ANNOTATION

For the dissertation work by Aigerim Abisheva, prepared on the topic "Technology of production a cultural vaccine against Rhinopneumonia of horses" submitted for the degree of Doctor of Philosophy (PhD) in the specialty 6D120100 – "Veterinary Medicine"

1. Relevance of the research topic

In our country, horse breeding has long been a traditional branch of animal husbandry and is now at a new stage of qualitative development: expensive pedigree horses are bred, and the number of working and improved-breed animals is increasing.

To increase the horse population and boost production, priority is given to measures against factors that hinder the development of the industry, especially infectious diseases, in particular equine rhinopneumonia.

Equine rhinopneumonia is a highly contagious disease characterized by abortions in mares during the second half of gestation and acute inflammation of the respiratory tract in young foals. Horses are susceptible regardless of breed, sex, or age; however, young animals are more sensitive. Purebred pedigree horses become ill more often, whereas local horses are more resistant. Among other animals, donkeys, mules, and ponies can also be affected.

The source of the pathogen is sick horses, animals in the latent period, and virus carriers. The virus is present in the blood and nasal discharges of infected horses and spreads into the air as an aerosol during sneezing. Large amounts of virus accumulate in the placenta of a pregnant mare and are released into the environment during abortion. The aborted fetus, fetal membranes, and placenta are the main sources of infection. Transmission via stallion semen has not been fully proven.

The disease is widespread in America, Europe, and CIS countries, including the Republic of Kazakhstan.

This disease causes substantial economic losses to farms due to increased mortality among foals and pedigree horses and the costs associated with veterinary and sanitary measures.

If a farm has pregnant mares, rhinopneumonia may cause abortions; in the respiratory form, the disease is observed in foals and young horses and, with good management, proceeds relatively mildly. The upper respiratory tract is mainly affected and body temperature rises slightly. In older horses, this form is rare due to naturally acquired immunity. The disease can cause abortions in up to 90% of animals, lasting up to 8-10 months.

Because of the contagious nature of rhinopneumonia, prevention is based on strict compliance with general and specific anti-epizootic measures. To prevent the introduction of the virus, importing horses from affected farms, as well as from locations where abortions occurred within the last 2 months, is prohibited. All incoming horses are kept in preventive quarantine for 30 days.

Based on the above, the aim of our research was to develop a technology for creating a virus vaccine against equine rhinopneumonia.

As a result of the studies, experimental batches of a dry, cell-culture-derived virus vaccine against equine rhinopneumonia were produced and its immunobiological properties were tested.

2. The purpose of the dissertation research: The aim of the scientific studies was to investigate the culture and immunogenic properties of the causative agent of equine rhinopneumonia and to develop a technology for preparing a vaccine against this disease.

3. Research objectives:

- To conduct an epizootological study on equine rhinopneumonia;
- To study the susceptibility of equine rhinopneumonia virus by passaging on various primary and continuous cell cultures;
- To adapt equine rhinopneumonia virus to active replication in the selected cell culture through continuous passaging and to select the most optimal cultivation regime;
- To produce experimental batches of a dry virus vaccine against equine rhinopneumonia and to test their immunobiological properties;
- To develop recommendations for using the virus vaccine to prevent equine rhinopneumonia in veterinary practice in the Republic of Kazakhstan.

4. Research methods:

The work was carried out in 2018–2021 at the Department of Biological Safety of the Kazakh National Agrarian University, and the experimental part was conducted in the laboratory of the Research and Production Center “DiaVak-ABN” LLP.

An epizootological study of equine rhinopneumonia in the Republic of Kazakhstan was performed using statistical data of the Ministry of Agriculture of the Republic of Kazakhstan for 2011–2022.

The study used the “AK-2011” strain isolated from a sample obtained from an aborted fetus of a horse affected by rhinopneumonia.

The biological activity of equine rhinopneumonia virus (viability of strain “AK-2011” at 6.0 lg $TCID_{50}/cm^3$) was determined in calf trachea cells, continuous calf kidney cell culture, sheep kidney, continuous cattle kidney cell culture, continuous green monkey kidney cell culture, continuous Syrian hamster kidney cell culture, and in a primary trypsinized horse kidney culture grown in tubes and flasks.

The virus was passaged by continuous inoculation in cell cultures. For this, the growth medium was removed from flasks containing cell cultures, a viral suspension was added at the appropriate dose, and adsorption onto the monolayer surface was carried out at 37°C for 1 hour with gentle rocking. Then the inoculum was removed, maintenance medium was added to the infected monolayer, and incubation continued at 37°C.

The viral mass was harvested when a clear cytopathic effect was observed in the monolayer or at an appropriate time after infection, depending on the experimental purpose. The cell culture in vessels was frozen at −20°C, thawed at room temperature, vigorously shaken, dispensed into 0.5 L bottles (200–300 mL each), and stored frozen at −20°C until use.

Virus titer in the virus-containing cell fluid was determined by titration on primary trypsinized horse kidney tube cultures or calf trachea tube cultures. Tenfold dilutions (10-1–10-7) were prepared in maintenance medium, and each dilution (1 cm3) was inoculated into 4–6 tube cell cultures.

The cytopathogenic activity of the virus was assessed by the intensity of cytopathic effects, the onset time and rate of development of cytopathic changes, and the titer of accumulated virus. All tubes (flasks) were examined daily under low magnification and compared with uninfected controls. Virus titer was calculated by the Reed–Muench method.

To confirm the molecular genetic properties of the "AK-2011" viral strain, PCR analysis was performed. Viral DNA was isolated using a commercial QIAGEN kit. Amplification of the extracted DNA was carried out using a Gene Amp PCR 9700 thermocycler.

The amplification reaction was performed in 50 µL and included 5 µL of 10× PCR buffer (Qiagen, USA), 1 µL of 10 mM dNTP (NEB, USA), 0.5 µL of DNA (100 ng/µL), 1 µL of primers seeH-F 5'-AGC ATG ATT CTA ACT TAA TTG AAG CCG -3' (20 pmol/µL) and seeH-R 5'-TAG CAT GCT ATT AAA GTC TCC ATT GCC-3', and 0.25 µL (1.25 Units) of Taq DNA polymerase (Qiagen, USA). Amplification conditions: 95°C – 5 min; 20 cycles: 95°C – 20 s, touchdown 60°C (−0.5) – 20 s, 72°C – 30 s; 20 cycles: 95°C – 20 s, 50°C – 20 s, 72°C – 30 s; 72°C – 7 min.

Sequencing was performed on a next-generation "MiSeq" sequencer (Illumina, USA) using the MiSeq Reagent v. 3 kit (Illumina, USA).

Bioinformatic analysis of the sequencing data was carried out using Geneious 11.0 (Biomatters, New Zealand).

Alignment of nucleotide sequences and phylogenetic analysis of genes with reference sequences from GenBank were performed in MEGA 6.0 using the maximum likelihood method with 500 replicates, GTR model.

The developed vaccine was evaluated (approbation) by the following parameters: appearance, color, absence of foreign impurities and mold, absence of cracks in vials, stopper tightness and correctness of labeling, presence of vacuum, pH value, resuspension time and concentration, moisture mass fraction, sterility, biological activity, safety in rabbits and white mice, and immunogenicity of the virus vaccine in rabbits and horses.

The level of specific antibodies against the causative agent of equine rhinopneumonia was determined by the complement fixation test and ELISA according to the approved guidelines.

5. The main provisions submitted for the defense of the dissertation:

- The epizootic situation of equine rhinopneumonitis in Kazakhstan.

- As a result of the experimental studies, the main biological and molecular-genetic characteristics were investigated, and an avirulent strain of equine rhinopneumonitis virus, "AK-2011," was obtained as a promising candidate for the development of a vaccine against equine rhinopneumonitis.

- Through continuous passaging of the equine rhinopneumonitis virus in various primary and continuous cell cultures, its susceptibility was assessed; the

virus was adapted to active replication in the selected cell culture, and the most optimal cultivation regime was determined, enabling the production of a consistently high-titer, highly active viral biomass.

- Experimental batches of a lyophilized (dry) viral vaccine against equine rhinopneumonitis were developed and their immunobiological properties were evaluated.

- Recommendations for the prophylactic use of the viral vaccine against equine rhinopneumonitis in the veterinary practice of the Republic of Kazakhstan were developed and proposed for implementation in production.

6. Description of the main results of the study.

Analysis of epizootological data indicates an increase in the spread of equine rhinopneumonia in 2011–2022. During this period, 10 regions (oblasts) were unfavorable: Aktobe, Zhambyl, Almaty, East Kazakhstan, North Kazakhstan, Atyrau, Karaganda, Kostanay, Akmola, and Pavlodar. West Kazakhstan, Mangystau, Kyzylorda, Ulytau, Zhetysu, Abai, and Turkistan oblasts are considered free of equine rhinopneumonia, with no cases recorded in 2011–2022.

According to genomic characteristics of the "AK-2011" viral strain, the virus showed 99.9% similarity to the RacL11 strain described in Germany in 1963.

The genome of the RacL11 reference strain consisted of 147,469 nucleotides, whereas a number of deletions were identified in the ORF67 protein-coding region of the rhinopneumonia virus genome. It is assumed that these deletions may be artificially introduced to attenuate a wild strain for vaccine purposes.

The virus isolated in Kazakhstan was found to be phylogenetically closest, based on the ORF68 gene widely used to determine evolutionary relationships, to the T-953 and 2222-03 strains isolated in the USA and Australia, respectively. These data indicate a close genetic relationship between the studied strain and internationally circulating viral isolates. A screening of the most sensitive primary and continuous cell cultures capable of supporting continuous replication of rhinopneumonia virus was conducted: TT, PT-80, PO, DVK, VERO, and BHK-21. Under optimized cultivation conditions, incubation at 37°C for 2–3 days and variation of the infectious dose from 0.01 to 1.0 $ТЦД_{50}/см^3$ ensured a high level of virus accumulation in the TT cell culture. In TT cells, virus titer increased gradually from the first passage and reached 6.00 lg $ТЦД_{50}/см^3$, remaining stably high thereafter. The most active viral material was obtained in the TT culture; therefore, this culture was characterized as the most sensitive and optimal replication system for the virus.

When determining the dose and route of administration of the prepared vaccine, intramuscular injection produced a markedly higher result than subcutaneous injection (80% vs 60%). A 2.0 mL dose, which ensured the highest antibody titer (1:400), was recognized as optimal, and the route and volume were optimized taking into account physiological characteristics of the animals.

To assess immunogenicity, antibody titers in vaccinated rabbit groups were tested by CFT on days 7, 14, and 30. In the vaccinated group, the titer on day 7 was 1:300, reaching the highest value of 1:500 on day 14. On day 30, the titer

decreased slightly to 1:400. These results indicate a rapid and pronounced immune response after vaccination.

The dynamics of antibody titers in foals after vaccination were determined by ELISA. After primary immunization, the titer was zero on day 0, increased to 25 on day 7, reached 60 on day 15, and peaked at 85 on day 30. Thereafter, titers gradually decreased: 80 on day 45, 70 on day 60, and 65 on day 75. This indicates a rapid and pronounced immune response after primary vaccination, followed by gradual weakening over time.

After revaccination (booster), the antibody titer reached 60 on day 90 and 70 and 80 on days 120 and 150, respectively. On day 200, the titer increased again to 85, followed by a gradual decrease: 75 on day 250, 70 on day 300, and 60 on day 360. These data demonstrate that booster vaccination not only enhances the immune response but also helps maintain it for a long period. Thus, stable antibody titers indicate high immunogenic and protective properties of the vaccine

7. Justification of the novelty and importance of the obtained data

Equine rhinopneumonia has been registered in the country in private and horse-breeding farms. Currently, there is a gradual increase in horse imports from regions of Russia, CIS countries, and a number of European states, which creates a risk of introducing rhinopneumonia from affected countries. The growth of the horse population and expansion of imports from neighboring countries require strengthened monitoring of the epizootic situation for rhinopneumonia.

The experiments showed that among the tested cell cultures, the TT cell culture was the most sensitive to the “AK-2011” equine rhinopneumonia virus strain and was the only system that produced a high titer. Virus replication in this culture remained stable during ten consecutive passages.

The studies demonstrated that the following conditions are important for obtaining a culture virus-containing fluid with an infectious activity of (5.75±6.00) lg $ТЦЭ_{50}/см^3$ for the production strain “AK-2011” of equine rhinopneumonia virus:

- The infectious dose should be within 0.1–0.5 $ТЦЭ_{50}$/mL;
- The concentration of TT cells should be 150–200 thousand cells/mL;
- Under these conditions, the monolayer forms within 40–72 hours, and the highest virus titer (5.25–6.0 lg $ТЦЭ_{50}$/mL) is determined during this period.

Within this work, it was planned to prepare experimental vaccine batches, refine the main technological stages of industrial production, and comprehensively evaluate the immunogenicity and safety of the final product.

The technological process of vaccine preparation consisted of two stages:

I - obtaining the matrix (seed) virus series, II - obtaining the production vaccine series.

Immunogenicity is the ability of a vaccine to induce an immune response in the body. The onset of immunity is the appearance of an immune response after a certain time following vaccination; this indicator characterizes the rapidity of vaccine effect.

After studying biological properties, the development of a vaccine against equine rhinopneumonia was initiated and further approbation trials were carried out under the name “RinoVac”.

The quality of experimental series of the developed equine rhinopneumonia vaccine was assessed by the following indicators: bacterial contamination (presence/absence of bacterial impurities), fungal and mycoplasma contamination, sterility, safety, and areactogenicity.

Intramuscular administration of the virus vaccine at 2 cm^3 enhances the immune response. Revaccination (booster) was performed in test animals after 90 days to strengthen the primary immune response and ensure long-term protection, thereby improving vaccine immunogenicity.

8. Compliance with scientific development directions or state programs:

The dissertation work corresponds to the priority areas of strengthening measures to prevent livestock diseases, improving the vaccination and diagnostic system, ensuring the safety of livestock products, and the development of veterinary biotechnology.

On the basis of the Law of the Republic of Kazakhstan dated 4 December 2015 “On Public Procurement” (hereinafter — the Law) and the results of public procurement conducted through the “Auction (since 2022)” method dated 24 April 2024 No. 11983185-1, the Parties have entered into this public procurement contract for goods.

9. Description of the doctoral student's contribution to the preparation of each publication

Based on the dissertation materials, 7 scientific works were published, including 1 article in the journal “Transbound Emerg Dis.” indexed in Scopus (Q1, percentile 99), 3 articles in journals recommended by the Committee for Quality Assurance in Science and Higher Education of the Ministry of Science and Higher Education of the Republic of Kazakhstan, and 4 articles in proceedings of international conferences, congresses, and symposia.

The results were presented and published in conference proceedings: at the International Scientific and Practical Conference “Biotechnology and Biological Safety: Achievements and Prospects”, dedicated to the 65th anniversary of the Research Institute for Biological Safety Problems (6–8 September 2023); at the International Scientific and Practical Conference “Current Status and Future Prospects for the Development of Veterinary Medicine and Animal Husbandry in the Republic of Kazakhstan”, dedicated to the 80th anniversary of Academician of the NAS of the Republic of Kazakhstan, Doctor of Veterinary Sciences, Professor T. Saiduldin, held at the Kazakh National Agrarian Research University (Almaty, 15–16 March 2023); at the International Conference “Prospective Research in Psychology, Sports and Healthcare” held at the Lomonosov International Institute for Prospective Research (Saint Petersburg, 11 November 2022); and at the International Scientific and Practical Conference of Young Scientists “Youth and Innovations–2020” held at the Belarusian State Agricultural Academy (Gorki, 14–16 May 2020).

10. Volume and structure of the dissertation

The dissertation is presented on 104 pages of computer-typed text and contains 17 tables and 23 figures. It includes the following sections: introduction, review of scientific literature, research materials and methods, experimental part, conclusions, and references list comprising 228 sources.