and 7.

Dilution of vaccine	Titer of neutralizing antibody
1/5	19.8
1/25	7.0
1/125	2.6
1/625	1.7
Control	0.1

izing antibodies contents are shown in Table 1. The results of the vaccine potency determination using NIH test are shown in Table 2a, 2b. The reference vaccine with a potency of 2.5 IU/ml gave a 50% end-point dilution (DE_{50}) of 1/93.3. The test vaccine gave a DE_{50} of 1/61.6. Thus the relative potency of the test vaccine was equal to $2.5 \times 61.6/93.3 = 1.65$ IU/ml.

Rabies vaccines produced by cell culture method, show greater potency and safety, compared to those produced using neural tissue [1, 16]. In 1996, 51378 human cases of rabies exposures were recorded in Iran, and the post-exposure treatment costs for

vaccine and serum for these cases were more than 2 million dollars.

According to statistics compiled at the Pasteur Institute of Iran, of the 51378 human post-exposure cases reported in 1996 in Iran, dog transmission accounted for 45140 (almost 88%) and for cats 1823 (3.5%), wolf 303 (0.5%), fox 186 (0.3%), Jackal 169 (0.3%) and the remainder 3757 (7.3%). Of this the total number of fatalities was 6 (0.01%). In the United States of America following widespread vaccination of dogs, the incidence of human post-exposure cases decreased from 43 in 1945 to one case in 1980 [13].

In consideration of the above and also of the fact that about 88% of human post-exposure cases were caused by dogs, it is proposed that mass production of cell culture veterinary vaccine, containing adjuvant, manufactured in roller bottles, for mass vaccination of dogs can be effective and economical for human prophylaxis. In this respect, we have produced rabies vaccine with potency of 1.65 IU/ml which compares favorably with the standard set by WHO for veterinary use (specified as at least 1 IU/ml) [5].

Table 2a. Determining the 50% end-point dilution (DE_{50}) of reference vaccine. 10 mice were used for each vaccine dilution.

Vaccine dilution	Died	Survived	Cumulative		Total	%Mortality
			Died	Survived	Died + Survived	
1/5	0	10	0	23	23	00.0
1/25	4	6	4	13	17	23.5
1/125	5	5	9	7	16	56.2
1/625	8	2	17	2	19	89.4

Table 2b. Determining the 50% end-point dilution (DE_{50}) of test vaccine. 10 mice were used for each vaccine dilution.

Vaccine dilution	Died	Survived	Cumulative		Total	%Mortality
			Died	Survived	Died + Survived	
1/5	2	8	2	20	22	09.0
1/25	3	7	5	12	17	29.4
1/125	5	5	10	5	15	66.6
1/625	10	0	20	0	20	100.0

ACKNOWLEDGEMENTS

Much gratitude is felt to Dr. Pierre Perrin of the Pasteur Institute of Paris, who graciously gave much valuable support and assistance.

REFERENCES

1. Bulletin of WHO (1985) Recommendations for production of cell culture rabies vaccines. 63: 661-666.
2. Smith, J.S., Yager, P.A. and Baer, G.M. (1996) A rapid fluorescent focus inhibition test (RFFIT), Manufacture requirements for cell-culture rabies vaccines and

- recommendations for their use. In laboratory techniques in rabies (Meslin, F.X., Kaplan, M.M., and Koprowski, H., eds) World Health Organization, Geneva, PP 181-188, 318-320.
3. Delagneau, J.F. (1981) Structure of the rabies virus. *Ann. Virol. (Inst. Pasteur)*. 132 E: 473-478.
4. MacPherson, I. and Stocker, M. (1962) Polyoma transformation of hamster cell clones an investigation of genetic factors affecting cell competence. *Virol.* 16: 147-155.
5. WHO Tech. Rep. Ser. (1981) Requirements for rabies vaccine for veterinary use. Annex 3, 658: 96-130.
6. WHO Tech. Rep. Ser. (1981) Requirements for rabies vaccine for human use. Annex 2, 658: 54-95.
7. WHO. Expert Committee on Biological Standardization. (1986) Geneva. 1-18.
8. Bussereau, F. (1982) Reproducible plating system for rabies virus. *Vaccine*. 11: 82-87.
9. Consales, C.A. (1988) The preparation of cultured rabies virus and the production of antiserum for human use. *Journal of Biological Standardization*. 16: 27-32.
10. Swanson, C. (1987) The effects of 13-Propiolacton. *The Journal of Infectious Diseases*. 5: 155-163.
11. Perrin, P.(1995) Inactivation of DNA by 13-Propiolacton. *Biologicals* 23 : 207-211.
12. Morgeaux, S. and Tordo, Noel. (1993) 13-propiolacton treatment impairs the biological activity of residual DNA from BHK-21 cells infected with rabies virus. *Vaccine*. 1: 11.
13. Tordo, N. and Poch, O (1988) structure of rabies virus. In: *Rabies* (Campbell, J.B. and Charlton, K.M., eds) Kluwer Academic Publishers, Boston, USA, PP 35-36.
14. Perrin, P. (1990) *In vitro* rabies vaccine potency appraisal by ELISA. *Biologicals* 18: 321-330.
15. Barth, R. (1988) The NIH test for potency. *Vaccine* 4: 364-377.
16. Perrin, P., Lafon, M. and Sureau, P. (1990) Rabies vaccines from Pasteur's time up to experimental subunit vaccines today. *Viral Vaccines* 12: 325-345.