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DETERMINING OPTIMAL CONDITIONS FOR GROWING RECOMBINANT VECTORS TO BE USED IN DEVELOPING A BOVINE TUBERCULOSIS VACCINE

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Abstract
Two recombinant influenza A virus vectors expressing the ESAT 6 and TB10.4 mycobacterial proteins from the non-structural (NS) gene were constructed via reverse genetics technique to develop a specific means of prophylaxis for bovine tuberculosis.
We experimented to determine optimal conditions for growing recombinant vectors in Vero cell culture and chick embryos.
This study established that the maximum amount of virus builds up in a Vero cell culture with the Dulbecco′s Modified Eagle′s Medium (DMEM) serum-free medium. However, using cell culture to produce vector vaccines is labour-intensive and inefficient. An alternative way, a traditional, time-tested technique, is provided by growing samples in chick embryos. One of the advantages of this technique is its affordability and availability, enabling easy scale-up of vaccine production.
In the optimization experiments, the FLU-ΔNS_ESAT 6 and FLU-ΔNS_TB10.4 viruses constructed were inoculated into 10-day-old chick embryos. It was determined that the optimal incubation temperature that led to the highest virus build-up was 37 ± 0.5 °C. And the infectious activity level of the FLU-ΔNS_ESAT 6 recombinant vector was at 8.95 ± 0.07 log10EID_{50} 0.2 cm^{-3}, while that of the FLU-ΔNS_TB10.4 was at 9.20 ± 0.07 log10EID_{50} 0.2 cm^{-3}, what was provided by infectious doses of 1000–10000 EID_{50}, which makes it possible to create a virus-containing material with a hemagglutination activity level of 1:64.
The size of recombinant vector amplicons expressing proteins TB10.4 and ESAT 6 was 1170 bp and 1175 bp, respectively. Electron microscopy images confirm that the developed virions are morphologically similar to the avian influenza virus.
Key words: vector vaccines, *M. bovis*, proteins, recombinant vector, Vero cell culture, chick embryos.

Introduction

Vector vaccines are a safe type of vaccine used for preventing infections both in humans and animals (Buzitskaya *et al*., 2022). The use of an influenza vector's genetic structure is characterized by a truncation of the translated region of the NS1 protein by 50% and a replacement of its carboxyl portion with a sequence that encodes tuberculosis proteins. The choice of the target proteins is determined by the fact that these proteins are expressed at different phases of the mycobacterium's life cycle and contain characterized epitopes recognizable by CD4+ and CD8+ cells for a balanced cell-type protective immune response (Sergeeva *et al*., 2017).

Out of a great variety of proteins encoded by *M. tuberculosis* epitope genomes, only a small group is directly involved in the onset and development of the disease. The ESAT 6, TB10.4, and HspX mycobacterial proteins are highly noteworthy among this group. These proteins are the most effective immunogens widely used as potential candidates for heterologous vaccination worldwide. They are predominantly utilized in vector and DNA vaccines. These proteins are present in the *M. bovis* strain of the Bacillus Calmette-Guérin (BCG) vaccine and can be administered as a booster vaccine. Additionally, they are not incorporated into contemporary diagnostic test systems (Kuchur, 2018).

Until today, Kazakh researchers have not undertaken studies to develop a bovine tuberculosis vaccine. This poses a real nationwide biosecurity threat of a potential panzootic. The Research Institute for Biological Safety Problems has started working on a vaccine for bovine tuberculosis of the *M. bovis-8* strain to tackle this. Determining optimal conditions for growing recombinant vaccine vectors and enhancing their build-up is an important step in these efforts.

There are known techniques utilized to increase the amount of influenza virus in cell cultures during standard processes of producing influenza vaccines (Jawinski *et al*., 2019). However, influenza vaccines produced using cell cultures have significant limitations in production upscaling. This requires an alternative technique, such as one involving chick embryos (Sedova *et al*., 2012).

This study aims to determine optimal conditions for growing recombinant vectors in Vero cell culture and various days-old chick embryos to develop a vector vaccine for bovine tuberculosis.

Materials and Methods

Recombinant vectors construction

The recombinant vectors expressing the ESAT 6 and TB10.4 *M. bovis* proteins were constructed through reverse genetics in proportion genes with 5:3 H5N1 reassortants. Interferon-deficient Vero cells were transfected with a set of 8 plasmids expressing avian influenza proteins by using a Cell Line Nucleofector® Kit (Lonza, USA) (Hoffmann *et al*., 2002). Influenza virus genes were cloned into the pHW2000 plasmid. These included PB1, PB2, PA, NP, and a chimeric NS gene expressing *M. bovis* proteins ESAT 6 and TB10.4 from the A/Puerto-Rico/8/34 (H1N1) strain; hemagglutinin (HA) and neuraminidase (NA), as well as the M gene taken from the recombinant A/chicken/Astana/6/05(H5N1) strain. The synthesis and cloning were conducted at Evrogen LLP, Moscow.

Growing influenza vectors in Vero cell cultures

Vero cell cultures were acquired from the American Type Culture Collection (Sereinig *et al*., 2006).

To determine the optimal incubation temperature, the Vero cell cultures were inoculated with the FLU-ΔNS_ESAT 6 and FLU-ΔNS_TB10.4 viruses at a dose of 1000 log10$TCID_{50}$ cm^{-3} at 37 ± .5 ℃. They were then incubated at 37 ± 0.5 ℃, 33 ± 0.5 ℃ and 35 ± 0.5 ℃ and relative air humidity of 55 ± 5% with an access of 5% carbon dioxide for 24, 48, 72 and 96 hours (Ferko *et al*., 2001; Sergeeva *et al*., 2017).

Growing influenza vectors in chick embryos

Influenza vectors were grown in 10-, 11-, and 12-days old chick embryos delivered from local poultry farms free from avian influenza. Chick embryos were inoculated into the allantoic cavity with a virus in a volume of 0.2 cm^3 and checked daily by ovoscopic method using an ovoscope. The death of an embryo within the first 24 hours was considered non-specific, and such embryos were discarded. At the end of the incubation period, inoculated eggs were cooled at 2–8 °C for 16–18 hours. Embryos were inoculated via the allantoic cavity in doses of 100 to 1000000 EID_{50} to determine the optimal inoculating dose.

Determining biological activity of recombinant vectors in Vero cells

Biological activity was determined by limited dilution assay in Vero cells. The cells were seeded into 96-well plates and left to grow for 24 hours until they formed a complete monolayer. Afterwards, the cell culture medium was removed and washed 3 times with this same medium. Samples were applied to the wells in four repeats with a 10-fold dilution (0.2 cm^3 $well^{-1}$). Titers were calculated each day using the Reed-Muench method, and the results were reported as 50% tissue infectious dose ($TCID_{50}$ cm^{-3}) Reed & Muench, 1938; Korochkin *et al*., 2010).

Assessing infectious activity of recombinant vectors grown in chick embryos

The infectious activity was determined by titration on 10-12 days-old chick embryos. Embryos were infected into the allantoic cavity with a 10-fold dilution of 0.2 cm^3 in four repetitions. Infected embryos were incubated for 72 h at 26, 34, 35, 36 and 37 ± 0.5 °C, then cooled at 2–8 °C for 16–18 h. The infectious activity was calculated using the Reed-Muench method, and the results were reported as a 50% embryonic infectious dose of the virus (EID_{50} 0.2 cm^{-3}). The hemagglutination titer was determined by the hemagglutination assay. The hemagglutination titer shows the limiting dilution of the virus that causes complete agglutination of rooster erythrocytes (Reed & Muench, 1938; WHO, 2002).

PCR

The molecular weight of the chimeric NS segment of the generated recombinant vectors was analyzed by RT-PCR using a direct primer – AGCAAAAGCAGGGTGACAAAG and reverse primer – GAAACAAGGGTGTTTTTTATTA TTAAAT (Abay *et al*., 2023).

Electronic microscopy

Samples were adsorbed on Formvar-coated grids stabilized with carbon. Negative contrasting was carried out using a 2% phosphorus-tungsten water solution, and a microscopy examination was done using a transmission electron microscope JEM-100 CX-II (JEOL, Japan) at an accelerating voltage of 80 kV at 100000 magnifications. Photos were made from developed and fixed negatives on an Azov enlarger.

Statistical analysis

In this study, average values and the standard error of the examined parameters were determined. The significance of variance between results was determined in GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) statistics software. A $P < 0.05$ was considered as significant.

Results and Discussion

A reverse genetic engineering technique was used to construct the FLU-ΔNS-ESAT6 and FLU- ΔNS_TB10.4 recombinant vectors (5:3 H5N1 reassortants with a modified HA cleavage site) with an NS chimeric genome fragment that encodes protective *M. bovis* proteins within the NS1 truncated protein's reading frame. Influenza virus genes were cloned into the pHW2000 plasmid (Figure 1).

Figure 1 shows a map of the pHW-NS-ESAT 6 plasmid encoding a modified NS gene of the A/Puerto Rico/8/34 virus (H1N1), into which a sequence of *M. bovis* protein ESAT 6 was inserted (after 124th aminoacid) (A); and the pHW-NS-TB10.4 plasmid encoding a modified NS gene of the A/Puerto Rico/8/34 virus (H1N1), into which a sequence of *M. bovis* proteins TB10.4 was inserted (after 124th amino acid).

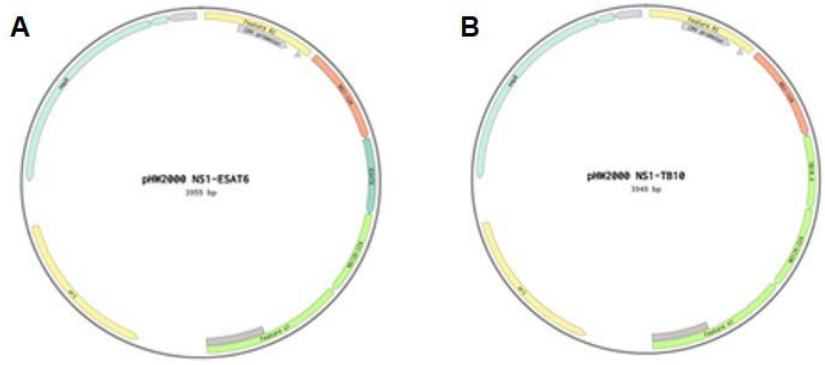

Figure 1. Map of FLU-ΔNS – ESAT 6 (A) and FLU-NS_TB10.4 (B) recombinant vectors in the pHW2000 plasmid.

A portion of transfected material was passed into cell cultures while the other portion was used to inoculate chick embryos. The culture and allantoic fluid samples obtained at this stage contained a 'zero' passage of viral samples.

The first signs of cytopathic activity in the Vero cell culture for both samples began to show 36 hours after transfection. In 48 hours, the cell monolayer was completely destroyed as a result of viral cytopathic activity (Figure 2). The presence of recombinant viruses in culture fluid samples through a hemagglutination assay (HA) were confirmed.

Then, an assessment of optimal conditions for growing viruses after transfection was performed. A day after inoculating a cell culture with the viruses, no significant cytopathic activity was observed. The first signs of cytopathic activity in the Vero cell cultures appeared after 48 hours of incubation. In 72–96 hours, the Vero cell monolayer in both cultures was completely destroyed due to viral cytopathic activity (Figure 2).

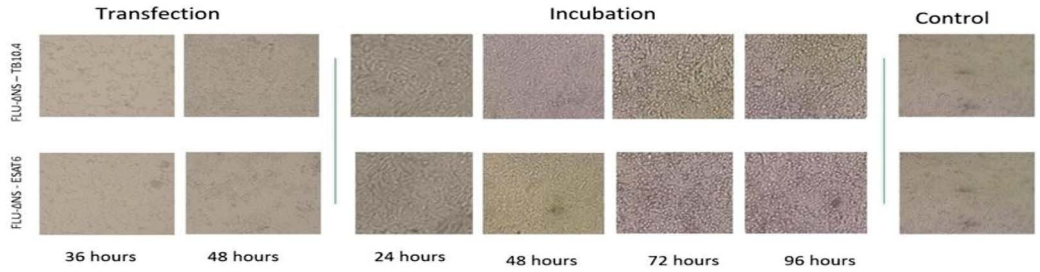

Figure 2. The cytopathic activity of recombinant influenza viruses in a Vero cell culture after transfection. The image was made on a light MicroOptix microscope at a 10x magnification.

As seen in Figure 2, recombinant strains were inoculated into Vero cell culture. The activity of the virus manifested itself starting from 48 hours, and by 96 hours of incubation, destruction of the cell monolayer was observed.

Then, several passages of recombinant vectors were performed on the Vero cell cultures to assess the virus's biological activity at each passage and different incubation temperatures (33 ± 0.5 °C, 35 ± 0.5 °C, 37 ± 0.5 °C) were examined to establish the optimal one (Figure 3).

Thus, after the incubation of recombinant vectors at various temperatures, cytopathic activity was observed in the Vero cell culture at 37 ± 0.5 °C.

Recombinant vectors' infectious activity was observed at several temperatures. However, the temperature of 37 ± 0.5 °C was optimal for incubating the vectors in the Vero cell culture monolayer because it provided the highest infectious activity, which averaged 7.50 ± 0.08 log10TCID$_{50}$ cm^{-3} for FLU-ΔNS_ESAT 6 and 7.75 ± 0.08 log10TCID$_{50}$ cm^{-3} for FLU-ΔNS_TB10.4 (Figure 3, A).

Then, experiments were conducted to assess the optimal duration of incubating recombinant vectors in a Vero cell culture monolayer. Inoculated Vero cell cultures were incubated for 24, 48, 72 and 96 hours at 37 ± 0.5 °C with relative air humidity of 55 ± 5% (Figure 3, B).

Vector growth in the Vero cell cultures was analyzed, and cytopathic activity was observed to occur in 48 hours and last for 96 hours. Both vector samples had their biological activity peak at the 72nd

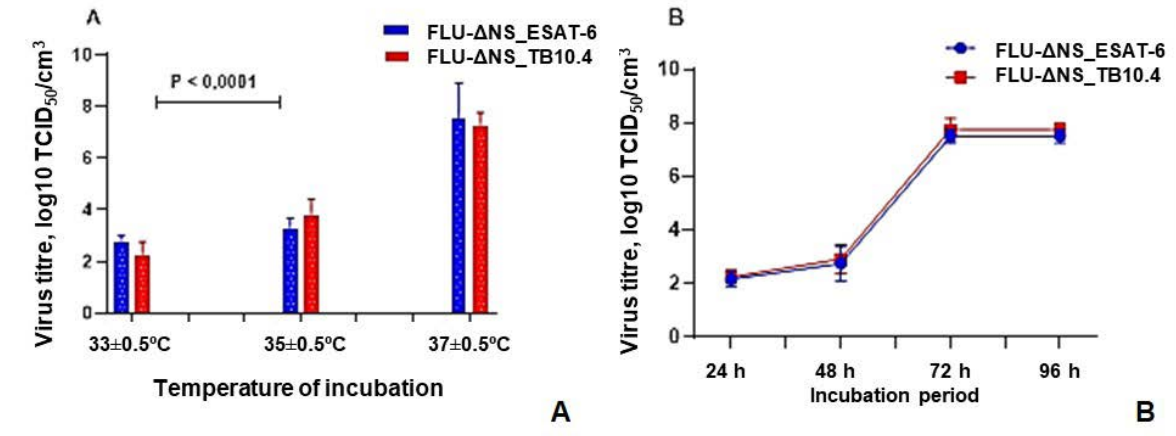

Figure 3. Selection of optimal cultivation parameters in Vero cell culture. (A) Optimal incubation temperatures (33, 35 and 37 ± 0.5 °C). (B) Biological activity levels vs duration of incubating recombinant vectors in a Vero cell at 37 ± 0.5 °C.

hour of the experiment and maintained it for 96 hours. The biological activity of FLU-ΔNS_ESAT 6 was at 7.50 ± 0.08 log10TCID$_{50}$ cm^{-3}, and that of FLU-ΔNS_TB10.4 was at 7.75 ± 0.08 log10TCID$_{50}$ cm^{-3}.

In the above experiments, the maximum damage to the monolayer in the Vero cell culture occurred after 72 hours of incubation in a DMEM serum-free nutritive medium in a CO_2 incubator at 37 ± 0.5 °C with 5% CO_2 access.

To assess the optimal conditions for incubating recombinant vectors in chick embryos, the optimal age of embryos had to be determined. At the same time, the impact of incubation temperature on the amount of recombinant vector build-up was assessed. Inoculated chick embryos were incubated at various temperatures (26, 34, 35, 36 and 37 ± 0.5 °C) in a relative air humidity of 55±5% and incubated for 72 hours (Figure 4). Experiments involving incubation at 26 ± 0.5 °C and 34 ± 0.5 °C yielded negative results as almost no viral growth was observed in these temperatures.

As shown in Figure 4, the FLU-ΔNS_ESAT 6 and FLU-ΔNS_TB10.4 recombinant vectors were inoculated into embryos, and the embryos did not die during the incubation. The highest build-up amounts were observed in 10 days old embryos incubated at 37 ± 0.5 °C. The FLU-ΔNS_ESAT 6 recombinant vector showed a high hemagglutination activity level (of 1:64) with the infection activity at 8.95 ± 0.07 log10EID$_{50}$ 0.2 cm^{-3}. The FLU-ΔNS_TB10.4 recombinant vector also had its hemagglutination activity level at 1:64, while its infectious activity was at 9.20 ± 0.07 log10EID$_{50}$ 0.2 cm^{-3}.

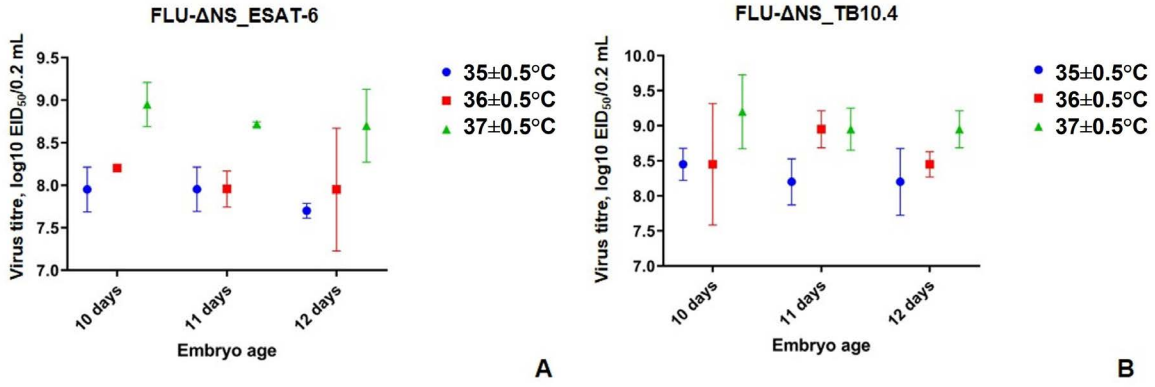

Figure 4. Characteristics of recombinant vectors' growth in 10-, 11- and 12-day' chick embryos and incubated at 35.0, 36.0 and 37.0 °C ± 0.5. (A) growth of FLU-ΔNS_ESAT 6. (B) growth of FLU-ΔNS_TB10.4. The infectious titres are expressed in EID$_{50}$ cm^{-3}.

The rate of virus build-up was determined to be associated with the inoculating dose. The experiment used the following incubation parameters: temperature of 37 ± 0.5 °C, relative air humidity of 55 ± 5%, and

chick embryos age of 10 days. The embryos were inoculated via the allantoic cavity in doses of 100 to 1000000 EID_{50} (Table 1).

All of the above doses (with the other incubating parameters remaining the same) led to an increase of both the infectious activity (from 7.45 ± 0.14 to 9.20 ± 0.12 log10EID_{50} cm^{-3}), and hemagglutination activity (from 1:16 to 1:64). However, the use of minimal doses (10 and 100 vs. ≥1000EID_{50}, with P value <0.0001 to 0.01) led to predominantly lower hemagglutination activity results, while the use of higher doses (1000000 vs. ≤ 100000 EID_{50}, P value <0.0001 to 0.02) led to lower infectious activity results. Based on these, 1000-10000EID_{50} was selected as the optimal dosage for inoculating chick embryos. Both doses provided equally high infectious activity titers for all of the recombinant vectors tested (difference between strains, P value >0.99 to 0.13). Also, with these doses, hemagglutination activity titers were comparable to those produced by higher doses (P value 0.99 to 0.24) (Table 1).

Table 1

Viral build-up rate based on the inoculation dose (X ± m, n=3)

Embryonated egg inoculation dose, EID_{50}	Hemagglutination		Infectious log10EID_{50}/cm3	
	FLU NS_ESAT 6	FLU NS_TB10.4	FLU NS_ESAT 6	FLU NS_TB10.4
~10	1:32	1:16	7,45 ± 0.14	8.95 ± 0.14
~100	1:32	1:32	7.95 ± 0.08	9.45 ± 0.08
~1000	1:64	1:32	8.45 ± 0.17	9.70 ± 0.17
~10000	1:64	1:64	8.95 ± 0.24	9.70 ± 0.24
~100000	1:64	1:64	8.45 ± 0.2	9.45 ± 0.2
~1000000	1:64	1:64	8.20 ± 0.12	9.20 ± 0.12

An assessment of the stability of the mycobacterial insertion in the NS gene was performed using RT-PCR. The NS gene of the studied recombinant vector samples was compared with that of a wild avian influenza virus. The RT-PCR investigation confirmed that the length of the NS gene's segment containing protein inserts was elongated and comprised 1170 b.p. for TB10.4 and 1175 b.p. for ESAT 6.

The recombinant vector samples were examined by electron microscopy (Figure 5).

The electron microscopy examination shows that the virus assembly was completed successfully. The virions are sphere-shaped and coated with a supercapsid formed by a lipid bilayer with 10 nm glycoprotein spikes responsible for hemagglutination activity.

The most economically viable means of preventing infections is immunization. Animal vaccines using causative agents' genetic material delivered by virus vectors is a new vaccine technology. The potential of virus vectors as candidates for new vaccines is based on their ability to express any foreign proteins in vivo with or without modification (de Vries & Rimmelzwaan, 2016).

Influenza viruses as a vector are safer than other viruses because of influenza's inability to integrate

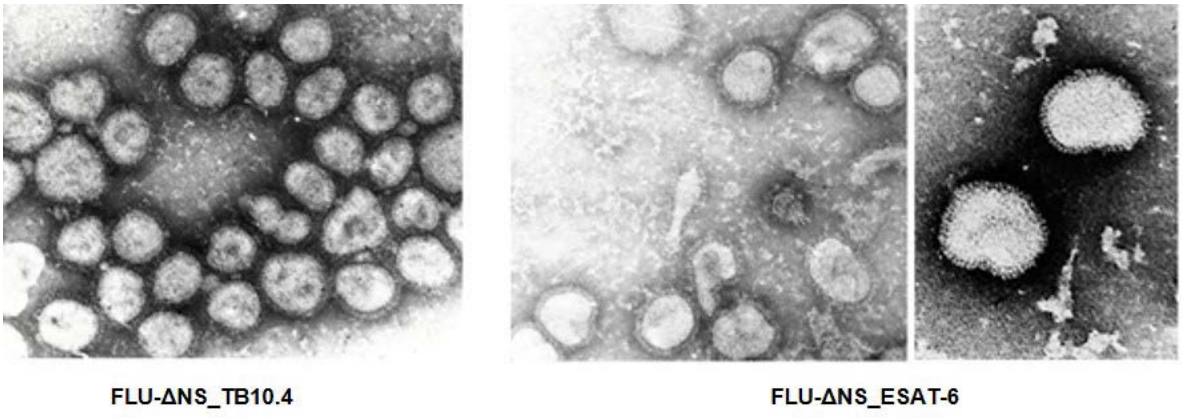

Figure 5. Electron microscopy image of recombinant vectors.
The photo was taken at 100000x magnification with a JEM-100 CX-II JEOL transmission electron microscope (Japan).

into the recipient's chromosomes (Ferko *et al*., 2001).

This study examines an influenza vector with a modified NS genome containing a sequence encoding a *M. bovis* protein.

In the study, the maximum amount of viruses built up when grown in a Vero cell culture using the DMEM serum-free medium at 37 ± 0.5 ℃ with an access of 5% carbon dioxide. Both recombinant vector samples peaked their biological activity at 72 hours of the experiment and maintained it for 96 hours, with the biological activity of FLU-ΔNS_ESAT 6 reaching 7.50 ± 0.08 $log10TCID_{50}$ cm^{-3} and that of FLU-Δ NS_TB10.4 reaching 7.75 ± 0.08 $log10TCID_{50}$ cm^{-3}.

It should be noted that RIBSP researchers successfully used this approach previously to create a human tuberculosis vaccine based on influenza virus vectors expressing proteins ESAT 6 and Ag-85 (*M. tuberculosis*). Viruses were also grown in a Vero cell culture in a DMEM serum-free medium to produce this human vaccine. Research on animal models, such as mice and guinea pigs, showed the vaccine to be completely safe (Buzitskaya *et al*., 2022). Moreover, it was established that vaccinated animals developed a strong antigen-specific T-cell immune response and a high level of protection, one that is not inferior to that offered by a commercial Bacillus Calmette–Guérin (BCG) vaccine (Shurygina *et al*., 2014; Stukova *et al*., 2014).

If the above incubating conditions are observed, it is possible to consistently produce virus-containing material with an infectious activity level not less than 7 $log10TCID_{50}$ cm^{-3}, which is well suited for preparing a vector vaccine for bovine tuberculosis. However, the production of vector vaccines using primary animal cell cultures is characterized by a high level of labour intensity and low efficiency and requires rigorous aseptic techniques and highly skilled personnel. Moreover, animal cells may not survive in isolation and, therefore, are incapable of independent sustainable existence without an artificially created complex environment. One of the key limitations of cell cultures is their high cost and a small number of resulting cells. In cell cultures, the heterogeneous cells undergo multiple manipulations in consecutive passages, eventually departing from the original composition (Grachev & Khapchaev, 2008; Anaya *et al*., 2013).

The use of chick embryos to grow viruses in vaccine production is a conventional process that has stood the test of time. The advantage of this culturing system is the fact that it is economically affordable and readily available whenever there is a need to grow viruses, which makes vaccine production highly scalable. Also, studies of A-type recombinant influenza viruses grown in chick embryos have established the stability of NA and HA genes (Nakowitsch *et al*., 2013; Rajaram *et al*., 2020).

In 10-day-old chick embryos, the optimal temperature for the highest virus growth was 37 ± 0.5 °C. At this temperature, the FLU-ΔNS_ESAT 6 recombinant vector's infectious activity level was at 8.95±0.07 $log10EID_{50}$ 0.2 cm^{-3} while that of FLU-ΔNS_TB10.4 was at 9.20 ± 0.07 $log10EID_{50}$ 0.2 cm^{-3}. According to the study, the optimal infectious dose for the recombinant FLU NS_ESAT 6 and FLU NS_TB10.4 vectors in 10 days old chick embryos is 10000 EID_{50}. This dose makes it possible to produce virus-containing material with a hemagglutination activity level of 1:64 (Figure 4 and Table 1).

M. bovis protein inserts in the NS gene remained stable when passaged in both the Vero cell culture and chick embryos.

The conditions for growing recombinant vectors in chick embryos determined by this study to be optimal are suited for preparing a safe and effective new-generation vaccine for bovine tuberculosis.

Conclusions

Developing a domestic vaccine for bovine tuberculosis remains a priority for Kazakhstan's veterinary biotechnology industry. Different vaccine production technology and composition options are available. Vector vaccines are considered to be the safest vaccines, especially those using influenza vectors. In this study, recombinant vectors were constructed and inoculated in cell cultures and chick embryos to grow viruses. The vectors produced were highly stable and biologically active and, therefore, could be used to develop a vaccine for bovine tuberculosis.

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