Perfusion filtration technology for the cultivation of VNK-21/13-13 cells for the manufacture of rabies vaccine

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Abstract. The possibility of practical use of perfusion cell culture technology VNK-21/13-13 was evaluated in the manufacture of rabies vaccine using bioreactors with a working volume of 5 liters and 100 liters. The purpose of this study is to develop a highly effective technology for perfusion continuous suspension cultivation of the transplanted BHK–21/13-13 cell line, suitable for use as a cellular substrate in the manufacture of rabies vaccine. Using Cytiva filter elements made of PES membrane material with a retention rating of 0.65 microns, a cell density above 7× 106 cells/ml was achieved for the subsequent production of a viral suspension. The glycoprotein content measured by the ELISA method reached a maximum on the 4th day after infection, was 5 IU/ml. The maximum cell accumulation and glycoprotein content in the 5 L bioreactor was more than 2-3 times higher than for the serial production of rabies vaccine. When reproducing the rabies virus in a 100 l bioreactor after perfusion cultivation, the cell concentration was 16-20 million/ml with a viability of 95.4%. The highest content of the virus glycoprotein was also achieved in a bioreactor with a working volume of 100 liters 4 days after infection and amounted to 8 IU/ ml. Sequencing of the glycoprotein gene revealed the genetic stability of the main immunogenicity protein, which is an important indicator for controlling the stability of the virus during many passages in vaccine production using a perfusion system.

1 Introduction

The intensification of the production process of viral vaccines based on cell cultures requires advanced technological strategies to overcome the limitations of traditional periodic cultivation. The use of perfusion or continuous modes of cultivation of suspension cell lines in order to obtain their high cell density has so far been little studied in the large-scale production of viral vaccines in the Russian Federation. For many years, foreign scientists in scientific research and industry have been considering the possibility of switching to more efficient processes for the production of viral vaccines. Compared with traditional periodic processes, perfusion cultivation strategies can lead to a ten- to a

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hundred-fold increase in product yield. Such cultivation technologies can be implemented to achieve cell concentrations exceeding 107 cells/ml or even 108 cells/ml, while maintaining low levels of metabolites that potentially inhibit cell growth and viral replication [1].

Suspension-adapted cell lines (such as PER. C6®, AGE1.CR ®, MDCC. SUS, EB66®, CAP® and BHK-21 cells) have shown promising cell growth in bioreactors and productivity for a wide range of viruses [2-6].

One of the promising approaches to overcome the limitations in the supply of nutrients to cells is the variable tangential flow system (ATF, Refine Technology, USA) [7]. This method is a combination of perfusion and filtration, the technology provides high cell density, can be scaled to vessels with a volume of 1000 liters by increasing the number of hollow fibers (the corresponding scale is indicated in the name of the device: from ATF2 to ATF10), is compatible with various types of bioreactors and provides some flexibility in choosing the type of hollow fiber membranes.

Rabies is an acute progressive encephalitis caused by rabies virus (Rabies virus, BB) of the family Rhabdoviridae, transmitted to humans mainly from domestic animals. The main direction of rabies prevention is vaccination of domestic and wild carnivores [8-9]. The development of a high-performance perfusion cell culture method based on the example of improving the industrial technology for obtaining a rabies vaccine is an urgent study. The aim of the research is to develop a highly effective technology for perfusion continuous suspension cultivation of a transferable BHK–21 cell line suitable for use as a cellular substrate in the manufacture of rabies vaccine.

2 Materials and methods

2.1 Virus strains, rabies vaccines

The work used the strain "Shchelkovo-51", the reference strain of the rabies virus "CVS" (Challenge Virus Standard, the center for rabies OIE Nancy Laboratory for Rabies and Wildlife ANSES, France), the Rabikan anti-rabies inactivated dry culture vaccine for dogs and cats (from the strain "Shchelkovo-51") produced by FKP Shchelkovsky Biocombinat (series 1020, 12.01.2021) and a national standard (reference) vaccine with a specific activity equal to 5.2 IU/ml: an industry standard sample of an anti-rabies vaccine (SRT-00494189-0042-2010 FKP Shchelkovsky Biocombinat). The reference vaccine was calibrated according to an international standard and its effectiveness (IU/ml) was evaluated using the NIH test [10] in comparison with the batch of the international reference drug (BRP) No. 5 (BRP) [8-9].

The rabies inactivated dry culture vaccine from the Shchelkovo-51 strain for dogs and cats is a rabies virus of the Shchelkovo-51 strain inactivated with β-propiolactone, lyophilized in a protective environment with the addition of 33.3% sucrose-peptone-gelatin stabilizer. The specific activity is 3.7 IU/ml.

BHK-21/13-13 cells were kindly provided by Shchelkovsky Biocombinat. Samples were taken with a syringe through the bioreactor sample port. Part of the sample was used to measure cell viability, the rest was centrifuged (3000 g, 10 min, 4 °C), and the infusion fluid was aliquoted and stored at -80 °C for subsequent analysis of infectious activity during rabies virus reproduction or for analysis of metabolites (Bioprofile 100 plus analyzer, Nova Biomedical).

2.2 Nutrient media and solutions

The standard culture media Igla, 199, DMEM, lactalbumin hydrolysate on Earle (GLAE) or Hanks (GLACH) solution obtained from the Institute of Polio and Viral Encephalitis; trypsin from Megsk, Fluka in salt buffer solutions of different composition were used in the work. Bovine blood serum and fetal serum were obtained from BIOLOT LLC (St. Petersburg, Russia).

2.3 Filter elements

Polyvinylidene fluoride filter (F=0.2 m2; 300 kDa); metal mesh filter with holes 75 microns, 15 kDa; PALL Microza filter, Part No. UJP-1047, PVDF membrane material, 0.65 microns, total filtration area 0.1 m2; Cytiva module, CFP-6-D model-5A, Part No. 56-4102-50, PES membrane material, 0.65 microns, total filtration area 0.16 m2; ultrafiltration cassettes manufactured by PALL with polyester fiber membranes (PES) and a filtration area of 0.1 m2 Part No. OS100T12, 100 kDa, ultrafiltration cassettes manufactured by PALL with PES membranes and a filtration area of 0.1 m2 Part No. OS300T12, 300 kDa.

Physicochemical, virological, biotechnological and immunological research methods were used at different stages of the work. The cultivation of the producer strain was carried out in bioreactors with a working volume of 5 liters (total volume of 7 liters) and 100 liters, in periodic mode, or in perfusion mode using a perfusion system of domestic production (LLC BIOTECHNO, Russia). Cell concentration and viability were determined by direct counting in the Goryaev chamber or using an automated cell counter.

Cell and virus cultivation in a bioreactor. BHK-21/13-13 cells were infected with a strain of rabies virus at a cell concentration of 1×106 ml−1 cells with a multiplicity of infection of 0.1 TCID50 per cell. Cultivation was carried out in a 5-liter bioreactor equipped with a perfusion system. At the stage of cell culture proliferation, the following conditions were created: pH=7.2, pO2=50% air saturation, temperature =37°C and mixing speed =30 rpm. At the stage of rabies virus production, the pH was maintained at 7.4, pO2 = 30% air saturation, temperature = 37 °C and mixing speed = 30 rpm. Perfusion was started after 2 days of cultivation. The perfusion rate was changed depending on the cell density. The recirculation mode was switched on after 24 hours of cultivation. Samples were taken daily to determine the density of cells, their viability, and the infectious activity of the virus.

The infectious activity of the viral suspension was also determined by titration in cell culture and expressed by TCID50/ml (50% tissue culture infectious dose) according to claim 2.1.3. Chapter 3.1.18. – Rabies (infection with rabies virus and other lyssaviruses), WOAH Terrestrial Manual 2023. The cells were stained using a direct immunofluorescence test, and each well in which specific fluorescence was observed was considered positive. The titer was calculated using the Spearman–Kerber formula [11].

The immunogenic activity of the rabies vaccine was determined by the volumetric method of the US National Institutes of Health (NIH-test) on white mice and expressed in international units (IU) [10].

2.4 Sequencing of the virus glycoprotein gene

The genetic affiliation of inactivated samples of the rabies virus strain Shchelkovo-51 was determined by sequencing using the Sanger method. The nucleotide sequence of fragments was determined using the AVI Prism 3100 Genetic Analyzer automatic sequencer (Applied Biosystems, USA) and a set of reagents for sequencing BigDye Terminator No.1 Cycle

Sequencing Kit (Applied Biosystems, USA), according to the manufacturer's recommendations.

3 Results and discussion

A filtration system with a variable tangential flow was designed to retain the cells. The supply of the nutrient medium was controlled by scales to maintain a constant working volume in the bioreactor. Thus, the consumption of the nutrient medium has always been equal to the consumption of permeate. The permeate flow rate was increased automatically throughout the process to maintain the rate of cellular perfusion.

The diagram of the perfusion filtration laboratory unit is shown in Figure 1. The idea was to implement laboratory perfusion on electromagnetic valves (8,9,10). One scale (1) was used to fix the volume of the nutrient medium at the entrance to the bioreactor (4) and other scales at the outlet to measure the volume of the spent nutrient medium (12). Two peristaltic pumps (3 and 5) were used. Pump 3 provided an automatic supply of nutrient medium to the bioreactor. Pump 5 supplies the medium with cells through a filter (6). The pressure (7) is regulated by means of a compressor, the values are transmitted to the operator panel. The installation used software from the Russian manufacturer BIOTECHNO LLC, which made it possible, as a result of a series of experiments, to carry out technical refinement of the automation system.

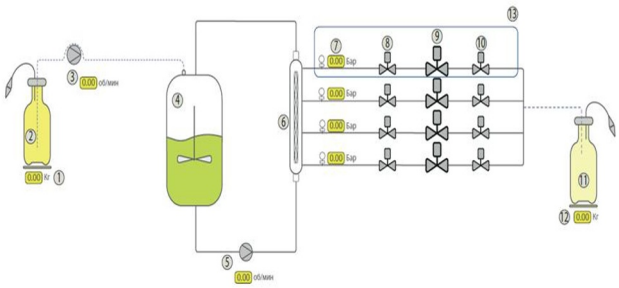

Fig. 1. Diagram of a laboratory installation for perfusion filtration

Four types of membranes with different characteristics (membrane material/pore size and filtration area) were investigated. And only in the experiment, when the Cytiva module, CFP-6-D-5A model, Part No. 56-4102-50, PES membrane material with a retention rating of 0.65 microns, a total filtration area of 0.16 m2, was used as a filter element in a perfusion filtration unit with a laboratory fermenter, encouraging results began to be obtained. This experiment ensured cell viability of more than 95% when a concentration of 8.5 million cells/ml was reached for 72-96 hours of cultivation. This filter completely trapped the cells, and the cells were well removed from the membrane surface and returned to the reactor, during the entire process the filter worked without contaminating the membrane or blocking it, which indicated that the selected membrane with such parameters is suitable for culturing cells with high density. In multiple cell culture experiments, the levels of metabolites were very comparable, and no inhibition by waste products was observed. Cell retaining membranes were compared for their ability to collect viral particles in a bioreactor or permeate. In this case, the cells were infected when the cell concentration reached 2 million cells/ml. Before the virus infected the cells, the medium was completely removed. The multiplicity of virus infection was 0.01 TCID50 per cell. Perfusion and permeate flow were suspended for 20-24 hours after infection in order to avoid loss of viral particles and ensure effective penetration of the virus into cells. During the replication phase of the virus,

a nutrient medium containing blood serum was used and a constant perfusion rate was maintained at a perfusion acceleration rate of 0.1* day-2. The experiment was carried out in four repeats, the cells continued to grow after infection with the virus and reached a maximum concentration of cells / ml. At the same time, cell viability did not decrease. The glycoprotein content measured by the ELISA method increased, reached a maximum on the 4th day after infection, and amounted to 5 IU/ml. The maximum cell accumulation was 8 x 106 cells/ml, which is more than 2-3 times higher than for the serial production of rabies vaccine. Specific viral productivity was calculated when the peak of the rabies virus glycoprotein content was obtained on the 4th-5th day after infection.

Depending on the characteristics of the membrane used for cell retention, viral particles can accumulate either exclusively in the culture vessel or additionally in the permeate, so it was important to select the membrane in accordance with our tasks. To accumulate the rabies virus, ultrafiltration cassettes manufactured by PALL with PES membranes and a filtration area of 0.1 m2 were tested. For comparison, the Part No. OS100T12 cassette with a delay rating of 100 kDa was used first, then the Part No. OS300T12 cassette with a delay rating of 300 kDa. The tangential flow was 0.6 l/min, the transmembrane pressure was 1 bar. In both cases, the cassettes successfully trapped the virus in the concentrate area, in the culture vessel. However, the further choice turned out to be in favor of a cassette with a delay rating of 300 kDa as more productive.

Most vaccines eventually need to be produced on an industrial scale. Therefore, experiments were conducted in a 100-liter bioreactor with stirring to confirm the scalability of the developed process.

A growth medium and a kidney cell line of a newborn Syrian hamster BHK-21/13-13 were introduced into the bioreactor at a seed cell concentration of 0.5±1.0 million/ml. Cells were cultured in continuous perfusion cultivation mode using a filtration system with variable tangential flow until a cell concentration of 2 ± 0.1 million/ml was reached and infected with rabies virus.

For the 100 L reactor, the choice fell on the Cytiva model, assembly of the cartridge in a steel case for in-line sterilization (CFP-6-D-55STM model No.56-4109-25, 0.65 microns, 2.5 m2 in area with a cartridge housing SS-55STM No. 56-4106-28).

To concentrate the virus in a 100 l bioreactor, two cassettes PALL Part No. OS300T06, 300 kDa with a filtration area of 0.5 m2 each (only 1m2), PES membrane material were taken. The perfusion rate was controlled by the installation software. In the next stage, the perfusion rate was monitored based on measurements of cell concentration using a capacitive probe. The perfusion rate was maintained at a perfusion acceleration rate of 0.1*day-2. The concentration of cells after perfusion cultivation was 16-20 million/ml with a viability of 95.4%. The highest glycoprotein content of the virus was also achieved in a bioreactor with a working volume of 100 liters 4 days after infection and amounted to 8 IU/ml. For the Shchelkovo-51 strain (access code ON181556), sequencing of the glycoprotein gene was obtained, registration number in GenBank for this nucleotide sequence: BankIt2569966 BSeq#1 ON181556.

The genetic stability of the virus strain during reproduction in the perfusion system for many passages was controlled by the level of identity of the sequence of the glycoprotein (G) gene. Samples of culture fluids of the vaccine strain of the 2nd and 16th passages were selected. The sequences of the glycoprotein gene of vaccine virus samples during reproduction in the suspension cell line BHK-21/13-13 in perfusion mode in the 2nd passage and the 16th passage are 100% identical when evaluated using the BLAST algorithm (2036 out of 2036 bp – 0 nucleotide substitutions). The data obtained confirm the genetic stability of the main immunogenicity protein, which is an important indicator for controlling the stability of the virus during cultivation in the perfusion system.

4 Conclusion

The possibility of practical use of the developed perfusion suspension culture technology was evaluated using a rabies virus model. During this study, the production of cellular biomass was carried out under perfusion conditions with a 5 L and 100 L bioreactor. A cell culture with a concentration of at least 2x106 cells/ml was used to infect the rabies virus.

As follows from the data obtained, the glycoprotein of the virus accumulates in the liquid phase of the culture. The high accumulation of viral glycoprotein of the virus, measured in ELISA, in the liquid phase of the culture was not accompanied by degeneration of the cell layer, the latter circumstance was of considerable practical interest, since it allowed for several collections of viral antigen from one producer culture.

As follows from the data, when growing the rabies virus in cells under perfusion conditions, it was possible to carry out at least 3 collections of a viral crop with a high content of a specific antigen - glycoprotein. The optimal timing of harvesting was 4 days after the infection of the crop.

Thus, we have selected the optimal parameters for cell culture and virus strain under perfusion conditions, ensuring stable accumulation of cultural antigen during at least 3 collections of virus-containing liquid.

The results of the specificity and genetic stability tests met the requirements for the vaccine.

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