Optimization Of New Recombinant Influenza Virus Expressing M. Tuberculosis Esat6 2a Ag85a Proteins Cultivation In Vero Cells

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Abstract: This paper presents the results of the development of the optimal conditions for new TB-FLU Esat6 2A Ag85A recombinant strain cultivation in Vero cell culture. The studies revealed that the maximum accumulation of the virus in cell culture can be achieved with 0,001 TCD_{50}/cell infecting dose, 34,0 ± 0,5 °C incubation temperature and 72 hours of incubation time. The highest reproductive activity of the virus was obtained using trypsin (Sigma # T4049, Lot10D169, # Lot020M7022T1426-1g) at a concentration 2 µg/ml. The TB-FLU Esat6 2A Ag85A recombinant strain expressing mycobacterial proteins Esat6 and Ag85A with open reading frame of NS1 protein of the influenza virus is genetically stable and capable to maintain enthetic inserts during 10 consecutive passages in Vero cell culture. Subject to the established cultivation parameters the recombinant virus accumulation level reached up to 7,75 ± 0,08 lg TCD_{50}/cm^3 with 7,0 log_2hemagglutinating titer, which is quite suitable for the development of a TB vaccine for human health care.

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1. Introduction

Tuberculosis (TB) is a severe chronic disease of humans and many animal species caused by various species of mycobacteria mainly Mycobacterium tuberculosis, characterized by the formation of tubercles exposed to caseous necrosis and calcification in various organs. [1]

According to WHO recommendations vaccination is the main way to prevent TB. And in this regard scientists from many countries conduct studies on the development of effective TB specific prophylaxis means.

TB vaccine Bacille Calmette-Guerin (BCG) has been used all over the world for decades. This vaccine has several disadvantages including variable efficacy in humans, the inability to protect against reactivation and re-infection and pathogenicity in a host with weakened immune system [2-4].

To solve this problem, the scientists of RIBSP CS ME&S RK are working on the development of a live influenza vector based vaccine. For this purpose a new recombinant influenza virus expressing the mycobacterial antigens TB-FLU Esat6 2A Ag85A and adapted to Vero cell culture was obtained.

Many research laboratories and pharmaceutical companies have developed the cultural inactivated influenza vaccines. The vaccines produced in Vero (Influject, Baxter) and MDCK (Influvac TC, Solvay) cell cultures have already been licensed for the prevention of seasonal influenza in Europe (Whilsshut e.a., 2006).

Today, scientists all over the world have developed the manufacture technology of many vaccines on the basis of recombinant strains. And different mammalian cell lines, including diploid (MRC- 5, WI- 38 and FRhl- 2) and continuous cell lines (PER.C6, NIH-3T3, BHK, CHO, Vero and MDCK) are used as cultivating systems for those vaccines. Practical application of continuous cell cultures certified in accordance with the WHO requirements is now considered as the most promising area of biotechnology. Continuous cells of green monkey kidney (Vero) have been successfully used as a substrate for the production of many vaccines.

This cell culture has been used to produce polio vaccines for more than 20 years. The safety of using the continuous Vero cell culture is confirmed by successful production and use of several billion doses of live polio vaccine, as well as millions of doses of inactivated injectable vaccines against rabies, polio and herpes simplex [5, 6]. Unlike many other cell lines Vero cell culture does not cause progressive metastatic tumor growth in athymic mice up to 169 passage. DNA isolated from Vero cells does not have oncogenic characteristics in the highest

concentrations in various applications to animals. Certified Vero cell line meets the safety and security criteria of WHO and European Pharmacopoeia by the absence of infectious and oncogenic viruses of primates such as SV- 40, SIV, retro -D virus, cytomegalovirus and others [7].

The use of recombinant viruses with reduced or deleted NS1 protein (delNS) which is antagonist of first type interferon system is a new trend in the creation of effective and safe live influenza vaccines[8, 9]. Due to the lack of NS1 protein expression these strains lose their ability to replicate in INF-competent organisms but can accumulate to high titers in INF-deficient systems, such as the Vero cell line, which is permitted for vaccine production [10, 11]. Inability of delNS virus to replicate in respiratory tract cells provides a high level of safety of the vaccine produced on the basis of genetic strains with elongated sequence encoding NS1protein. At the same time, delNS vaccine viruses are sufficiently immunogenic due to increased cytokines production in vaccine injection location. The release of such cytokines as first type interferon stimulates nasal mucosa local immunity mediated by secretory IgA antibodies, cytotoxic and system B and T cell immune response providing cross-protection without adjuvants [12, 13].

Vero cell culture is also widely used for cultivation and production of recombinant influenza vectors expressing mycobacterial antigens [14, 15]. Studies based on immunofluorescence analysis of PCR NS fragment constructions shows that ESAT6 is genetically stable and able to retain the recombinant protein even after several passages in Vero cell culture. As a result, a substantial amount of the tuberculosis antigen is accumulated in the cells during reproduction of a recombinant influenza virus. NS1 gene deletion results in the failure of the virus to replicate in interferon-competent cells such as continuous MDCK cell culture, chicken embryos, humans, etc., and provides reliable attenuation of the vaccine strains. At the same time such vaccine strains can grow to high titers in interferon-incompetent Vero cells creating the possibility for effective vaccine production on this substrate [14, 15].

The effectiveness of the design and production of the vaccine prototypes based on actual recombinant strains primarily depends on the choice of the most suitable bio-system and optimization of cultivation parameters for the virus, which are an integral part of the antiviral vaccines technology development.

Thus, the aim of this work is to optimize cultivation parameters of the new influenza virus recombinant strain expressing TB-FLU Esat6 2A Ag85A mycobacterial antigens in Vero cell culture to obtain biomaterial of high quality suitable for the TB vaccine development.

2. Material and Methods

The Vaccine Virus Obtaining

The recombinant strain was isolated in RGE RIBSP CS ME&S RK and Influenza Research Institute NWD RAMS, Russia. It is 7:1 reassortant of A/PR8/34 (H1N1) influenza virus A/duck/Singapore/F-119/97 (H5N3) and influenza virus. The recombinant strain was obtained by reverse genetics in Vero cell culture. It has recombinant NS gene containing nucleotide sequences encoding mycobacterial proteins ESAT- 6 and Ag85A.

The reverse genetics techniques were used for the recombinant vaccine strain design. It has 1 plasmid containing hemagglutinin from A/duck/Singapore/F-119/97 (H5N3) and 7 plasmids containing the remaining seven segments of the influenza virus genome taken from A/Puerto Rico/8/34.

These plasmids were used to transfect the Vero cell line, allowed for the vaccines production. Recombinant influenza virus is characterized by the presence of chimeric NS1 containing nucleotide sequences of the M. tuberculosis genes ESAT- 6 and Ag85A in the NS gene ORF.

Cell Culture and Media

For the cultivation of vector vaccine we used interferon-incompetent continuous Vero cell line certified by WHO and the European Collection of Cell Cultures (ECACC), obtained from AVIR Green Hills Biotechnology (Austria). Serum-free culture medium OPTI PRO SFM (Gibco, #12309, Invitrogen) was used as the supporting medium.

Trypsin from bovine pancreas (Sigma # Lot 020M 7022), a solution of 0,25 % Trypsin-EDTA (# T4049, Lot10D169) and the solution Accutase (# A6964-100ml SLBB5634) were used to activate the reproductive properties of the virus. The solutions were applied at various concentrations and diluted in a balanced Hanks saline to prepare 1 % solution which was stored at -20 °C.

The Virus Cultivation Optimization

Vector vaccine TB-FLU Esat6 2A Ag85A cultivation was performed in Vero cell culture. The cell culture infection was conducted at a dose from 0,00001 to 0,1TCD_{50}/cell, incubation temperature varied from 33 to 37 °C, incubation time was from 24 to 96 hours. When the lesion of cells monolayer was around 70-80 % it was frozen at -70 ^{0}C for 12-14 hours.

HA Reaction

Hemagglutinating activity of the resulting virus-containing fluid was determined by the common

method in HA reaction using 0, 87 % of rooster erythrocytes [16].

Determination of the Virus Infectivity in Cell Culture

The virus activity was determined by titration in cell culture. 10-fold dilutions from 10^{-1} to 10^{-8} were prepared on the support medium from primarily obtained biomaterial. The titration results were identified by the presence of cytopathic changes in the infected cells monolayer provided that it was absent in the control ones. The virus titer was expressed as lg TCD_{50}/cm^3, which was calculated by the Reed and Muench method [17].

Polymerase chain reaction for the identification of the NS gene

Isolation of viral RNA from the virus-containing fluid was implemented using a reagent Trisol, Invitrogen, and QIAampViralRNAMiniKit, Qiagen.

TB-FLU Esat6 2A Ag85A recombinant influenza virus cDNA was amplified with the use of Taq DNA polymerase 5U/µl (Promega) and Pfu Turbo DNA polymerase 2,5 U/µl (Stratagene) applying NS gene-specific primers:

- NS834, 10 pmol /µl 5'-CTCTTGCTCCACTTCAAGC
- RTlen 10 pmol /µl 5 - AGCAAAAGCAGGGTGACAAAG.

Temperature and time: 1 min at 95 °C; 30 cycles - at 95 °C for 30 sec, at 58 °C for 1 min, 72 °C for 3 min, then at 72 °C for 10 minutes.

cDNA Synthesis

cDNA was synthesized using ImProm-II ™ reverse transcriptase (Promega) and a primer Uni12 (Genxpress). 8 µl of the extracted 500 nM RNA was mixed with 10 µl of RT – Mastermix consisting of 5X buffer, 25 mM MgCl2, 10 mM dNTP's, Uni- 12 oligonucleotide (5'-agcaaaagc agg -3 '), RNasout ® RNaseingibitor, PT and sterile water. RT reaction was performed at 42°C.

DNA sequencing was performed by Sanger method in automatic 16-capillary sequencer Genetic Analyser 3130 xl, Applied Biosystems. POP-7 was used as the polymer for capillaries. Accumulation of DNA termination products was carried out by cycle sequencing.

Statistical Analysis of the Research Results

All experiments were independently repeated few times to ensure reliable results. The obtained results of the study were processed mathematically. Calculation of the arithmetic mean (M) and mean square error (m) was performed using "Microsoft Excel".

3. Results

Studies of the stability of the recombinant strain passaged in Vero cell culture

To determine the stability of TB-FLU Esat6 2A Ag85A strain we conducted 10 consecutive passages in Vero cell culture followed by determination of infectivity and stability of the strain after each passage.

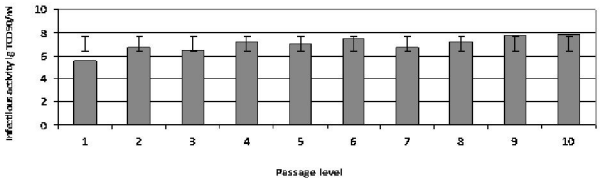
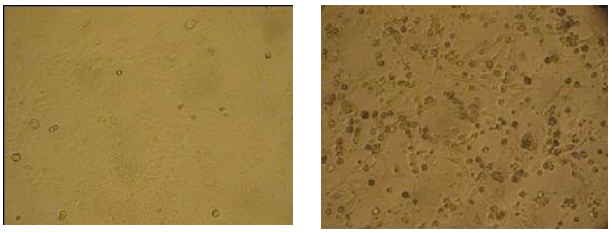


Figure 1 - The stability of TB-FLU Esat6 2A Ag85A recombinant strain passaged in Vero cell culture

Figure 1 shows that, during 10 consecutive passages infectious activity of TB-FLU Esat6 2A Ag85A recombinant strain was from 5,50 ± 0,14 to 7,83 ± 0,08 lg high TCD_{50}/cm^3 with a high level of confidence (P < 0,001-0,05), cultivation time was 72-96 hours. The nature of the cytopathic effect (CPE) was typical of influenza virus infection with the formation of rounded cells individual loci. Further development of the CPE led to total lesion of the monolayer and was accompanied by cytoplasmic granulation, vacuolization, nuclear pyknosis, shrinkage and degeneration of cells. Complete destruction of the monolayer was observed in 96 hours of cultivation depending on infection dose (Fig. 2).



A - Vero cell culture (control) B – The virus CPE (after 72 hours)

Figure 2 - Light microscopy of Vero cells culture before and after infection with TB-FLU Esat6 2A Ag85A strain

The recombinant virus A/PuertoRico/8/34-Esat 6 Ag85A was obtained by reverse genetics in Vero cell culture and characterized by recombinant NS1 gene containing M. Tuberculosis ESAT6 and Ag85A nucleotide sequence. Due to the insertion, the virus A/PuertoRico/8/34 Esat-6 Ag85A differs from wild influenza strain A/PuertoRico/8/34 by the presence of enlarged NS1 genomic fragment. A/PuertoRico/8/34

Esat-6 Ag85A virus was provisionally named as TB-FLU Esat6 2A Ag85A.

We conducted studies after different passages to determine the genetic stability of the recombinant strain of influenza A virus expressing mycobacterial protein with NS1 open reading frame. A/PuertoRico/8/34 NS-117 with the modified NS gene encoding 117 amino acids of the N-terminal protein site was taken as the parent strain to compare NS1 proteins after different passages (NB!: normal size of NS1 protein consists of 230 amino acid residues).The size of the recombinant strain NS gene after 1, 5 and 10 passages was determined by agarose gel electrophoresis after PCR amplification with specific primers:

Forward: NSLEN - 5'-AGCAAAAGCAGGGTGACAAAG- 3 'and

Reverse: NS834 - 5'-CTCTTGCTCCACTTCAAGC- 3 '.

Figure 3 shows that the size of amplified recombinant NS fragment exceeds wild type influenza virus NS gene.

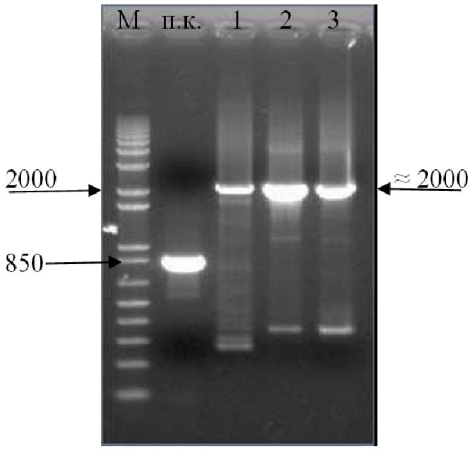


Figure 3 - Electrophoregram of PCR product of modified NS gene with mycobacterial ESAT- 6 and Ag85A proteins (Esat 6 Ag85A). M - DNA molecular weight marker (Invitrogen); P.C. - NS gene of wild-type influenza virus A/PuertoRico/8/34; 1 - TB-FLU Esat6 2A Ag85A NS gene (1 passage), 2 - TB-FLU Esat6 2A Ag85A NS gene (passage 5), 3 - TB-FLU Esat6 2A Ag85A NS gene (passage 10)

The experiments confirmed the presence of NS gene insertion in TB-FLU Esat6 2A Ag85A recombinant strain after different passages. Our studies based on the NS fragment constructs PCR showed that influenza vector expressing Esat6 Ag85A mycobacterial proteins with the open reading frame of influenza virus NS1 protein is genetically stable and capable of maintaining recombinant proteins during 10 serial passages in Vero cell culture.

Sequencing and TB-FLU-Esat6 2A Ag85A recombinant strain NS gene sequence comparative analysis confirmed the presence of the nucleotide sequence of M. Tuberculosis ESAT6 and Ag85 in the recombinant strain.

Thus, the results of the studies identified genetic stability of recombinant influenza virus vector expressing mycobacterial antigens in Vero cell culture without loss of antigen and reproductive characteristics during 10 passages (observation time).

Optimization of Cultivation Conditions

Infecting dose

Infecting dose from 0,1 to 0,00001 TCD_{50}/cell were tested to determine the optimal infecting dose of TB-FLU Esat6 2A Ag85A strain.

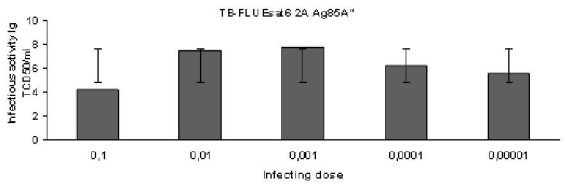


Figure 4 - Accumulation of the strain TB-FLU Esat6 2A Ag85A in Vero cell culture depending on the infecting dose

Our studies established that the level of TB-FLU Esat6 2A Ag85A strain accumulation in cell culture infected with 0,001 TCD_{50}/cell was higher as compared to other tested doses. The reliable difference was $P < 0.05$. Having applied the above mentioned infecting dose the cultivation period was 72-96 hours which contributed to the formation of full virions and time saving. The infectious activity titer was 7,83 lg TCD_{50}/cm^3. Change of the infecting dose below 0,001 TCD_{50}/cell and above 0,1 TCD_{50}/cell increased the incubation period, and significantly reduced the infectivity.

Thus, TB-FLU Esat6 2A Ag85A strain reproduction depends on the infecting dose. To obtain more active virus-containing material the cell culture should be infected with the dose of 0,001 TCD_{50}/cell.

The incubation temperature

In the next series of experiments we conducted studies to determine the effect of different incubation temperatures on the accumulation of TB-FLU Esat6 2A Ag85A recombinant strain with subsequent determination of infectious activity.

Figure 5 shows that the virus reproduction was observed at all tested temperatures. At the incubation temperature 37 °C the viral titer was reduced for not more than 2 lg TCD50/ml as compared to the titer at an optimum temperature 34 ^{0}C ($P < 0,01$). The results

of the conducted experiments established that the optimum temperature for TB-FLU Esat6 2A Ag85A recombinant strain incubation was 34° C (7,75 ± 0,08 lg TCD_{50}/cm^3).

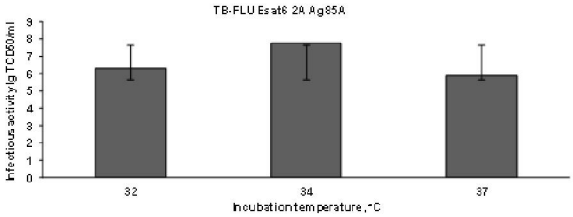


Figure 5 - The dynamics of accumulation of TB-FLU Esat6 2A Ag85A recombinant strain in Vero cell culture depending on the incubation temperature

Thus, the results of our experiments suggested that TB-FLU Esat6 2A Ag85A recombinant strain should be cultivated at the temperature (34,0 ± 0,5)°C for maximum virus accumulation.

Incubation time

To determine the period of incubation of TB-FLU Esat6 2A Ag85A recombinant strain, cell culture was infected at a dose of 0,001 TCD_{50}/cell at 34 °C with 5 % CO_2. After 24, 48, 72 and 96 hours of incubation viral suspension were frozen at -70°C for 12-14 hours.

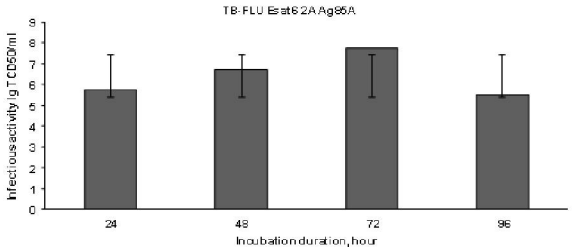


Figure 6 - Accumulation of TB-FLU Esat6 2A Ag85A recombinant strain in Vero cell culture depending on the incubation period

The research results presented in the figure 6 shows that TB-FLU Esat6 2A Ag85A recombinant strain accumulated in the largest titers at 72 hour of incubation. Infective activity was 7,75 ± 0,08 lg TCD_{50}/cm^3. Increase of incubation time up to 96 hours resulted in reduction of infective activity to 5,50 lg TCD_{50}/cm^3.

Thus, the optimal incubation period for TB-FLU Esat6 2A Ag85A recombinant strain in Vero cell culture is 72 hours.

Protease selection

Studies on the use of various proteases in the culture medium were conducted. Trypsin, from bovine pancreas, 0,25 % Trypsin-EDTA Solution produced by «SIGMA», and Accutase solution at different concentrations were used for the virus hemagglutinin proteolysis (Table 1).

Table 1 - Reproduction of TB-FLU Esat6 2A Ag85A recombinant strain in Vero cell culture with different concentrations of proteases

Strain Name	Protease	Ferment concentration	Infectious activity, lg TCD $_{50}/cm^3$, (X±m)	Titer in HA reaction, log_2
TB-FLU Esat6 2A Ag85A	Trypsin, from bovine pancreas (SIGMA)	2,0 µg/ml	7,75±0,14	7,0
		1,5 µg/ml	7,00±0,16	6,0
		1 µg/ml	6,00±0,12	3,0
		0,5 µg/ml	5,50±0,08	3,0
	0,25% Trypsin-EDTA solution SIGMA	1:50	6,25±0,16	4,0
		1:100	6,00±0,08	4,0
		1:200	6,25±0,14	5,0
		1:300	6,50±0,18	3,0
	Accutase solution	1:5	6,50 ±0,00	6,0
		1:10	6,00 ±0,12	4,0

Table 1 shows that the reproduction of TB-FLU Esat6 2A Ag85A strain in cell culture using medium containing the above mentioned proteases occurs at various degrees. The greatest accumulation of the virus was observed in a medium containing 2 mg/ml Trypsin with infective activity 7,75 ± 0,14 lg TCD_{50}/cm^3 with a titer 7,0 log_2 in HA reaction. Reliable difference was ($P < 0,2$).

When using trypsin complex the largest infectious (6,25 lg TCD_{50}/cm^3) and hemagglutinating activity (5,0 log_2) was observed at the dilution 1:200.

When we used Accutase solution at the dilution 1:5 the infective activity was (6,50 lg TCD_{50}/cm^3 and 6,50 log_2).

Thus, the results of the studies show that 0,25 % Trypsin-EDTA, Trypsin, from bovine pancreas (SIGMA) and Accutase solution are effective in certain concentrations for hemagglutinin proteolytic activation and destruction area of cell monolayer of TB-FLU Esat6 2A Ag85A recombinant influenza strain cultured in Vero cell culture.

Discussion

Currently, the traditional methods of isolation of Mycobacterium tuberculosis is less satisfying to the needs of the doctors, as microbiological research results are clearly insufficient. The applied methods are ineffective and do not enough information about the true state of mycobacterial population, vegetating inside the patient's organism. It is believed that the characteristics of the isolated strain remain unchanged over 5 passages [18, 19].

Therefore, the development of a method to preserve the biological characteristics of Mycobacterium tuberculosis during long-term cultivation is an urgent problem. According to WHO requirements influenza A virus strains cultured in embryonated chicken eggs or in cell culture of African green monkey kidney Vero (SU-614 837, 15.10.1997, 849716.) should be used for the biologicals' production.

Having used reverse genetics methods Russian scientists obtained influenza recombinant strains expressing the mycobacterial protective antigen ESAT-6 the treatment and prevention of tuberculosis. The invention is protected by the patent number 231887 [20]. Russian scientists also obtained recombinant strain of influenza virus A/Singapore/2A-ESAT6 (H2N2) using reverse genetics in MDCK cell culture obtained from ATCC (Manassas, Virginia, USA, Cat No CCL- 34) [21, 22, 23]. The cell line was maintained in DMEM medium, containing 10 % serum of cow embryos (Biolat, St. Petersburg) and 2 μg/ml of trypsin and presence of recombinant NS gene containing ESAT6 Mycobacterium tuberculosis nucleotide sequence, which was confirmed by RT-PCR product sequencing. The resulting virus is able to replicate in the MDCK cell culture up to 7,5 log at the optimum temperature of 37 °C. The virus is genetically stable for at least five serial passages in MDCK cell culture [24].

In connection with the above mentioned information, we conducted a series of experiments to develop cultivation parameters of recombinant influenza vector TB-FLU-Esat6 2A Ag85A, expressing mycobacterial antigens. We used Vero cell culture adapted to growth in serum-free medium OptiPRO at optimum temperature of 34°C. Experiments to determine the optimal conditions for virus cultivation (infection dose, temperature and incubation time) were implemented using standard methods. Stability of the virus passaged in cell culture is very important. In addition, change of the virus reproductive activity during serial passages in cell cultures is important for the development of the vaccine production technology. That is why we studied the reproductive characteristics of the recombinant strain in Vero cell culture during ten consecutive passages with the determination of biological activity after each passage. Infectious activity dynamic growth up to 7,75 lg TCD_{50}/cm^3 was observed.

TB-FLU-Esat6 2A Ag85A recombinant strain is genetically stable and able to keep recombinant proteins during 10 consecutive passages in Vero cell culture. It is characterized by the presence of recombinant NS1 gene containing the insertion of TB-FLU-Esat6 2A Ag85A M. tuberculosis nucleotide sequence between positions 400-401 of NS1 gene ORF which was confirmed by RT- PCR product sequencing.

According to Russian scientists an infecting dose of 0,001 – 0,0001 TCD_{50}/cell is the optimal dose of infecting $1*10^7$ Vero cells (flask volume 75 cm^2) which is consistent with our findings [25]. Virus accumulation in cell culture depends on a multiplicity of infection which mainly affects the duration of virus accumulation and not on the harvest. Normally $0,001\text{-}0,1TCD^{50}$ per cell (M = 0,001-0,1) is used for culture monolayer infection for virus accumulation. The virus accumulation in the cell culture is the result of multicycle reproduction. In case of dealing with cell cultures with rapid reduction of viability and viruses which do not have a short cycle of reproduction, the multiplicity of infection is more important. Therefore, we studied the effect of the multiplicity of recombinant strain infection dose in Vero cell culture. It is found that the optimal culture infective dose of the recombinant strain in Vero cell culture is 0,001 TCD_{50}/cell.

Temperature limits and temperature optimum of the virus replication is controlled by the viral genome, although the temperature also depends on the cell system. It is believed that 36-37 °C is the optimum temperature for the reproduction of most viruses. However, there are exceptions from this rule. For example, optimum temperature for rhinoviruses replication is 33-34 °C. Cattle herpesvirus 2 accumulates in primary calves cell culture at 32 °C. Influenza C virus, in contrast to influenza A and B, multiplies at 32-33 °C although it accumulates slowly. Several alpha viruses accumulate in permanent mosquito cell lines at 34,5 °C to significantly greater titer than at the optimum temperature required for the propagation of these cells. The optimal temperature for the virus replication may also be dependent on the temperature of the previous cultivation.

Studies of the incubation temperature effect on the virus accumulation showed that the optimum temperature for incubation of TB-FLU-Esat6 2A Ag85A recombinant strain was 34 °C. The

accumulation of infectious activity was up to 7,75 lg TCD_{50}/cm^3 with a term of cultivation - 72 hrs.

During virus cultivation the addition of proteases (usually trypsin) to the culture medium is necessary for the formation of highly infectious virus with accumulation of high concentrations of hemagglutinating antigens and its capacity to re-reproduction [20].

In this regard, we conducted studies to determine the influence of proteases on the process of virus cultivation.

The implemented study showed that trypsin (Sigma) at a concentration of 2 μg/ml was the most effective initiator of infection process in Vero cell culture. The proteolytic activation of hemagglutinin of TB-FLU-Esat6 2A Ag85A recombinant strain with trypsin resulted in increased virus reproductive characteristics.

Conclusion.

The results of TB-FLU-Esat6 2A Ag85A recombinant strain cultural characteristics studies showed the possibility of getting active viral biomass suitable for the development of the technology for manufacturing of TB vaccines and diagnostic means for human health care.

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