Laboratory tests of the horse strangles vaccine

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Abstract. An inactivated vaccine against horse strangles was developed based on the Streptococcus equi strain "N-5/1", which has sufficient efficacy and environmental safety. The vaccine was tested in accordance with the order of the Ministry of Agriculture of the Russian Federation No. 101 dated March 6, 2018 “On approval of the rules for preclinical studies of a medicinal product for veterinary use, clinical study of a medicinal product for veterinary use, and bioequivalence of a medicinal product for veterinary use”. According to the results of the tests, the absence of toxicity and irritating activity during intragastric administration of the vaccine to white mice at the maximum permissible dose (1 cm³) was established. The vaccine withstands the test for pyrogenicity, does not cause an increase or decrease in body temperature in animals during the observation period. A sufficiently high immunogenicity in laboratory animals of an inactivated vaccine was determined with a single injection (up to 80%), which meets the general requirements for the immunogenicity of microorganism strains in the composition of inactivated vaccines.

1. Introduction
Horse breeding is one of the main branches of animal husbandry developing in many countries of the world. Restoring livestock, increasing the productivity of herd horse breeding and obtaining high-quality products along with other factors, depend on the effectiveness of veterinary activities, including measures to prevent infectious diseases. The most common is the horse strangles, especially among young horses, which is caused by a streptococcus - Streptococcus equi. This disease has been known since ancient times as the cause of acute upper respiratory tract disease in horses [1]. This disease is characterized by fever, mucopurulent nasal discharge and acute edema, followed by the formation of an abscess in the lymph nodes of the head.

Horse strangles is widespread throughout the world and causes enormous economic damage to horse breeding. So, horse strangles is most common in the Novosibirsk, Altai, Irkutsk regions, Krasnoyarsk and Altai Territories, the Republic of Khakassia, Sakha (Yakutia) of the Russian Federation, as well as in Kazakhstan, Kyrgyzstan, Mongolia [2-7]. The available literature contains data on equine disease in Egypt [8], Korea [9] and Brazil [10].

In the Republic of Sakha (Yakutia), the incidence of young horses by horse strangles is 57.8 - 62.7% of the total population, mortality depending on the development of the epizootic process is 4.0 - 22.0% [5]. In the Republic of Kazakhstan, incidence is 30.1 - 46.7% of young animals, mortality reaches 16.0 - 28.3% [6]. In Mongolia and the Republic of Sakha (Yakutia), the spread of infection and an increase in the incidence of horse strangles are associated with a decrease in the...
immunobiological reactivity of animals in extreme climatic conditions and the preparation of koumiss, in the production of which young horses receive less milk from their mothers [5, 7].

The most effective and low-cost measure in the fight against horse strangles is vaccination. In the modern world, different types of vaccines are tested, manufactured and applied (inactivated, attenuated, living). Earlier (1943-1966) vaccines proposed from living, weakened and killed strains of S. equi did not find practical application [11-15]. Nevertheless, scientists from around the world continue to develop vaccines for horse strangles, which also did not find wide practical application. Thus, in the Netherlands, a live vaccine against horse strangles was developed from the deposited strain of S. equi TW 928 (No. CBS 813.95, Centraalbureau voor Schimmelcultures, P.O. box 273, 3740 AG Baam, The Netherlands). The authors of this vaccine claim that it can be used for animals from birth in various forms (parenteral, intramuscular, subcutaneous, intradermal, oral, intranasal) [16]. However, the vaccine is not registered in Russia. Currently, the USA has developed and is using a live vaccine from an attenuated strain that causes the production of serum antibodies in 7-10 days; and a dual-use modified Pinnacle IN intranasal vaccine. These vaccines are not used in Russia, and these vaccines require two- and three-fold administration with an interval of several weeks, which is also inconvenient for practice.

Kazakhstan has developed an inactivated subunit vaccine from the strain S. equi US-15, the “KazNIVI” vaccine and the “Akyntay” vaccine [17, 18], which contain antibiotics. In 1996-2000 at the Yakut Scientific Research Institute of Agriculture, an effective inactivated vaccine against horse strangles with the immunomodulator polyribonucleate [5] was developed and introduced into production. In the manufacture of the vaccine there was used strain S. equi “N-34” (certificate of deposit No. 988/27 of 12/17/1993). However, due to the relatively high cost of vaccine components, specific prophylaxis did not cover most horse breeding units and farms. The high cost of the vaccine was due to the high cost of the immunomodulator. Currently, the registration deadline for this vaccine has expired, as well as the strain of S. equi, from which the vaccine is made, has lost its specific properties. Therefore, in order to widely use the effective method for the prevention of horse strangles, it is necessary to isolate a new strain of S. equi and to find environmentally friendly, effective and relatively cheap components of the vaccine preparation.

According to the results of our previous studies, the horse strangles vaccine should contain an immunomodulator that enhances the immunogenicity of inactivated vaccines, stimulates the immunobiological reactivity and antibacterial component. It is known that the use of antibiotics in the prevention and treatment of horse strangles is one of the most common methods in the fight against strangles. This leads to the suppression of beneficial intestinal microflora, on which the work of the gastrointestinal tract, the immune system and all metabolic processes of the animal organism depends, as well as the appearance of antibiotic-resistant bacteria strains [5, 19]. Thus, the use of antibiotics becomes inappropriate during the development of organic animal husbandry.

Based on the foregoing, the aim is to develop a new vaccine against the horse strangles, which has sufficient efficiency and environmental safety.

2. Experimental part
In the manufacture of the vaccine, S. equi strain “N-5/1” was used, which was deposited in the All-Russian state collection of microorganisms strains used in veterinary medicine and animal husbandry FSBI VGNKI (registration number VKShM-B-141P, certificate of deposit dated May 22, 2018). The strain S. equi "N-5/1" isolated from the abscess of the submandibular lymph node of the foal with a horse strangles. The strain in its tinctorial, morphological, cultural, biochemical, antigenic properties and the ability to accumulate bacterial mass meets the requirements for vaccine strains.

To accumulate the bacterial mass for the purpose of manufacturing the vaccine, meat-peptone broth (MPB) with 1% glucose with the addition of horse serum was used. After growing the bacterial base, they were inactivated with a 0.04% formalin solution with an active formaldehyde content of at least 36%. Aluminum hydroxide was used as adjuvant.
An immunomodulator - culture fluid (CF) from *Bacillus subtilis* TNP-3 bacterial strain (registration number RCAM04759, certificate of deposit dated 12/27/2017) was added to the vaccine. The strain was cultured for 5 days in meat-peptone broth at a temperature of + 37 °C. To separate the culture fluid (CF), the bacterial mass containing 1 billion microbial cells was centrifuged at 7000 rpm within 15 minutes. It was filtered through membrane filters into sterile bottles, heated in a water bath at a temperature of 95 °C for 15 minutes. CF was added to the finished vaccine in a ratio of 2:1. The vaccine is a clear liquid of light yellow color with a white precipitate, which, when shaken, easily breaks into a uniform suspension.

Laboratory tests of the vaccine against horse strangles were carried out on outbred laboratory mice of both sexes of 5-8 week olds weighing 18-20 grams and healthy male rabbits weighing 1.5-1.6 kg according to the parameters of table 1.

All laboratory studies were carried out in compliance with ethical duty in relation to laboratory animals in accordance with GOST R 53434-2009 from 12.12.2009 “Principles of good laboratory practice”.

### Table 1. Parameters of preclinical studies of the experimental series of vaccine against strangles.

| Indicator                                      | Characteristic and norm                                      |
|------------------------------------------------|-------------------------------------------------------------|
| Appearance, color                              | The vaccine is a clear liquid of a light yellow color with a white precipitate, which, when shaken, easily breaks up into an even suspension. |
| The presence of mechanical impurities, mold, cracked vials, violation of the corking of vials | Not allowed                                                  |
| Sterility                                      | Must be sterile                                              |
| Harmlessness                                   | Must be harmless                                             |
| Toxicity, allergenic ability                   | Not allowed                                                  |
| Pyrogenicity                                   | Not allowed                                                  |
| Immunogenicity                                 | Must be immunogenic                                          |

The vaccine was tested in accordance with the order of the Ministry of Agriculture of the Russian Federation No.101 dated March 6, 2018 “On approval of the rules for preclinical studies of a medicinal product for veterinary use, clinical study of a medicinal product for veterinary use, and bioequivalence of a medicinal product for veterinary use”.

The vials with the preparation were examined in transmitted light to establish the presence of mechanical impurities, mold, cracked vials and breach of corking. The stability of the vaccine was checked in the conventional manner by examining the appearance, sterility, safety and immunogenicity every 3 months for 24 months. Sterility was determined in accordance with GOST 28085-2013 “Biological preparations. The method of bacteriological control of sterility”. The determination of harmlessness was carried out on white mice with a body weight of 18-20 g. The vaccine was injected subcutaneously in the back at a dose of 1.0 cm³ to three white mice, according to GOST 9380-067-00008064. They were observed for 10 days. The acute toxicity of the vaccine against horse strangles was studied in laboratory outbred mice, by pathomorphological and histological studies after administration of the preparation intragastrically. The pyrogenicity test was carried out in 2 groups of 3 heads of healthy male rabbits with a body weight of 1.5-1.6 kg. For three days before testing the preparation and during the experiment, rabbits were measured body temperature. Body temperature was measured using a medical mercury thermometer. The thermometer was administered rectally for 5 minutes.

The lethal dose of the production strain *S. equi* “N-5/1” (LD₅₀) was previously determined to assess the immunogenic properties of the horse strangles vaccine. Determination of the virulent activity of LD₅₀ strain *S. equi* “N-5/1” was performed according to the Kerber method using application MS
EXCEL spreadsheet formulas for data processing in the study of the pathogenic properties of microorganisms [20]. The effectiveness of immunization was determined by the number of mice resistant to morbidity and mortality to infection in comparison with animals of the control group.

3. Results and discussion
A preclinical study of the experimental series of the vaccine against the horse strangles was carried out according to the parameters presented in table 1.

Definition of appearance
Vials with the finished vaccine were examined visually in transmitted light, and they were also checked for impurities, the integrity of the container and the tightness of the closure were checked.

At the end of the observation period (24 months), no change in the appearance of the vaccine, mechanical pollution, violation of the integrity of the vials and the tightness of the closure was established.

Determination of sterility
The sterility control of the vaccine was determined in accordance with GOST 28085-2013 “Biological preparations. The method of bacteriological control of sterility”.

As a result of studies on sterility, it was found that the vaccine against horse strangles remains sterile for 24 months, which exceeds the shelf life of the vaccine by 2 times.

Determination of harmlessness
The safety of the vaccine against strangles (GOST 31926-2013) was determined on laboratory outbred mice weighing 18-20 g (10 animals). A combined vaccine sample of 3 vials was prepared for testing. The mice were injected subcutaneously in the back with the finished vaccine preparation at a dose of 1.0 cm$^3$ per head.

Within 10 days of observation did not note a single case of disease and death of animals. When dissecting experimental animals at the end of the observation period, at the injection site, a pronounced inflammatory reaction was not detected. The vaccine against horse strangles has been found to be completely harmless to animals.

The study of acute toxicity and allergenic ability
The study was conducted in accordance with GOST 31926-2013 "Medicines for veterinary use. Methods for determining harmlessness."

The procedure for conducting studies on the toxicity and allergenic ability of immunobiological preparations in laboratory animals is mandatory in order to ensure the safety of the first clinical trials involving naturally susceptible animals. Studies of acute toxicity and the allergenic ability of the body are aimed at identifying toxic effects in an acute experiment with a single vaccine.

The experiments were carried out on white outbred mice weighing 18-20 g. Animals were divided into 2 groups of 3 heads. The group 1 was given a vaccine against horse strangles once; in the 2 control group - once sterile distilled water. The studied materials were administered at the maximum permissible dose (1.0 cm$^3$) intragastrically on an empty stomach.

Within 7 days after the introduction of preparations there were conducted clinical observation of animals. At the same time, daily weight gain was introduced. The recorded data are shown in table 2.

| Groups | Days of observation/body weight, g |
|--------|------------------------------------|
|        | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 1$^a$  | 17.2±1.34 | 16.8±2.5 | 16.8±2.4 | 16.6±2.3 | 16.6±1.69 | 16.4±1.27 | 16.6±1.69 |
| 2$^b$  | 17.1±0.84 | 17.1±0.84 | 17.4±1.06 | 18.1±1.2 | 17.7±0.98 | 17.5±0.63 | 17.6±0.63 |

$^a$1 group (experimental).
$^b$2 group (control).
Within 7 days after the administration of the preparations, the general condition was satisfactory in white mice of both groups, there were no signs of a violation of the central nervous system, appetite was not disturbed, and the coat remained smooth. The data in table 2 indicate that with a single administration of the maximum allowable dose of vaccine against strangles in laboratory mice does not affect the general condition of the animals. So, in mice of the control group there is an increase in body weight by 0.5 g, and in the experimental group, a decrease in body weight by 0.6 g. In pathomorphological studies of mice in both groups, no changes in parenchymal organs were detected, which would indicate acute toxicity of the body. Thus, studies on white mice showed that the vaccine against strangles in the studied doses does not have irritating activity and toxicity in relation to laboratory animals and can be classified as practically non-toxic preparations. Changes in body mass indicators in both groups of white mice remained within the physiological norm.

A pathomorphological examination of the parenchymal organs of mice of both groups did not reveal visually noticeable morphological changes. Histological examination of internal organs in laboratory white mice of the experimental group revealed: in the lungs and heart, expansion of blood vessels, unchanged; in the liver, hepatocytes with clear boundaries are observed, the blood vessels are evenly filled with blood, the bile ducts are not dilated; spleen - large cells are visible in the red pulp, similar to monocytes and blood cells; in the kidneys - granular degeneration, renal corpuses and tubules without changes. In the control group of laboratory white mice, the blood vessels in the lungs and heart are dilated, erythrocyte hemolysis; in the liver - dystrophic changes; the spleen is unchanged, in the kidneys - granular degeneration. Thus, the studies carried out on white mice showed that the vaccine against the horse strangles in the studied doses does not have irritating activity and "acute toxicity" in relation to laboratory animals and can be attributed to practically non-toxic preparations. The studies were conducted according to generally accepted methods in compliance with ethical standards.

The study of pyrogenicity

The pyrogenic properties of the vaccine were determined on healthy male rabbits with a body weight of 1.5-1.6 kg. There were formed 2 groups of 3 heads.

Body temperature was measured for three days before testing the preparation on rabbits. The measurement was carried out daily in the morning before feeding with a medical mercury thermometer. The thermometer was administered rectally for 5 minutes. The temperature measurement results were immediately recorded.

18 hours before the test, rabbits were deprived of food without water restriction. During the experiment rabbits were not fed or watered.

Before the experiment, with an interval of at least 30 minutes, body temperature was measured twice for each rabbit.

Before injection rabbit ears were treated with 70% alcohol. The vaccine against strangles (group 1) was injected into the ear vein of rabbits at a dose of 0.1 cm³. As a control, a similar dose of sterile saline was introduced in group 2 of rabbits. After preparation administration, body temperature was measured at an interval of 30 minutes for 3 hours. The recorded measurements of body temperature in rabbits are presented in table 3.

The data in table 3 show a slight fluctuation in body temperature in animals of both groups over 3 hours, which does not cause inhibition of the general condition of rabbits. On clinical examination of rabbits of both groups, no signs of the disease were observed, the general condition remained normal, the mucous membranes were pale pink, there were no discharge from the nose and eyes. Thus, a slight increase in body temperature was interpreted as a stressful condition due to multiple rectal measurements of body temperature.
The study of immunity in laboratory animals

We used an experimental series of inactivated vaccines against horse strangles (Yakut Scientific Research Institute of Agriculture), which as an immunomodulator includes culture fluid from the bacterial strain *B. subtilis* TNP-3.

The immunogenic activity of the inactivated horse strangles vaccine was studied on white outbred mice weighing 18-20 g. Two groups of animals of 10 heads were formed: one experimental and one control. The vaccine was administered subcutaneously at a dose of 0.5 cm$^3$, physiological saline in the same dose was used for control. After immunization, the state of oppression, decreased appetite, and ruffled coat were not observed in mice. For 10 days, vaccinated and control mice were kept in special cages with dry litter, water and food were given daily in the morning and evening.

The lethal dose of the *S. equi* strain “N-5/1” (LD$_{50}$) was previously determined. For this, the strain culture after reseeding on meat-peptone agar (MPA) with blood was brought to a concentration of 2 billion microbial bodies in 1 cm$^3$. Then serial dilutions were prepared on sterile saline solution (200 million, 20 million, 2 million, 200 thousand, 20 thousand, 2 thousand, 200 m.b.). From each serial dilution, 4 heads were infected at a dose of 0.2 cm$^3$ subcutaneously in the back. As a control, 4 mice at the same dose were injected with saline. Observations were carried out for 15 days. The lethal dose, which causes 50% death of animals, was calculated by the Kerber method using MS EXCEL spreadsheet formulas proposed by S.A. Donkov [20]. According to the results of studies, the absolute lethal dose that causes 100% death of animals was (Dcl) - 11247 microbial bodies per 1 cm$^3$, the minimum lethal dose that causes 95% of animal deaths (Dlm) was 10024, the 50% lethal dose was (LD$_{50}$) - 3557 microbial bodies in 1 cm$^3$ (table 4).

Further, to assess the immunogenic properties of the vaccine against horse strangles, a challenge was carried out 10 days after vaccination of the immunized and control animals with a dose of 5LD$_{50}$ of the virulent *S. equi* strain “N-5/1”.

After infection from the experimental group, on day 2 and 5 died 2 mice, and the death of all mice was noted in the control group.

Thus, the studies on immunogenicity have shown that a single administration of the vaccine to laboratory animals can provide up to 80% protection against the horse strangles pathogen.

The high efficiency of the inactivated vaccine, in our opinion, can be explained by the antigenic activity of the vaccine strain and the immunomodulating component - the culture fluid (fugate) of the bacterial strain *B. subtilis* TNP-3. According to the results of our previous studies, the bacterial strain *B. subtilis* TNP-3 can induce the synthesis of interferon and stimulate the body's immunobiological reactivity to enhance the immunogenicity of inactivated bacterial and viral vaccines [5, 19].

### Table 3. Results of temperature measurement before and after injection.

| Groups | Body temperature, °C | Before injection | After injection |
|--------|----------------------|------------------|-----------------|
|        | 1 measurement | 2 measurement | After 30 min | After 1 hour | After 1.5 hours | After 2 hours | After 2.5 hours | After 3 hours |
| a1     | 38.5±0.49 | 38.2±0.35 | 38.9±0.91 | 38.1±0.07 | 38.4±0.49 | 38.5±0.56 | 38.7±0.14 | 38.8±0.56 |
| b2     | 38.5±0.21 | 38.5±0.14 | 38.6±0.21 | 38.4±0.42 | 38.5±0.14 | 38.5±0.07 | 38.7±0.14 | 38.7±0.07 |

*a1* group (experimental).

*b2* group (control).
Table 4. Determination of lethal dose (LD\textsubscript{50}) on laboratory mice.

| Fell ill | Died | Calculation results | Mass results |
|----------|------|---------------------|--------------|
| 2 000 000 000 | 4 | 4 | 4 | Dcl\textsuperscript{a} | 100 | 11246.8265 | 5623.413 |
| 2 000 000 | 3 | 3 | 4 | Dlm\textsuperscript{b} | 95 | 10023.74467 | 5011.872 |
| 200 000 | 3 | 3 | 4 | LD\textsubscript{50}\textsuperscript{c} | 50 | 3556.55882 | 1778.279 |
| 20 000 | 2 | 2 | 4 | ID\textsubscript{50}\textsuperscript{d} | 50 | 3556.55882 | 1778.279 |
| 2 000 | 1 | 1 | 4 | saline | 50 | 3556.55882 | 1778.279 |
| 200 | 0 | 0 | 4 |
| 20 | 0 | 0 | 4 |

\textsuperscript{a}Absolute lethal dose.  
\textsuperscript{b}Minimal lethal dose.  
\textsuperscript{c}50\% lethal dose.  
\textsuperscript{d}50\% infective dose.

The immunogenicity vaccine that we developed is not inferior to preparations developed in the Netherlands, the USA and Kazakhstan [15-18]. And it is superior in environmental friendliness and harmlessness, since it does not contain antibiotics in the composition, as suggested by the authors of the “Akyntai” vaccine [17, 18]. Vaccines from live cultures, which were previously developed in Russia and tested in the USA and other countries, can cause complications in conditions of herd keeping of horses in countries with extreme climates [5, 12, 15, 16]. We have proposed the use of immunomodulators and probiotics to increase the immunogenicity of inactivated vaccines against the horse strangles [5, 19].

The absence of toxicity of the vaccine with the culture fluid from the bacterial strain \textit{B. subtilis} TNP-3 is consistent with previous results that showed the safety of the preparation “Sakhabactisubtil”, consisting of bacteria strains \textit{B. subtilis} TNP-3 and \textit{B. subtilis} TNP-5, in linear rats in the development of a drug - probiotic [19, 21].

4. Conclusion

Thus, a new inactivated vaccine against horse strangles with a single-use immunomodulator has been developed. The absence of toxicity, pyrogenicity, and a sufficiently high immunogenicity of the inactivated vaccine with a single injection (up to 80\%) was established, which meets the general requirements for the immunogenicity of inactivated vaccine strains.

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