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Features of *B. anthracis* Vaccine Strains for the Production of Anthrax Vaccines

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Abstract

This article presents the results of a comparative study of the cultural, morphological, biological and protective properties of current or proposed vaccine strains against anthrax *B. anthracis* 34F2, 55-VNIIVViM, STI-1, 1190-R - Stamatin, Pasteur, Shuya-15, 916- 1, No. 94, Mato, Ihtiman. All strains are attenuated. The mass fraction of spores of strains after cultivation on beef-peptone agar (MPA) was 90–98%. ImD50 of vaccine strains for guinea pigs was: 55-VNIIVViM - 0.34; 34F2 and STI -1 - 0.58; Ihtiman - 0.76, Shuya-15, 916-1, No. 94, Mato- 1.71, 1190-R - Stamatin - 3.14; Pasteur - 0.05 million spores. *B. anthracis* strains 55-VNIIVViM, 34F2, STI-1 at a dose of 10 ImD50 and *B. anthracis* Pasteur strain at a dose of 5 ImD50 provided protection against infection by a culture of the control weakly virulent *B. anthracis* M-71 capsular strain at a dose of 1.0 million live spores of 100% immunized guinea pigs.

Discipline: Veterinary bacteriology.

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1 Introduction

The epizootic situation of anthrax in the territory of the Russian Federation in the last decade remains without significant changes. In 2013, 2014, 2015, 2016 and 2018 registered 3, 3, 2, 6, and 2 unfavorable points, respectively [5].

In 2019, 5 cases of anthrax in humans were registered in the Russian Federation, including 4 in the Republic of Dagestan and 1 in the Stavropol Territory [10]. In 2019, anthrax was noted in

Armenia, Kyrgyzstan, Azerbaijan, Kazakhstan and Tajikistan, 2 epizootic foci each, and 1 in Belarus [6].

In 2020, in the Russian Federation, an anthrax outbreak with the registration of the disease of one head of cattle and five cases of infection among people was recorded in the subject of the North Caucasus District - the Republic of Dagestan [11]. In 2020, 5 outbreaks were recorded in Kyrgyzstan in 3 regions anthrax, in Kazakhstan and Georgia - 1 each, in Azerbaijan - 4 [7].

For three quarters of 2021, one unfavorable point was identified on the territory of the Russian Federation in the Republic of Tyva. In 2021, there were two unfavorable points in Armenia [8, 9].

Anthrax is currently a potentially important agent of biological terrorism and a threat to civilization [12,13,14].

To date, the fight against anthrax in animals around the world is still based on preventive vaccination. For this purpose, in veterinary practice in most foreign countries, a live vaccine from a capsuleless culture of the B. anthracis strain Stern ($34F_2$) is used. In the Russian Federation, a vaccine from a capsuleless strain of B. anthracis 55-VNIIVViM is used to prevent disease in animals.

For the immunization of people in a number of European countries and the United States, a vaccine from the B. anthracis V 770-NP1-R strain is used. The filtrate of this culture, which does not contain bacterial cells, is adsorbed on an aluminum hydroxide adjuvant (amphogel) and contains all three components of the toxin: a protective antigen (PA), lethal factor (LF) and edema factor (EF) [15].

In Russia, for human immunization, a vaccine from a capsular strain of B. anthracis STI-1 is used [16].

The formation of a post-vaccination immune response correlates with the genetic characteristics of B. anthracis vaccine strains, which determine their antigenicity, reactogenicity, and residual virulence, which in turn determines the ability of microorganisms to multiply and persist for a long time in the animal body.

Despite the high protective properties, many modern live vaccines against anthrax from attenuated strains used in veterinary practice can cause post-vaccination local and general manifestations of reactogenicity (erythema, induration, soreness, edema, fever, sometimes death) in certain animal species, in particular in goats and horses [16].

Periodically, after vaccination, mainly in goats and less often in horses, an increase in body temperature and the development of edema at the injection site are observed, which spreads to the breast area (Figure 1), submandibular space and head, regional lymph nodes increase and the general condition worsens (up to lethal outcome) [8].

Figure 1: Swelling at the injection site

There is no unified public database for the registration of cases of post-vaccination complications in animals in the Russian Federation, however, it is known that in 2013, 2014 and 2015. 5, 4 and 9 cases of post-vaccination complications in goats were identified, respectively.

In 2016, it was reported that 16 out of 60 breeding Alpine goats vaccinated against anthrax had complications in the form of edema of a doughy consistency, without fever. In the same year, post-vaccination complications were reported in 28 goats out of 35 immunized, 3 goats died.

In 2017, the death of 4 Cameroonian goats was established after the introduction of the anthrax vaccine. In animals, edema in the dewlap area, pain on palpation, anemic mucous membranes, and signs of intoxication of the body were noted. In the same year, adverse post-vaccination reactions were registered in 153 and the death of 2 out of 159 goats who were vaccinated against anthrax.

In 2018, post-vaccination complications were registered in 20 Alpine goats, one of them died.

In 2021, after immunization against anthrax of 1,000 Saanen goats imported from abroad, 200 of them had post-vaccination complications that required the use of antibiotics and the administration of anthrax globulin.

Post-vaccination complications after the use of anthrax vaccines are typical not only for our country. There is evidence of severe reactions in goats and llamas, presumably associated with the action of saponin, which is part of the adjuvant of the applied vaccine [17].

In the Russian Federation, animals with severe post-vaccination reactions are treated with anthrax immunoglobulin, antibiotics, and symptomatic agents. As a rule, they recover, but they do not develop post-vaccination immunity. As a result, non-immune animals remain in the farms, susceptible to infection with anthrax.

The solution to this problem requires additional testing in order to establish the possibility of improving the currently used anthrax vaccines, both in terms of optimizing the value of immunizing doses for highly reactive animals, and in terms of increasing the duration of maintaining tense immunity, through the use of adjuvants and the development of a new vaccine.

In this regard, the purpose of this work is a comparative study of the cultural-morphological, biological and protective properties of the above and other relevant vaccine strains and the

selection of strains for the creation of a harmless and immunogenic vaccine for animals of different species.

2 Method

We used in our work:

- cultures of strains B. anthracis $34F_2$, 55-VNIIVViM, STI-1, 1190-R - Stamatin, Pasteur, Shuya-15, 916-1, No. 94, Mato, Ikhtimani M-71, which were obtained from the All-Russian State Collection of Strain's microorganisms used in veterinary medicine and animal husbandry (FGBU "VGNKI");

- laboratory animals (white mice and guinea pigs);

- nutrient media - Columbian blood agar, MPB, MPA, Kazan medium.

The quality control of these strains was carried out in accordance with regulatory documents and passport data.

Plasmid composition and attenuation of cultures of vaccine strain 55-VNIIVViM, 1190-R-Stamatin, $34F_2$, STI-1, Pasteur, Shuya-15, 916-1, No. 94, Mato and Ihtiman to Bacillus were established by genetic typing using the test system "SIB-DIF" (FGBUN TsNIIE Rospotrebnadzor).

Tinctorial and morphological properties and motility of bacteria were assessed by microscopy. Cultural properties - by the nature of growth in liquid and solid nutrient media; hemolytic activity - according to the growth of their cultures on MPA containing 5% defibrinated sheep blood. Phagosensitivity of the cultures was tested using the anthrax diagnostic bacteriophage Fah-VNIIVViM, taking into account the presence of a zone of strain culture growth inhibition around the site of bacteriophage application.

The mass fraction of spores was determined in the “crushed drop” preparation under a microscope. At least 200 anthrax spores and rods were counted separately in each field of view. Further, the mass fraction of C1 spores, %, was calculated by the formula:

$$C_1 = \frac{N_4 \times 100}{N_3} \quad (1)$$

where N_4 is the number of anthrax spores counted; 100 - constant coefficient; N_3 is the total number of counted cells (spores and rods).

Capsule formation was studied using the Kazan medium, which consisted of 60% natural mineral (bicarbonate magnesium-calcium-sodium) sterile water and 40% sterile unpreserved bovine blood serum. After 18 hours of incubation at 37 ± 1 °C, smears were made from microbial cultures, stained with Loeffler's blue and viewed under an immersion microscope system.

After titration of cultures of vaccine strains in the experiment on guinea pigs, ImD_{50} was determined using suspensions of lyophilized cultures of strains B. anthracis 34F2, 55-VNIIVViM, STI-1, 1190-R-Stamatin, Pasteur, Shuya-15, 916-1, No. 94, Mato, Ihtiman in a sterile isotonic 0.9% sodium chloride solution with a pH of 7.2. For this purpose, successive dilutions of suspensions with different concentrations of live spores were prepared.

Suspensions of cultures of the studied strains in each dilution were administered to 6 clinically healthy guinea pigs in a volume of 0.5 cm^3 subcutaneously in the abdomen. After 14 days, immunized and 6 control (intact) animals were infected with a culture of a weakly virulent capsular strain Bacillusanthracis M-71 at a dose of 1.0 million live spores, which was also injected subcutaneously into the abdomen in a volume of 0.5 cm^3. The animals of the experimental and control groups were observed for 10 days.

ImD_{50} for each strain was calculated by the formula:

$$lgIMD_{50} = lgD - lg\,\sigma\,(\Sigma\ Li - 0.5) \quad (2)$$

where D is the maximum of the tested doses; lg σ is the logarithm of the ratio of each subsequent dose to the previous one, i.e. logarithm of dilution number; Li is the ratio of the number of animals that survived after infection to the total number of guinea pigs that were injected with each dose of the strain culture; Σ Li is the sum of Li values found for all tested doses; 0.5 is a constant coefficient.

Residual virulence of anthrax vaccine strains B. anthracis $34F_2$, 55-VNIIVViM, STI-1, 1190-R - Stamatin, Pasteur, Shuya-15, 916-1, No. 94, Mato, Ihtiman was determined in the experiment on white mice (18 - 20 d) and guinea pigs (body weight 350 - 400 g). A suspension of strain cultures was administered to animals intraperitoneally and subcutaneously, respectively, at a dose of 10 ImD50, with the exception of the Pasteur capsular strain, which was administered at a dose of 5 ImD_{50} in a volume of 0.5 cm^3. The animals were observed for 10 days.

The immunogenicity of vaccine strains was tested on guinea pigs (body weight 350–400 g). A suspension of strain cultures was injected subcutaneously into animals at 10 ImD_{50}, except for the Pasteur capsular strain, which was injected at a dose of 5 ImD_{50} in a volume of 0.5 cm^3. 21 days after vaccination, all immunized and control (intact) animals were infected with a culture of weakly virulent capsular strain Bacillusanthracis M-71 at a dose of 1.0 million live spores. Which was also injected subcutaneously into the abdomen in a volume of 0.5 cm^3. The animals of the experimental and control groups were observed for 10 days.

3 Result and Discussion

According to the results of the studies, all tested strains, with the exception of Pasteur's Bacillus anthracis, revealed the presence of only one pXO1 plasmid containing the genes that determine the synthesis of a three-phase toxin (pXO1+/pXO2-), and in Pasteur's Bacillus anthracis, the pXO2 plasmid containing genes that determine the synthesis of the capsule (pXO1-/pXO2+). The results of a comparative study of the cultural, morphological and immunobiological properties of cultures of anthrax vaccine strains are presented in Table 1.

As can be seen from the table, the cultures of all B. anthracis vaccine strains used in the work were immobile.

The mass fraction of spores in all strains after cultivation on meat-peptone agar (MPA) at a temperature of 35 °C for 7 days was 90–98%.

Table 1: Comparative characteristics of cultures of anthrax vaccine strains

Name of B. anthracis strains	Quality indicators of vaccine strains										
	Mobility	Mass fraction of spores, %	Capsulation	Phage sensitivity	Hemolytic activity	Gram stain	Growth per MPA	Growth in BCH	ImD50, million	Residual virulence in white mice and guinea pigs survived/died	Immunogenicity of the strain,%
55-VNIIVViM	-	95	-	-	-	+	R- form	1	0,34	6/4; 10/0	100
34F2	-	95	-	+	-	+	R- form	2	0,58	9/1; 10/0	100
STI-1	-	98	-	+	-	+	R- form	3	0,58	9/1; 10/0	100
1190-R tamatin	-	90	-	+	-	+	R- form	2	3,14	7/3; 10/0	80
Ihtiman	-	95	-	+	-	+	R-RO-forms	4	0,76	9/1; 10/0	80
Pasteur	-	98	+	+	-	+	R-и RO-forms	4	0,05	0/10; 10/0	100
Shuya -15	-	95	-	+	-	+	R- и RO-forms	4	1,71	10/0; 10/0	70
№94	-	97	-	-	-	+	R- form	2	1,71	10/0; 10/0	60
916-1	-	95	-	-	-	+	R- form	2	1,71	10/0; 10/0	60
Mato	-	95	-	+	-	+	R- form	2	1,71	9/1; 10/0	80

Note - phagosensitivity: (+) - transparent zone of lysis, (-) - the absence of a zone of lysis; growth on MPA: (R-form) - grayish colonies, rough with processes, (RO-form) - grayish-white colonies with processes, elevated center); growth on the BCH (1) - transparent, on the surface of the medium - a parietal ring, at the bottom of the tube - loose sediment, (2) - transparent, at the bottom of the tube - loose sediment, (3) - transparent BCH, at the bottom of the tube - filamentous formations, (4) – BCH with slight opalescence, loose sediment at the bottom of the tube

All strains, with the exception of the Pasteur strain, did not form a capsule.

In the test for phage sensitivity, the cultures of most strains grew on a dense nutrient medium with the formation of a zone of lysis around a drop of bacteriophage, with the exception of strains 55-VNIIVViM, 916-1 and No. 94, which gave a continuous gentle growth of anthrax culture.

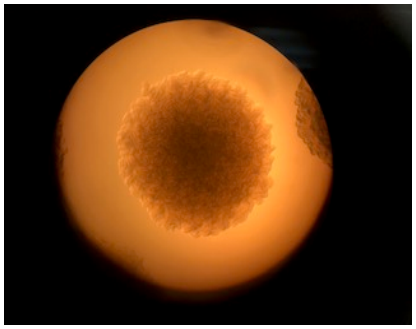

Figure 2: Culture colony of B. anthracis strain 55-VNIIVViM grown on MPA (Magnification 100x).

All the studied strains did not cause hemolysis of erythrocytes when cultivated on blood agar for 24 hours.

The typical growth of cultures of strains on nutrient media in different tests is also shown in Figures 2-6.

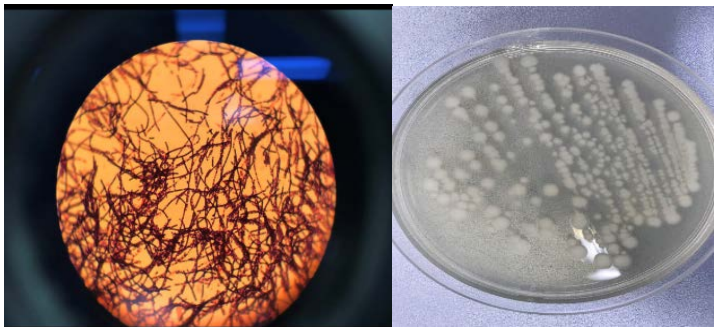

Figure 3: Microscopic appearance of a Gram-stained smear (Magnification 1000X) and typical culture growth of B. anthracis 34F2 strain on MPA.

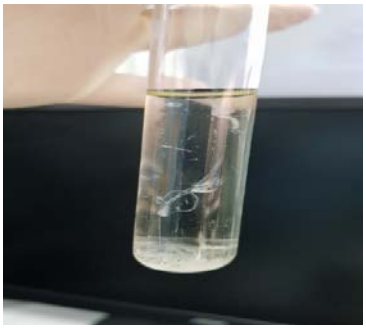

Figure 4: Typical culture growth of B. anthracis STI-1 strain in BCH.

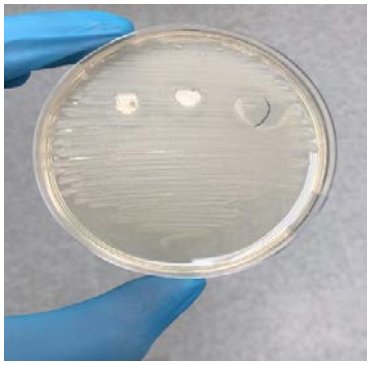

Figure 5: Phagosensitivity of B. anthracis STI-1 strain culture

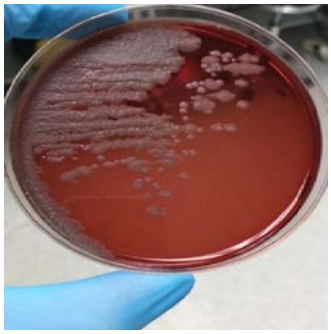

Figure 6: Absence of hemolysis when cultured with B. anthracis $34F_2$ strain on blood agar

The values of ImD_{50} of vaccine strains for guinea pigs were established: 55-VNIIVViM - 0.34; $34F_2$ and STI -1 - 0.58; Ihtiman - 0.76, Shuya-15, 916-1, No. 94, Mato- 1.71, 1190-R - Stamatin - 3.14; Pasteur - 0.05 million spores.

The residual virulence of the strains was determined using them at a dose of 10 ImD_{50}, with the exception of the Pasteur-5 ImD_{50} strain, on white mice and guinea pigs. Of 10 white mice in each group, which were injected with cultures of strains 55-VNIIVViM, $34F_2$, STI-1 and 1190-R - Stamatin, Ikhtiman and Mato at a dose of 10 ImD_{50}, 6, 9, 9, 7, 9, 9, respectively, survived individuals, and with the introduction of cultures Shuya-15, 916-1, No. 94, the death of animals was not observed. After the injection of a culture of the Pasteur strain at a dose of 5 ImD_{50}, all out of 10 mice died.

In the experiment on guinea pigs, the death of animals was not observed within 10 days when they were injected with the studied cultures of vaccine strains.

According to the results of monitoring the immunogenic activity of strains, 100% of guinea pigs immunized with cultures of B. anthracis 55-VNIIVViM, $34F_2$, STI-1 vaccine strains at a dose of 10 ImD_{50} and B. anthracis Pasteur strain at a dose of 5 ImD_{50} remained alive after infection with a culture of a control weakly virulent capsular strain B. anthracis M-71 at a dose of 1.0 million live spores. With a similar infection in groups of animals immunized at a dose of 10 ImD_{50} cultures of strains of B. anthracis 1190-R, Stamatin, Ihtimani Mato, 80% survived; Shuya-15 - 70%; Nos. 94 and 916-1 - 60% of individuals.

4 Conclusion

When performing these comparative studies, the main properties and immunobiological characteristics of anthrax vaccine strain B. anthracis 55-VNIIVViM, $34F_2$, STI-1, Pasteur, 1190-R - Stamatin, Ikhtiman, Shuya-15, No. 94, 916-1 and Mato. This confirms the presence in the institution's collection of cultures of the most known and relevant attenuated strains of B. anthracis currently used for the manufacture of vaccines or proposed as vaccines.

The results of the performed studies allow us to conclude that it is expedient to use four vaccine strains of Bacillus anthracis $34F_2$, 55-VNIIVViM, STI-1, Ikhtiman in further work and, as a first step, conduct a whole genome sequencing of these strains.

When choosing a vaccine strain for the production of anthrax vaccines, we consider it appropriate, along with the control of immunogenic activity, to evaluate the strains by residual virulence, and, in accordance with the results obtained, to vary the amount of the immunizing dose for animals of different species.

5 Availability of Data and Material

Data can be made available by contacting the corresponding author.

6 Acknowledgement

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