HERALD OF SCIENCE

OF S. SEIFULLIN KAZAKH AGROTECHNICAL
RESEARCH UNIVERSITY:

VETERINARY SCIENCES

№ 1 (009)

Astana 2025

COMPOSITION OF THE EDITORIAL BOARD

ISSN 2958-5430
eISSN 2958-5449
Index publications – 75830

Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 1 (009). – P. 4-14. - ISSN 2958-5430, ISSN 2958-5449

VETERINARY SCIENCES

doi.org/ 10.51452/kazatuvc.2025.5(009).1791
UDC 619:616.98.578.636 **Research article**

Immunogenic activity of a prototype activated moraxella vaccine under experimental conditions

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Received: 01-10-2024 **Accepted:** 06-02-2025 **Published:** 31-03-2025

Introduction

Background and Aim. The spread of moraxellosis in cattle among Kazakhstan has become a pressing issue in modern agricultural markets. Preventive measures are essential to mitigate economic losses. This study aimed to assess the immunogenic activity of a prototype inactivated autogenous antimoraxellosis vaccine developed from a local inactivated strain of the pathogen.

Materials and Methods. An outbreak culture was isolated from an animal showing signs of infectious eye disease, which was identified as *Moraxella bovis* B-2017/44 based on its culture and morphological characteristics. This strain was used to produce an autogenous vaccine using Montanide ISA 70 VG oil adjuvant (France). The vaccine's immunogenicity was tested via a prolonged complement fixation reaction in Aberdeen Angus calves. The immunogenic properties were evaluated in comparison with a vaccine for infectious bovine keratoconjunctivitis, which utilizes *Moraxella bovis* antigens and herpesvirus type I, created in Kazan, Russia.

Results. The autogenous anti-moraxellosis vaccine with Montanide ISA 70 VG adjuvant was effective against moraxellosis in cattle. Sterility and safety were evaluated in laboratory animals, whereas immunogenicity was assessed in calves over 12 months. The highest antibody titers were recorded on day 120 after vaccination.

Conclusion. The developed vaccine, based on the locally inactivated strain *Moraxella bovis* B-2017/44, enhances antiepizootic efforts and reduces economic losses from infectious keratoconjunctivitis in cattle.

Keywords: immunogenicity; *Moraxella bovis*; prevention; pinkeye; vaccine.

Introduction

Infectious bovine keratoconjunctivitis (IBK; Pink eye) is one of the most prevalent diseases affecting cattle worldwide, including in the Republic of Kazakhstan. During the epizootiological monitoring of infectious keratoconjunctivitis of Moraxella etiology in Kazakhstan from 2016 to 2019, the disease was identified in nine regions [1, 2, 3]. Moraxella strains affected imported and local cattle across a range of ages, sexes, and breeds, including Kazakh white-headed and Auliekol [4]. The etiology of contagious keratoconjunctivitis can be bacterial, viral, or parasitic. To better define Moraxella-related infections, researchers have introduced the term “ocular moraxellosis” [5].

In addition to pathogens, mechanical damage to the eye can lead to disease. Tiny particles such as dust or plant awns can enter the eye, cause corneal injury, and promote *Moraxella* attachment. Flies can act as potential vectors [6]. The clinical signs of moraxellosis include conjunctival inflammation, photophobia, corneal edema, and corneal ulceration. If left untreated, it can result in total blindness. Economic damage of includes reduced productivity, loss of offspring, diminished breeding value, and treatment costs.

Numerous studies have focused on preventing moraxellosis in cattle. Researchers have assessed the effectiveness of recombinant subunit vaccines for *Moraxella bovoculi* cytotoxin [7, 8, 9], as well as vaccines containing antigens from *Moraxella bovis* and *Moraxella bovoculi* bacteria [10, 11]. In some cases, autogenous vaccines incorporating *Moraxella bovis, Moraxella bovoculi,* and *Mycoplasma bovoculi* antigens have shown reduced rates of IBK compared with commercial vaccines. This study aimed to evaluate the immunogenic activity of a prototype autogenous inactivated anti-Moraxella vaccine compared with a combination vaccine targeting IBK caused by *Moraxella bovis* and herpesvirus type I.

Materials and Methods

Ethical approval

This study was approved during a meeting of the Local Ethical Commission at the Kazakh Research Veterinary Institute, part of the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan, on August 29, 2017.

Study period and location: This research was conducted from February 2018 to November 2020 at the Laboratory of Bacteriology of the Kazakh Research Veterinary Institute and on a farm located in the Talgar district of Almaty, Kazakhstan. The Almaty region has a continental climate with cold winters and hot summers. This necessitates the creation of comfortable conditions for livestock. The animals were kept in pens under canopies in uniform conditions. Seasonal protection of cattle from flies and other dipteran insects was provided by "Flectron" ear tags.

Reference strains: For the development of an experimental series of anti-Moraxella vaccine, the epizootic strain *Moraxella bovis* B-2017/44 [13] was utilized, and its cultural, morphological, and biochemical characteristics were analyzed for comparison [14, 15] using the reference strain *M. bovis* (American type culture collection [ATCC] 17948). Identification was conducted solely using bacteriological methods. The epizootic strain *Moraxella bovis* B-2017/44 was isolated from a 3-month-old calf with clinical signs of keratoconjunctivitis (Fig. 1) in the Akmola region of the Republic of Kazakhstan (Fig. 2).

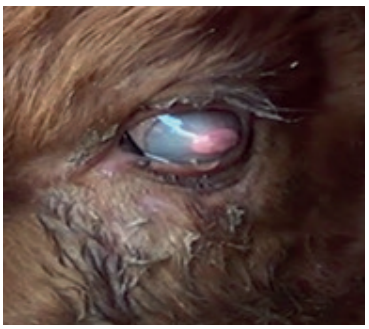

Figure 1 – Abundant ingrowth of superficial blood vessels into the cornea

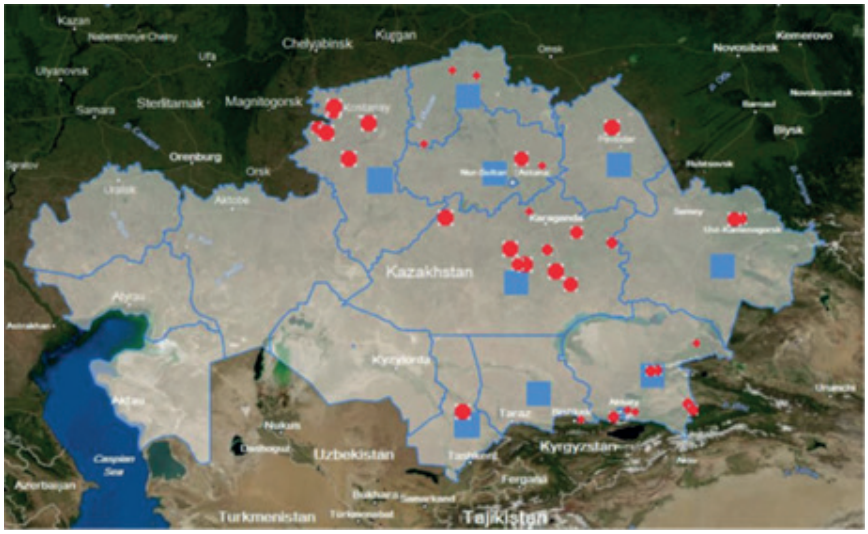

Figure 2 – Epizootic map visualizing the quantitative and qualitative indicators of the moraxellosis epizootic process across various regions of the Republic of Kazakhstan for 2019

Sample collection: A comparative study was conducted to evaluate the immunogenic activity of a prototype inactivated anti-Moraxella vaccine and an associated vaccine for IBK based on antigens from *Moraxella bovis* bacteria and herpesvirus type I. This study was conducted under experimental conditions in the vivarium of the Kazakh Research Veterinary Institute and on a meat farm in the Talgar district of Almaty. All animal studies were approved by the relevant ethical committee/institutional review board and were conducted in compliance with the biological safety standards and the ethical principles of animal experimentation outlined in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1987).

The experiment included nonvaccinated bulls and heifers aged 6 months to 1 year, all of whom exhibited no clinical signs of keratoconjunctivitis and were kept under identical conditions. The first group comprised 18 calves that were subcutaneously immunized with an experimental autogenous anti-Moraxella vaccine at a dosage of 5.0 cm^3 in the upper third of the neck. The second experimental group, also consisting of 18 calves, received the associated vaccine against IBK, which is based on antigens from Moraxella bovis and herpesvirus type I (developed by the Federal State Budgetary Scientific Institution "Federal Center of Toxicological, Radiation and Biological Safety" and the Russian Academy of Sciences in Kazan, Russian Federation). A control group of 18 calves was injected with a sterile adjuvant (Montanide ISA 70 VG) using the same method.

Cultivation of an epizootic strain: The epizootic strain *Moraxella bovis* B-2017/44, isolated in the Republic of Kazakhstan, was used for culturing and biomass production. The causative agent of moraxellosis was identified via bacteriological examination the conjunctival sac washes from the eyes. Sterile swabs with plastic handles were used to collect the biomaterial in transport tubes containing Amies medium (Italy), which were individually packaged. Discharges from the affected eye were collected using a sterile applicator in a rotating motion. The clinical pathological samples were transported to the bacteriology laboratory within 3-4 h in a thermal case packed with ice.

Laboratory analyses were conducted in accordance with the "Methodological Guidelines for the Diagnosis, Treatment, and Specific Prevention of Infectious Keratoconjunctivitis in Cattle Caused by *Moraxella bovis* and *Moraxella bovoculi*" [16].

The culture purity was assessed through microscopy of Gram-stained smears using a MEIJI TECHNOmicroscope (Japan) equipped with a digital camera, at magnifications of ×10 and ×100. The immune response to the experimental vaccine was validated conducted using the long-term complement fixation test (LCFR) [17, 18].

The epizootic strain was grown on a solid nutrient medium – (Hottinger agar) with the addition of 10% defibrinated ram blood in bacteriological test tubes for 24 h. The resulting biomass layer was removed using a physiological solution, and the cell suspension was transferred to a Tartakovsky flask-

incubation. For this purpose, Hottinger agar (HiMedia, M1425-100G, India) supplemented with 5% defibrinated ram blood was poured into sterile 1.5 L mat flasks with a volume of 250-300 cm^3. Once the medium solidified, the flasks were placed in a thermostat at 37 °C ±1.0 °C to ensure sterility. The specified strain was inoculated by adding 5.0 cm^3 of a bacterial suspension containing 1 billion colony-forming units (cfu) per cm^3 to each flask. The cultures were then incubated at 37 °C ±1.0 °C. After 36-48 h, the resulting colonies of *Moraxella bovis* were washed with a sterile 0.85% sodium chloride solution, preparing a suspension with a concentration of 10 billion cfu per cm^3, in accordance with the bacterial or optical turbidity standard of the DEN-1B densitometer (BioSan, Latvia). The inactivation of the bacterial suspension was carried out by heating it in a water bath at a temperature of 80 ºC for 30 minutes. To do this, the container with the suspension was placed in a preheated water bath. Once the temperature of the bacterial suspension reached 80 ºC, the time was recorded. After maintaining the temperature for 30-40 minutes, a sample was taken to assess the completeness of inactivation, which was done by inoculating the inactivated microbial suspensions onto Hartinger blood agar. If there was no growth observed, the suspension was considered inactivated. To assess the safety and shelf life of the vaccine, laboratory animals were used, specifically white mice weighing 20-30 g and rabbits weighing 3.0-3.5 kg. The experiments and methodologies adhered to the requirements of biological safety and the ethical principles of animal experimentation outlined in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1987). The statistical analysis of the serological reactions was performed according to *T. Saiduldin* [19].

Production of an inactivated vaccine: An inactivated bovine moraxellosis vaccine was developed using the antigen of *Moraxella bovis* B-2017/44. The inactivation of the bacterial suspension was carried out by heating it in a water bath at a temperature of 80 ºC for 30 minutes. In the absence of growth, the suspension was considered inactivated. Tht ultrasonic lysate was prepared by subjecting a suspension of 50.0 billion live microbial cells to an ultrasonic disintegrator UZDN-1 (Ukraine) for 15-20 min at an oscillatory frequency of 22 kHz and a power of 80–100 W/cm^2. This was followed by centrifugation for 30 min at 18,000 g to separate the undestroyed bacterial cells. The supernatant was then used to prepare the vaccine after inactivation at 80-°C for 30 min. The completeness of inactivation was monitored by seeding ultrasonic bacterial lysate on Hottinger agar (HiMedia, M1425-100G, India) with the addition of 5% defibrinated ram blood. In the absence of growth, the lysate was considered inactivated. The resulting ultrasonic lysate was thoroughly mixed under sterile conditions in a 1:1 ratio with the oil adjuvant Montanide ISA 70 VG (France) in a homogenizer (Bandelin, Germany) according to the attached documentation (Technical Bulletin Montanide tm ISA 70M VG Ready to use oil adjuvant for veterinary vaccines).

Determination of vaccine sterility. We determined sterility by inoculating vaccine samples onto the following microbiological media: MPA, MPB, MPPPB, Sabouraud, and Kitta-Tarozzi. Test tubes and flasks with inoculations onto all media except Sabouraud medium were kept in a thermostat at a temperature of (37 °C ±1 °C), and Sabouraud medium – at a temperature of (22.5 °C ±1.0 °C) for 7 days (14 days for anaerobic preparations). After the specified period, the samples were reseeded, except for inoculations into MPA. The samples were reseeded into the same nutrient media and in the same volumes as during inoculation. The secondary inoculations were kept for 7 days (14 days for anaerobic preparations).

The safety of a vaccine. The safety of the vaccine was determined via subcutaneous administration to 10 white mice at a dose of 0.5 cm^3 in the shoulder blade area, and observation was performed for 10 days.

Antigenic activity: The vaccines were studied in an experiment on rabbits weighing 2.5-3.0 kg, to which the vaccine was administered subcutaneously at a dose of 2.0 cm^3.

Vaccine stability during storage: The experimental sample was assessed under refrigerated conditions using 16 rabbits divided into four groups of four animals each. The first group received the preparation after 7 days, the second group after 3 months, and the third and fourth groups received it 6 and 12 months after production, respectively. Laboratory animals were subjected to serological testing before administration and on the 21st and 30th days after vaccine injection.

Immunogenic activity. The test vaccines were studied under experimental conditions in three groups (two experimental and one control) of 30 heads of intact calves. The animals were immunized using a

randomized method according to the explanation and clarification of CONSORT 2010 [28]. Animals in the first experimental group were administered an autogenous anti-Moraxella vaccine based on the lysate of the epizootic strain *Moraxella bovis* B-2017/44.

The second experimental group received an associated vaccine against infectious keratoconjunctivitis in cattle, which is based on antigens from Moraxella bovis and herpesvirus type I (Kazan, Russian Federation). Both vaccines were administered subcutaneously in the upper third of the neck at a volume of 5.0 cm^3. Serological testing of the animals was conducted on the 7th and 21st days following the initial administration of the antigen. On the 21st day after the preliminary study, which revealed a low titer of antibodies specific to *Moraxella bovis*, the animals were preimmunized. The titers of specific antibodies in the blood serum were analyzed on the 30th, 120th, 210th, and 360th days (Table 2).

The animals of the third control group were injected subcutaneously into the third part of the neck with the sterile adjuvant Montanide ISA 70 VG instead of the vaccine. The experimental and control groups of animals were maintained under the same conditions of keeping and feeding.

Results

In vaccine cultures on MPA, MPB, MPPB, Sabouraud and Kitta-Tarozzi nutrient media, there was no growth of bacteria or fungi, indicating sterility.

When assessing the safety of the vaccine in white mice, we observed that the animals remained healthy throughout the observation period, showing no signs of pathological local or systemic reactions.

The antigenic activity of the vaccine was evaluated by measuring the titers of specific antibodies in the blood serum of the vaccinated animals. Initial studies of antibody titers after the first administration of the vaccine yielded negative results. Consequently, revaccination was performed on the 21st day after the first vaccination, and the serum titers of specific antibodies were analyzed on the 21st, 28th, 120th, 240th, and 365th days following revaccination (Table 1).

Table 1 – Titers of specific antibodies in the blood serum of vaccinated rabbits

Laboratory animals/ administration method	Dosage of the drug, cm^3	Observation period/specific antibody titers						
		7	14 (revaccination)	21	28	120	240	365
10 rabbits/ subcutaneously	2.0	1:10	1:10	1:40	1:40	1:80	1:40	1:10

Table 1 shows that following revaccination, antibody titers in rabbits blood serum increased significantly. On day 28, the titers reached 1:40, with the highest titer of 1:80 observed on day 120. By day 240, the titers decreased to 1:40 by day 365, they were at 1:10. This indicates that Moraxella bacterial lysate treated with the Montanide ISA 70 VG adjuvant remained antigenic for a full year. Table 2 presents the results of the vaccine shelf– life determination.

Table 2 – Results of determining the shelf life of an autogenous vaccine

Vaccine dose, cm^3	Vaccine storage conditions, °C	Average antibody titer in rabbit blood serum after antigen administration											
		7 days			90 days			180 days			365 days		
		0	21	30	0	21	30	0	21	30	0	21	30
2.0	4-6	-	1:60	1:80	-	1:60	1:80	-	1:60	1:80	-	1:60	1:80

From the data in Table 2 it is evident that the storage mode of the antigen in a mixture with the oil adjuvant ISA 70 V at a temperature of 4-6 ºC does not reduce its activity over 12 months (observation period).

In addition the immunogenic activity of the vaccine was compared with that of a vaccine for infectious keratoconjunctivitis in cattle, which uses antigens from *Moraxella bovis* bacteria and herpes virus type I (from Kazan, Russia). This study was conducted on Aberdeen Angus calves which are an economic entity within the Talgar district of the Almaty region. The results of the serological analisis of blood from vaccinated LCFTs are presented in Table 3.

Table 3 – Data on the titers of specific antibodies in the blood serum of cattle after immunization with different vaccines

Animal groups/types of vaccines	Vaccine dose cm^3/ methods of administration	Serum titers of animals at different times after vaccine administration					
		1	7	21 revaccination nation	120	210	360
I	5.0 / subcutaneously	0 (0.0%; -0.0%)	24.5 (24.8% -19.9%)	14.5 (14.1%;-12.3%)	171 (48.5% -32.7%)	19 (14.1%; -12.3%)	17.1 (14.1% -12.3%)
II	5.0 / subcutaneously	0 (0.0%; -0.0%)	23 (14.1% -12.3%)	13 (14.1%-12.3%)	162 (30.1% -23.1%)	20 (14.1%; -12.3%)	17 (14.1% -12.3%)
III	5.0/ subcutaneously	0 (0.0%; -0.0%)	0 (0.0%; -0.0%)	0 (0.0%;-0.0%)	0 (0.0%; -0.0%)	0 (0.0%; -0.0%)	0 (0.0%; -0.0%)
Note: Group I received an autogenous vaccine (KazNIVI); Group II was vaccinated with a combined vaccine from the Federal Center for Traumatology and Microbiology - All-Russian Research Institute of Virology and Microbiology; Group III served as the control and was immunized with an adjuvant.							

From the data given in Table 3, it is evident that antibodies after the introduction of the autogenous vaccine, in the first group of animals, were detected on the 7th day in a titer of 24.5 (24.8%; –19.9%), then, on the 21st day, the amount of complement-binding substances decreased to 14.5 (14.1%; –12.3%), which was the reason for the decision to revaccinate the animals. On the 120th day after the first vaccination, the amount of antibodies increased to 181 (48.5%; –32.7%) and by the 240th day, they were 19 (14.1%; –12.3%) and on the 360th day, the titers of complement-binding substances were 8.1 (14.1%; –12.3%) (observation period).

In animals of the second group, which were administered the associated vaccine, specific antibodies were detected on the 7th day at a titer of 23 IU (±13.2%). Then, on the 21st day, the amount of complement-binding substances decreased, reaching 13 IU (±13.2%). After revaccination, on the 120th day the amount of antibodies increased to 162 IU (30.1%; –23.1%) and by the 360th day they amounted to 17 IU (±13.2%), and on the 258th day the complement-binding substances amounted to 6.5 IU (14.1%; –12.3%). Observation period.

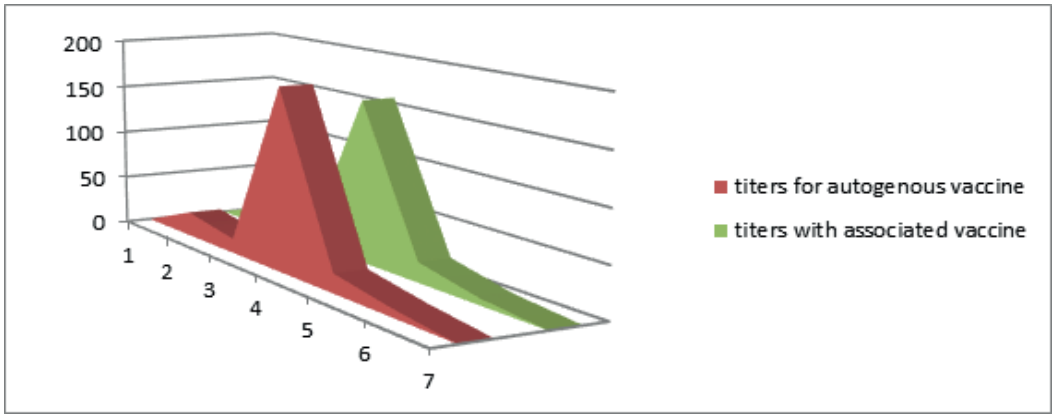

Figure 3 – Titers of specific antibodies in blood serumcattle after immunization with various vaccines

The data in Figure 3, show that there was no significant difference in antibody titers after immunization with autogenous and associated vaccines.

During the experiment, a clinical picture of keratoconjunctivitis was observed in 5 (8.3%) of the 60 immunized calves. In the experimental group, which were immunized with an autogenous vaccine,

conjunctivitis was observed in two calves (6.6%), with one animal having clouding of the pupil of the left eye and one animal having hyperemia and lacrimation of both eyes. In another experimental group, immunized with an associated vaccine, clinical signs of keratoconjunctivitis were manifested in 3 (10.0%) animals. In the control nonvaccinated group, the clinical signs of keratitis and conjunctivitis were observed in sevsn calves, which accounted for 23.3%. In this case, conjunctivitis developed in one or both eyes. The clinical image was accompanied by hyperemia, photophobia, lacrimation, pupil clouding, decreased appetite, and depression state. Five animals had purulent exudate discharge from the eyes. Coccal microflora - *Staphylococcus spp., Streptococcus spp.* dominated among the identified concomitant microorganisms. *Escherichia spp., Proteus spp., Pseudomonas spp.* were also isolated. In animals with clinical signs of eye diseases, swabs were taken from the conjunctival cavity for further microscopic examination. As a result of the microscopy, thelazia were not detected.

Bacteriological examination of eye washes from animals in the experimental groups immunized with autogenous and associated vaccines yielded one culture of Moraxella bovis, whereas laboratory studies yielded three epizootic strains of *Moraxella bovis* cultures from calves in the control group. Bacteriological studies of the isolated cultures were compared with the reference strain *M. bovis* (American type culture collection [ATCC] 17948).

Discussion and Conclusion

The objective of this study was to conduct a comparative analysis of the immunogenic activity of a prototype autogenous inactivated anti-Moraxellosis vaccine and a combined vaccine against infectious keratoconjunctivitis in cattle, using antigens from *Moraxella bovis* bacteria and herpes virus type I under experimental conditions. Various vaccines have been developed and used for the specific prevention of moraxellosis in cattle in nearby and distant countries. [10, 11, 21]. However, not in all cases the expected positive effect is achieved [7]. Calves immunized with the local strain vaccine showed a reduced incidence of infectious keratoconjunctivitis throughout the study compared with calves vaccinated with the commercial vaccine [12]. Positive clinical signs and high antibody titers were observed in pilin-MbxA vaccinated calves compared with control calves. Poor vaccine efficacy against *M. bovis* has also been noted in herds where *M. bovoculi* widespread [8]. There is evidence of a significant increase in the concentration of ocular antigen-specific IgA after intranasal immunization with recombinant *M. bovis* cytotoxin with a polyacrylic acid adjuvant [22]. Positive results were achieved when vaccinating cattle against infectious conjunctivitis using a vaccine that targets *Moraxella bovis* bacteria (strain “Chelyabinsk-2008”) and herpesvirus type I, specifically the drug “Kerokonvitin” [11]. Available literature data [20] indicate an increase in the immunogenic activity of bacterial antigens when lysates are added to microbial suspensions, one of which is an ultrasonic disintegrate. In connection with the aforementioned discussion, we developed a prophylactic drug from an ultrasonic disintegrate with the addition of montanide ISA 70 VG adjuvant. The difference in antibody titers between the autogenous and associated vaccines was not significant, and the presence of clinical manifestations of keratoconjunctivitis in immunized animals indicates the presence of concomitant pathogens. A high prevalence of *Mycoplasma bovoculi* and *Moraxella bovoculi* was found in cattle on livestock farms in the East [4, 26] *Moraxella bovoculi, Moraxella ovis* and *Moraxella bovis* in the North [23, 24] and *Moraxella bovis* in the South Kazakhstan [25]. In addition, *Moraxella bovis,* as the main causative agent of infectious bovine keratoconjunctivitis [29, 30], the etiologic role of *Moraxella bovoculi, Mycoplasma bovis* and *Mycoplasma bovoculi, Chlamydia, Listeria monocytogenes,* and some viruses, including BHV [31] has been proven. It is likely that to develop of an effective prophylactic immunization agent, it is desirable to combine the possible causative agents of ICC.

For the first time in the Republic of Kazakhstan, experimental testing was conducted on a product designed specifically prevent IBK. This product is based on the locally inactivated strain *Moraxella bovis* B-2017/44, and its immunogenic activity was studied in comparison with a related vaccine that incorporates antigens from *Moraxella bovis* bacteria and herpes virus type I. It was found that the drug meets all the requirements for vaccines, i.e., it is sterile, harmless, and has antigenic and immunogenic activity for a year. The immunogenic activity of the vaccine was studied in 60 calves for 12 months. Specific antibody titers in vaccinated animals were maintained for 12 months. During the observation period, the percentage of clinically ill calves in the experimental groups was 8.5%, and in the control

group, in which the animals were administered only the Montanide ISA 70 VG adjuvant, the disease was observed in 23.3% of calves, which is 36.4% more than in the immunized groups. Bacteriological studies identified 66.6% more epizootic strains in the control group than in the experimental group.

Authors' Contributions

RS, FB and GS: Conceptualization, methodology. KS: Investigation and formal analysis. RS, FB: Writing-original draft. AI, AKh and BI: Writing-review and editing. RS: Project administration. All authors have read, reviewed, and approved the final manuscript.

Acknowledgments

This study was financially supported by the Ministry of Agriculture of the Republic of Kazakhstan 2018-2020 (AP05131952).

Competing Interests

The authors declare that they have no competing interests.

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 1 (009). – P. 15-27. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2025.5(009).1816
UDC 571.27; 619 **Review article**

Effects of interleukin-15 on bovine natural killer and CD8+ T cells and its potential in treating viral infections

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Received: 08-01-2025 **Accepted:** 04-03-2025 **Published:** 31-03-2025

Abstract

Interleukin-15 (IL-15) is a promising cytokine for immunotherapy of human cancers and bovine viral infections. IL-15 indirectly has a negative effect on tumor and virus-infected cells by activating the signaling pathways of proliferation and activation of natural killer (NK) and memory CD8+ T cells. IL-15 therapy may have widespread clinical use, particularly when combined with chimeric antigen receptor (CAR) T or CAR NK cells. Additionally, the use of IL-15 in combination with checkpoint inhibitors and other therapies holds promise as a treatment for cancer and viral infections. Understanding the biological characteristics of IL-15 is crucial for developing effective therapeutic strategies. This study aimed to explore recent advances in IL-15 research, focusing on its antitumor mechanisms within the tumor microenvironment, advances in IL-15-based therapies for bovine viral infections, and its integration with other treatment approaches, including monoclonal antibodies.

Keywords: cancer; cytokine; immunotherapy; interleukin-15; natural killer cells; viral infection.

Introduction

Interleukins (ILs) are biologically active protein molecules that regulate immune and inflammatory responses in the body. They activate signaling pathways in immune cells and coordinate their interactions to defend against infections, diseases, and tissue damage. ILs promote immune cell growth, differentiation, and survival while stimulating phagocytes to eliminate pathogens [1, 2, 3]. ILs exert paracrine or autocrine effects on target cells by binding to specific receptors [4]. A notable characteristic of ILs is their self-limiting nature, which is driven by the instability of most mRNAs, resulting in shortterm synthesis and rapid protein secretion [3, 4].

ILs are classified based on their genetic sequences and functional activities. More than 40 types of ILs have been identified and categorized into several families. The IL-1 family, including IL-1α and IL-1β, plays an important role in inflammatory processes and immune cell activation [5]. The IL-2 family, including IL-4, IL-7, IL-9, IL-15, and IL-21, regulates the growth and differentiation of T, B, and natural killer (NK) cells [6, 7]. The IL-6 family, including IL-6 and IL-11, plays a role in inflammatory processes and immune response regulation [8]. The IL-10 family, including IL-10, IL-19, and IL-20, exhibits anti-inflammatory properties by reducing the activity of macrophages and other immune cells [9]. The IL-12 family, including IL-12, IL-23, and IL-27, contributes to T-helper cell activation and adaptive immune response modulation [10]. The IL-17 family, including IL-17A-F, is a critical inflammatory mediator that activates neutrophils and promotes inflammatory disease [11]. IL-18 and IL-33 are involved in the inflammatory response and pathogenesis of allergic diseases [12]. Each

family fulfills specific functions to maintain the balance of the immune system, protect the body against infections, and regulate inflammatory and autoimmune responses.

The functions of ILs have attracted attention as potential therapies for viral infections in farm animals, including bovine leukemia virus (BLV) [13]. BLV-infected cows exhibit impaired cytokine production, reduced T cell proliferation, and increased T cell apoptosis. They exhibit elevated levels of transforming growth factor-β and IL-10 and decreased levels of gamma interferon (IFN-γ), IL-12, IL-2, and IL-4. The increase in IL-10 levels promotes the expansion of regulatory B and T cells, leading to the suppression of the immune system and reduced ability to combat infection [14].

IL-15 and the representatives of the related family play an important role in effector T cell proliferation and longterm immune response formation. The expression of this cytokine contributes to the increase in the number of memory CD8+ T cells and maintenance of their activity. *Ex vivo* and *in vivo* experiments have shown that IL-15 increases the proliferation of T cell precursors in mice with chronic viral infections. Furthermore, IL-15 combined with anti-programmed cell death 1 (PD-1) antibodies has been reported to significantly promote the development of infection via the activation of CD8+ T cells, and IL-15 combined with anticancer monoclonal antibodies (mAbs) significantly enhances antibody-dependent cellular cytotoxicity, thereby increasing the effectiveness of the therapy [15, 16].

The aim of this study was to analyze the latest advances in IL-15 research, focusing on its antitumor mechanisms within the tumor microenvironment, its application in the treatment of viral infections in cattle, and its integration with other therapeutic approaches, including monoclonal antibodies.

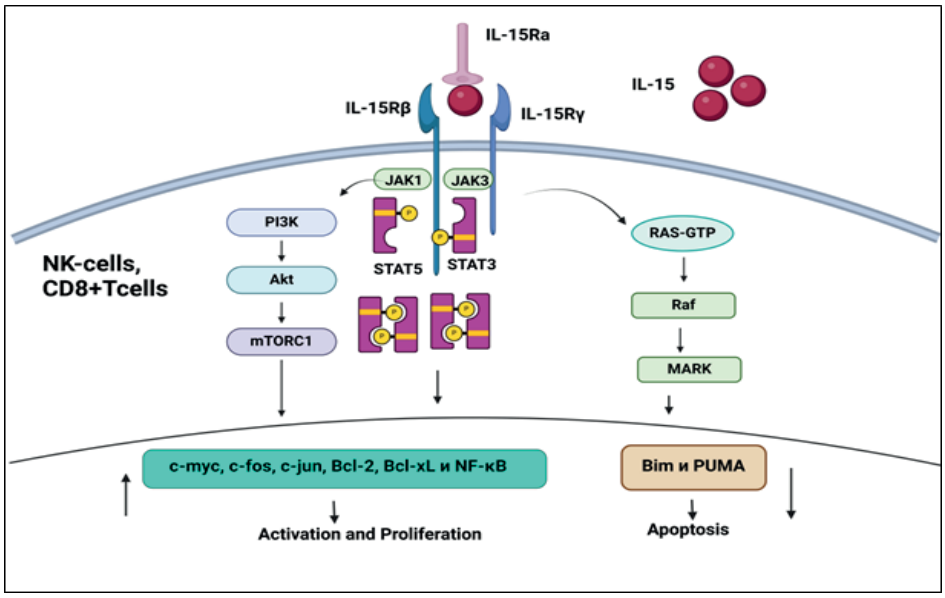
Biological functions of IL-15

IL-15 is synthesized by various cells, including macrophages, fibroblasts, epithelial cells, and endothelial cells, and plays a critical role in various biological processes. It plays a role in immune system regulation by activating and enhancing the survival of NK cells. IL-15-activated NK cells exhibit increased proliferation and functionality, whereas the combined use of IL-15 and IL-18 enhances their survival, maintains phenotypic properties, and increases the expression of the anti-apoptotic Bcl-2-like protein 1 [17]. IL-15 combined with IL-12 more effectively induces the production of cytokines, such as IL-10, MIP-1α, MIP-1β, and TNF-α, while slightly increasing IFN-γ levels. Previous studies have shown that the maximal production of granulocyte-macrophage colony-stimulating factor is achieved with a combination of IL-15 and IL-18 [18].

In addition to promoting the proliferation and activation of NK cells, IL-15 stimulates the activation and proliferation of various T-lymphocyte subsets, including $CD8^{-}CD4^{-}$, $CD8^{+}CD4^{+}$, $CD8^{+}$, and $CD4^{+}$ T cells. The proliferative effects of IL-15 are observed at all stages of $CD8^{+}$ T-lymphocyte development, primarily due to the increased expression of the anti-apoptotic molecule Bcl-2. Additionally, IL-15 enhances the induction of the anti-apoptotic protein Bcl-2 in exhausted $CD4^{+}$ T cells and Bcl-2 and Bcl-xL in activated $CD4^{+}$ T cells and memory $CD8^{+}$ T lymphocytes [19, 20]. Like IL-2, IL-15 activates the intracellular signaling pathways JAK1/STAT3 and JAK3/STAT5 by binding to the IL-2Rβ/γc receptor. These pathways subsequently trigger additional cascades, including PI3K/AKT/mTOR, Ras/Raf/MAPK, and AKT-XBP1s, which promote the expression and activation of key factors, such as c-myc, c-fos, c-jun, Bcl-2, Bcl-xL, and NF-κB. Concurrently, IL-15 reduces the expression of pro-apoptotic molecules such as Bim and PUMA (Figure 1). These effects enhance the survival, proliferation, and activation of effector T cells [20, 21, 22]. Unlike IL-2, which supports the proliferation of regulatory T (Treg) cells, IL-15 exerts minimal effects on Treg cells. This distinction is significant because Treg cells expressing FOXP3 inhibit effector T cells, thereby suppressing immune responses [23].

In addition to maintaining the balance of the immune system, IL-15 plays a key role in T cell activation in various inflammatory conditions. In viral and bacterial infections, signals from Toll-like receptors or type I interferons stimulate the production of IL-15 in antigen-presenting and epithelial cells [24]. IL-15 activates memory $CD8^{+}$ T cells, promoting their proliferation and effector functions in the absence of T cell receptor stimulation. In viral infections, elevated IL-15 levels promote the polyclonal expansion of memory $CD8^{+}$ T cells with diverse receptor repertoires. Although some studies have reported the protective role of IL-15, others have shown its potential to cause host tissue damage in bacterial infections [25, 26]. This pathological effect is evident in acute hepatitis A virus infection, in which elevated IL-15 levels stimulate NK-like cytotoxic activity in $CD8^{+}$ T cells, targeting liver

hepatocytes [27, 28]. Genetic variations in IL-15 or its receptor IL-15Rα are associated with an increased risk of autoimmune diseases, even in the absence of infection [29, 30]. Additionally, IL-15 activates the PI3K/AKT pathway, thereby maintaining chronic inflammation [31, 32, 33, 34].



Upon binding to its high-affinity receptor IL-15Rα and subsequent presentation to the IL-2/15Rβγ heterodimer, IL-15 triggers the activation of effector cells through three primary pathways: (1) the JAK-STAT pathway; (2) the PI3K-AKT pathway; and (3) the Ras-Raf-MAPK pathway.
Figure 1 – IL-15 signaling pathways in effector cells

Furthermore, IL-15 has been shown to enhance the effectiveness of antitumor mAbs and stimulate the secretion of the XCL1 chemokine and IFN-γ in activated NK and $CD8^+$ T cells. XCL1 facilitates the recruitment of type I dendritic cells to tumor tissues, whereas IFN-γ provides positive feedback to strengthen immune surveillance against tumors [30, 35, 36]. Additionally, IL-15 can stimulate memory $CD8^+$ T cells by upregulating Cpt1a expression [19, 37]. The Cpt1a enzyme catalyzes the transfer of long-chain acyl groups from acyl-CoA to carnitine, thereby allowing the entry of fatty acids into the mitochondrial matrix, where β-oxidation occurs [37]. This metabolic function of IL-15 highlights its potential application in combating obesity and metabolic disorders [37, 38].

IL-15 has tremendous antiviral and antitumor potential, making it a promising candidate for immunotherapy. In oncology, IL-15 holds therapeutic promise due to its ability to activate NK and $CD8^+$ T cells, enhance their antitumor activity, stimulate the recruitment and activation of dendritic cells, and establish a positive feedback loop to sustain immune surveillance against tumors.

Clinical significance of IL-15

IL-15 is a promising therapeutic agent for treating cancer and viral diseases. However, its clinical application is limited due to insufficient data on its systemic effects [39]. Two main approaches have been proposed to address these challenges: the first involves modifying the structure of IL-15 itself, and the second focuses on developing IL-15/IL-15Rα complexes [22]. Several IL-15-based therapeutics have been successfully developed and evaluated in clinical trials [40].

SO-C101 is a recombinant protein consisting of IL-15 fused with the NH_2-terminal (amino acids 1–77, $Sushi^+$) domain of IL-15Rα. This fusion extends the protein's half-life and promotes NK cell development and differentiation. Preclinical studies in a mouse model of colorectal cancer have shown that SO-C101 exhibits enhanced antitumor activity when combined with anti-PD-1 agents. This effect is mediated by the stimulation of $CD8^+$ T cell proliferation and activity in vivo. Additionally, SO-C101 promotes tumor cell killing and reduces metastasis by increasing NK cell infiltration, maturation, and proliferation while decreasing neutrophil infiltration in the lungs. Phase 2 clinical trials have evaluated the efficacy and safety of the combination of SO-C101 and the PD-1 inhibitor pembrolizumab in patients with advanced solid tumors and revealed clinical benefits and an encouraging safety profile [4, 41].

HetIL-15 (NIZ985) is a recombinant isomer of IL-15 and IL-15Rα, resembling IL-15 found in human plasma. Several studies in mice and macaques have shown that HetIL-15 has favorable pharmacokinetic properties and promotes cytotoxic lymphocyte proliferation. Additionally, a study on pancreatic adenocarcinoma showed that HetIL-15 therapy promoted tumor growth suppression, prolonged survival, and enhanced sensitivity to chemotherapy [42, 43].

SHR-1501 is a recombinant IL-15 engineered to enhance its activity via conjugation with IgG1-Fc. This modification extends the protein's half-life and stimulates T, B, and NK cell proliferation. To date, two phase I clinical trials have been conducted in China and Australia to evaluate the safety and efficacy of SHR-1501 in patients with advanced tumors [44].

NKTR-255 is a conjugate of two rhIL-15 proteins linked via polyethylene glycol that can interact with IL-15 receptors while retaining its biological properties. With its extended half-life and high potency, NKTR-255 induces the sustained activation and proliferation of NK cells and CD8+ T cells, thereby enhancing antitumor activity, including significant effects in multiple myeloma [45, 46].

HCW9201 is an IL-15 agonist consisting of a fusion protein that targets IL-12, IL-15, and IL-18 receptors. It activates and sustains memory NK cells while boosting their antitumor function. HCW9201 is currently used to treat leukemia and has been shown to enhance short- and long-term NK cell cytotoxicity and IFN production against leukemia cells [47].

ALT-803, also known as N-803, is an IL-15/IL-15Rα tetramer formed by fusing two IL-15 mutants (IL-15 N72D) with two IL-15Rα Su-IgG1 Fc fusion proteins. Phase I clinical trials have confirmed the tolerability and potential efficacy of a once-weekly dosing regimen of ALT-803 [22, 48].

Given its positive effects on the proliferation of NK cells and memory CD8+ T cells, IL-15 has attracted interest as a potential treatment for bovine leukemia. The combination of IL-15 and immune checkpoint inhibitors is a promising therapeutic approach. Combining IL-15 with anti-PD-L1 and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies significantly improved survival rates in mouse tumor models [49]. Currently, cytokines are being studied in conjunction with other forms of immunotherapy. Various formulations of IL-15 may demonstrate enhanced efficacy when combined with anti-CTLA-4 and anti-PD-L1 antibodies, as these combinations have shown highly effective antitumor responses [50]. This treatment strategy can significantly increase the numbers of NK and memory CD8+ T cells. Several preclinical studies have shown that the combination of IL-15 and anti-PD-L1 and anti-CTLA-4 antibodies markedly improves antitumor immunity, reduces tumor growth, and increases survival rates [50, 51].

Based on these findings, we conducted experiments to evaluate the synergistic effect of recombinant bovine IL-15 (rbIL-15) with mAbs targeting CTLA-4 and PD-L1 on IFN-γ production. No statistically significant increase in IFN-γ levels was observed in peripheral blood mononuclear cells (PBMCs) from healthy cows treated with mAbs and rbIL-15 compared with rbIL-15 alone. In PBMCs from BLV-positive cows, the triple combination significantly enhanced IFN-γ production compared with the other groups. Interestingly, no differences in IFN-γ levels were observed between healthy and BLV-positive cows treated with rbIL-15 alone. Despite these findings, the in vitro results are insufficient to justify the practical application of combination therapy in BLV-positive cattle. Therefore, further studies are needed to analyze the effects of IL-15 on T and B cells both *in vitro* and *in vivo*. These studies will provide valuable insights into using IL-15 as a molecular adjuvant to enhance the intensity and duration of immune responses [20].

The ability of IL-15 to stimulate the key mechanisms of innate and adaptive immunity significantly enhances the resistance of farm animals to viral infections. This property is particularly valuable in environments with high housing densities and high risks of epizootics. IL-15 stimulates the proliferation and cytotoxic activity of NK cells, serving as the first line of defense against viruses. This function is especially critical for combating infections, such as African swine fever, highly pathogenic avian influenza, porcine reproductive and respiratory syndrome, and classical swine fever. Additionally, IL-15 promotes the survival and activation of cytotoxic T lymphocytes, which recognize and eliminate virus-infected cells. IL-15 also induces the production of interferons, such as IFN-γ, thereby enhancing cellular resistance to viral replication and mobilizing other immune system components. These findings indicate that IL-15 is a promising therapeutic agent for managing viral infections in farm animals, particularly when used in conjunction with modern vaccination strategies and biosecurity measures.

Directions for future research

The potent immunomodulatory properties of IL-15 make it a promising candidate for treating oncological diseases and viral infections. Modifying IL-15 or developing IL-15-based therapeutics is one of the most promising research directions. In the initial stage of developing IL-15-based therapeutics, recombinant human IL-15 (rhIL-15) was produced using recombinant DNA technology. This protein was expressed as a non-glycosylated monomer (molecular weight 13 kDa) in *Escherichia coli* [40]. Early clinical trials demonstrated that rhIL-15 modestly increased the number of NK cells and CD8+ T cells in patients with metastatic melanoma and metastatic renal cell carcinoma. However, the results highlighted some limitations, including the short half-life of rhIL-15 and the necessity for daily administration. Despite these limitations, rhIL-15 remains a potential therapeutic option for cancer and viral infections. Further studies are needed to address these limitations and provide more precise data on its safety and efficacy to enable its successful clinical application [22, 52].

Another promising approach for developing IL-15-based therapeutics involves modifying T cells expressing chimeric antigen receptors (CARs) with IL-15 genes. This strategy involves generating T cells capable of secreting proinflammatory cytokines, thereby enhancing their functionality and antitumor activity [53]. *In vitro* studies have shown that introducing IL-12 into CAR-T cells increases their antitumor efficacy. Similarly, IL-15 expression in CAR-T cells has demonstrated multiple advantages, including enhanced expansion, reduced cell death rates, decreased PD-1 receptor expression, and significantly improved antitumor effects in vivo compared with unmodified cells. However, the use of proinflammatory cytokines, such as IL-12 and IL-15, is associated with toxicity risks, which presents a challenge for clinical applications. Regulating cytokine secretion by limiting CAR binding to antigens is a promising approach to mitigating these risks. This strategy can reduce the adverse effects associated with the continuous expression of proinflammatory molecules. These findings indicate that equipping CAR-T cells with cytokine-secreting capabilities is a promising approach to improving their therapeutic efficacy. However, further studies are needed to assess the safety of this approach and to address its potential limitations before clinical implementation [54].

Currently, IL-15 combined with mAbs and bispecific antibodies is under investigation in several clinical trials [55, 56, 57]. rhIL-15 administration has been shown to significantly increase the number of activated NK cells. However, this increase alone is insufficient to achieve robust antitumor effects. Preclinical studies have shown that IL-15-based therapies enhance antibody-dependent cellular cytotoxicity (ADCC) and improve antitumor activity. For example, the combination of IL-15 and rituximab and alemtuzumab has demonstrated increased therapeutic efficacy in B-cell leukemia models. NK cells and macrophages play critical roles in this enhanced effect, contributing to elevated ADCC and improved therapeutic responses. Notably, ADCC has been reported to be further elevated in NK cells after their interaction with macrophages. These findings have provided the foundation for clinical trials investigating the use of IL-15 combined with humanized mAbs for treating acute leukemia, chronic lymphocytic leukemia, T cell lymphoma, and renal cell carcinoma [49].

Conclusion

IL-15 is a promising immunomodulator due to its ability to activate NK and T cells, thereby enhancing their antitumor and anti-infective activities. IL-15 administration stimulates the production of cytokines such as IFN-γ, which activate macrophages and increase their phagocytic activity, which is a critical component of the body's defense against pathogens. Despite its promise, the use of IL-15 in veterinary medicine requires further studies to establish optimal dosages and administration regimens because excessive immune activation can lead to inflammatory reactions and other side effects. IL-15 may become an integral part of combination therapies for infectious diseases in cattle, particularly in cases of pathogen resistance to conventional antibiotics. Thus, IL-15 is highly relevant in the context of combating antimicrobial resistance.

Contemporary research has been increasingly focused on exploring less studied ILs, investigating their roles in emerging pathophysiological processes, and developing drugs targeting their modulation. These advances open new avenues for personalized medicine. The significance of ILs cannot be overstated, as they are key regulators of numerous biological processes. A deeper understanding of their mechanisms can enable a more effective management of immune and inflammatory responses.

Authors' Contributions

KM and KT: developed the concept and design of the study, drafted the manuscript. ZA, DK and LT: conducted a comprehensive literature search, analyzed the collected data. MN and BA: performed final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript.

Acknowledgments

This research was funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan (Grant No. AP19674577).

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 1 (009). – P. 28-37. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2025.5(009).1830
UDC 578.7 **Research article**

The immunity and sanitary conditions among people regarding the Covid-19 infection in the post-pandemic period

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Received: 16-01-2025 **Accepted:** 20-03-2025 **Published:** 31-03-2025

Abstract

Background and Aim. A year after the official declaration of the end of the pandemic, this study aimed to assess the epidemic situation by testing a small group of individuals for the presence of the SARS-CoV-2 virus and neutralizing antibodies.

Materials and Methods. Nasopharyngeal swabs were collected to isolate SARS-CoV-2 virus in cell culture, while venous blood samples were tested for neutralizing antibodies using a quantitative neutralization reaction. The study included 20 participants (approximately 10% of the team), of whom 16 had been vaccinated against COVID-19, and 4 remained unvaccinated. Vaccinated individuals included 4 who received Sputnik V (over 30 months prior) and 12 who received QazCOVID-in.

Results. All 20 participants demonstrated neutralizing antibodies against the Wuhan variant of the virus. For the Delta and Omicron variants, 19 participants showed neutralizing antibodies. Average antibody titers were 6.4 ± 1.39 $\log_2$ for the Wuhan variant, 6.2 ± 1.89 $\log_2$ for the Delta variant, and 4.75 ± 1.84 $\log_2$ for the Omicron variant.

Conclusion. The study highlights the persistence of antibodies against SARS-CoV-2 among both vaccinated and unvaccinated participants, though variation in neutralization efficacy was observed among different virus variants. These findings emphasize the importance of monitoring immune responses to track population-level immunity and guide future vaccination strategies.

Keywords: Antibodies; immune status; post-pandemic period; SARS-CoV-2 virus; titer; virus-neutralizing.

Introduction

After the end of the pandemic, there was a relative lull in the number of cases of coronavirus infection COVID-19 among the population. Large-scale immunization campaigns with vaccine preparations ceased and only in rare cases, except for the risk group, people voluntarily underwent vaccination and were subjected to diagnostic studies using laboratory tests. However, cases of respiratory diseases with a benign course were recorded. The etiology of these cases was not confirmed by medical studies. Therefore, in most cases, except in cases of laboratory testing, the presence and intensity of circulation of the SARS-CoV-2 virus in the community remained unknown [1]. The epidemic situation regarding coronavirus infection was assessed based on cases of official visits of sick people to medical institutions and diagnosis through laboratory tests. The decrease in cases of clinical disease and the absence of

mass laboratory tests indicated a real decrease in the intensity of the epidemic situation. However, cases of clinical coronavirus infection among the population continued to be identified, and the mutating causative agent of the disease spread on an interstate and intercontinental scale [2]. According to WHO and national health data, information regarding the number of people who fell ill, died, and recovered, as well as those vaccinated against the pandemic disease, was available [3, 4]. But there was no information about the population's immunity. This situation did not make it possible to predict the dynamics of the epidemic situation and evaluate the effectiveness of immunoprophylactic measures and immunization agents. In this connection, the purpose of the studies presented in this work was to establish the epidemic state among the population. This was done through indicator testing of a group of people for immune and sanitary status in relation to the coronavirus infection COVID-19 in the postpandemic period.

Materials and Methods

Virus

To set up the neutralization reaction, the SARS-CoV-2 viruses of the "Wuhan", "Delta", and "Omicron" variants were used, adapted in Vero cell culture with a biological activity of 6.00 lg $TCD_{50/cm3.}$

Cell culture

To isolate, titrate, and perform a neutralization reaction, we used the Vero (WHO certified) cell culture line grown in a monolayer in plastic mattresses and tablets.

Cells were grown in DMEM nutrient medium containing 5-10% fetal bovine serum (FBS). To maintain cell viability, the same nutrient medium was used, but containing 1-2% FBS.

Volunteers

20 employees of a research institute located remotely from large cities in the Zhambyl region acted as volunteers for the research. The volunteers included 11 men aged 22 to 52 years and 9 women aged 22 to 49 years. Volunteer serum samples collected on October 17, 2023 were used as research objects.

According to the anamnesis, volunteers constantly live in the locality where the research enterprise is located, and from time to time on weekends and days of labor leave they travel to different cities and towns of the Republic of Kazakhstan, as well as foreign countries.

Assessment of the immune state of volunteers

The immune status of volunteers was assessed based on the level of specific seropositivity and antibody titers to the SARS-CoV-2 virus detected in human blood serum. Specific antibodies were detected and their titer was determined using a quantitative neutralization reaction.

Setting up the neutralization reaction

The neutralization reaction was performed on a monolayer of Vero cell culture prepared in 96-well plastic plates. As a reaction mixture, we used two-fold dilutions (1:2, 1:4, etc.) of the tested blood serum of volunteers in a maintenance medium and a cultural suspension of the SARSCoV-2 virus of the "Wuhan", "Delta" and "Omicron" variant with a titer of 100 TCD50, taken in equal volumetric ratios. The resulting mixture was kept at a temperature of 37 °C for 60 minutes and added in equal doses to at least 4 wells of a 96-well plate with a test cell culture. As a dose control, the virus suspension of each variant was titrated on the same cell culture using its tenfold (10-1, 10-2, 10-3, 10-4) dilutions in a maintenance medium. To control the quality of the cell culture, at least 4 wells were left without adding the reaction mixture and virus, but replaced with a supporting medium. The cell culture in plates with a neutralization reaction was kept at a temperature of 37 °C for 5 days, after which the results of the virus CPE (cytopathic effect) were recorded. The absence of CPE in the cell culture, if it was present in the control wells with a dose of the virus and absent in the wells with the quality control of the cell culture, was considered to be neutralization of the virus or the presence of antibodies, and the presence of CPE, under the specified conditions in the listed controls, was considered to be the absence of neutralization and specific antibodies. The antibody titer was taken to be the highest dilution of blood serum that neutralized virus reproduction in at least 50% of cases. Antibody titers were given in logarithms of twofold dilutions of the blood serum. The virus and antibody titers in the blood serum were calculated according to Reed and Muench [5]. The reliability of the difference in antibody titers between groups (model animals) was determined using Student's t-test [6].

Assessment of the sanitary status of volunteers

The sanitary status of study participants was determined based on information provided by the volunteers themselves, external clinical signs and the results of virological testing for the presence of the virus by isolation in cell culture.

Virus isolation in vitro

Human infection with the SARSCoV-2 virus was determined by isolating the virus in cell culture from nasopharyngeal swab samples. The swabs were collected using sterile cotton swabs on a plastic stick, which were placed in a transport liquid and stored at minus 40 °C until the study. To isolate the virus, swab samples were thawed at room temperature, the cotton swabs were wrung out and removed, and the remaining liquid was centrifuged at 3000 g for 20 minutes, the sediment was transferred to a sterile tube, mixed with antibiotics and used to infect a Vero cell culture prepared in a monolayer in mattresses, seed area 25 cm^2. Before adding clinical samples, the cell monolayer was freed from the growth medium and washed twice with the DMEM nutrient medium without blood serum. The clinical sample under study, diluted with a nutrient medium without serum in a dilution of 1:2-1:4, was added to a monolayer of cells and kept at a temperature of 37 °C for 60 minutes. The inoculum was removed, the cell monolayer was cleared from the clinical sample by rinsing 3-4 times with the nutrient medium. Then, a monolayer of cells infected with a clinical sample was cultured at a temperature of 37 °C for up to 5 days. The presence of the virus was determined by the CPE, manifested in a monolayer of infected cells. In case of development of CPP, its etiology was identified using a neutralization reaction. In the absence of CPE, at least two blind passages in cell culture were performed. The isolated virus was identified using PCR and neutralization reaction.

Immunogenicity study design

Those wishing to undergo the study were selected from 10 divisions of the enterprise, 2 people from each on a voluntary basis. Before collecting blood samples, their medical history was recorded, including vaccination history against COVID-19, pandemic disease, and clinical condition at the time of blood donation. Each volunteer provided blood samples from a vein and nasopharyngeal swabs, collected using cotton swabs.

Serum was isolated from the blood, which was aliquoted in 1.0 ml portions and frozen at minus 40 °C until the study. Oropharyngeal swabs along with cotton swabs in a transport medium were also frozen at the same temperature and stored until the study. Before testing in the pH, blood serum samples were subjected to heat treatment at a temperature of 56 °C for 30 minutes.

Before performing the neutralization reaction, virus samples were titrated in a Vero cell culture prepared in 96-well plates. In the neutralization reaction, a cultural suspension with a virus titer of at least 106.0 TCD50/ml was used.

Each blood serum sample was tested in a neutralization reaction in parallel with three variants of the SARS-CoV-2 virus.

Statistical processing

The statistical data processing was carried out using the GraphPad Prism program, Version 8.

Results and Discussion

Anamnestic data and laboratory results for the detection of virus-neutralizing antibodies in serum samples and virus isolation from clinical samples collected from volunteers are shown in Table 1.

Table 1 – Immuno-sanitary status of people for coronavirus infection COVID-19

No	Subjects of the study	Vaccination period and vaccine against COVID-19	Anamnesis	Virus-neutralizing antibody titers for SARS-CoV-2 virus variant			Results of isolation of the SARS-CoV-2 virus
				Wuhan	Delta	Omicron	
1	EG no.1	QazVac, 27.05.21 (29 month)	Clinically healthy	7	7	5	-

Continuation of table 1

2	EG no.1	QazVac, 10.06.21 (28 month)	Clinically healthy	5	5	3	-
3	EG no.1	QazVac, 14.06.21 (28 month)	Clinically healthy	6	6	5	-
4	EG no.1	QazVac, 31.01.22 (21 month)	Clinically healthy	6	5	5	-
5	EG no.1	QazVac, 17.01.22 (21 month)	Malaise	6	8	5	++ (1-passage)
6	EG no.1	QazVac, 07.06.21 (28 month)	Clinically healthy	5	5	4	-
7	EG no.1	QazVac, 11.06.21 (28 month)	Clinically healthy	8	7	6	-
8	EG no.1	QazVac, 22.04.22 (18 month)	Clinically healthy	5	4	3	-
9	EG no.1	QazVac, 01.02.22 (20 month)	Clinically healthy	7	6	5	-
10	EG no.1	QazVac, 01.02.22 (20 month)	Clinically healthy	8	8	6	-
11	EG no.1	QazVac, 04.08.21 (26 month)	Clinically healthy	7	8	5	-
12	EG no.1	QazVac, 14.03.22 (19 month)	Clinically healthy	5	7	5	-
	Average data	**18-29** month	**1/12 (8.33 %)**	**6.25+1.09**	**6.33+1.52**	**4.75+2.96**	**1/12 (8.33%)**
13	EG no.2	Sputnik-V, 04.21 (30 month)	Clinically healthy	8	8	7	-
14	EG no.2	Sputnik-V, 04.21 (30 month)	Clinically healthy	8	8	6	-
15	EG no.2	Sputnik-V, 04.21 (30 month)	Clinically healthy	7	6	5	-
16	EG no.2	Sputnik-V, 04.21 (30 month)	Clinically healthy	8	7	5	-

Continuation of table 1

	Average data	**30** month	**0/4 (0%)**	**7.75+0.43**	**7.25+0.83**	**5.75+0.83**	**0/4 (0 %)**
17	CG	Not vaccinated	Clinically healthy	5	6	4	-
18	CG	Not vaccinated	Got sick in 2 weeks	8	8	6	-
19	CG	Not vaccinated	Loss of taste, smell	6	5	5	++ (1-passage)
20	CG	Not vaccinated	Clinically healthy	3	0	0	-
	Average data		**2/4 (50%)**	**5.5+1.80**	**4.75+2.95**	**3.75+2.28**	**1/4 (25%)**
	According to virus variants			**6.4+1.39**	**6.2+1.89**	**4.75+1.84**	

Note: In the numerator, the number of positive results; in the denominator – the total number of samples tested; "++" - positive result; "-" - negative result; antibody titer is given in log2 dilutions of serum; in parentheses, the percentage of positive results; CG - Control group; EG no.1 - Experimental group number 1; EG no.2 - Experimental group number 2

As can be seen from the data in Table 1, out of 20 volunteers studied, according to the anamnesis, 12 people were vaccinated with the inactivated vaccine “QazCOVID-in” (KZ) over 18-29 months, 4 people over 30 months were vaccinated with the vector vaccine “Sputnik-V” (RU), and the remaining 4 people did not take the vaccine against coronavirus infection COVID-19 during the pandemic and post-pandemic period.

The survey and clinical examination showed that at the time of collecting blood serum samples, among those vaccinated with the QazCOVID-in vaccine, one person felt unwell without an increase in body temperature. Other members of the group vaccinated with this vaccine did not experience illness resembling coronavirus infection during the post-vaccination period. Among the group of people vaccinated with the Sputnik-V vaccine, no illness resembling the COVID-19 coronavirus infection was observed during the post-vaccination period. Among the third group of people, at the time of the study, there was one case of clinical illness with symptoms of loss of taste and smell, and the second suffered a clinical illness of no more than moderate severity 2 weeks before the study.

During virological testing using Vero cell culture, the SARS-CoV-2 virus was isolated from clinical samples of one volunteer from the first group, consisting of those vaccinated with the QazCOVID-in vaccine, and one volunteer from the group without vaccination.

The results of the neutralization reaction showed that in both groups of people vaccinated against COVID-19 coronavirus infection, there are virus-neutralizing antibodies in 100% of cases. The identified antibodies in both groups of people vaccinated against COVID-19 coronavirus infection had the ability to neutralize all three variants of the SARS-CoV-2 virus used. The average antibody titer in the QazCOVID-in vaccine group was 6.25+1.09 $\log_2$ against the Wuhan variant, 6.25+1.09 $\log_2$ against the Delta variant and 4.75+2.96 $\log_2$ against the variant "Omicron" of the SARS-CoV-2 virus. In the group of people vaccinated with the Sputnik-V vaccine, antibody titers against the Wuhan, Omicron and Delta variants were 7.75+0.43 $\log_2$, 7.25+0.83 $\log_2$ and 5.75+ 0.83 $\log_2$, respectively. Antibodies specific to the SARS-CoV-2 virus were also present in people from the group who did not receive the COVID-19 vaccine. In three out of four people, antibodies were detected for all three variants of the Wuhan, Omicron and Delta virus in titers of 6.33 + 1.25 $\log_2$, 6.33 + 1.25 $\log_2$ and 5.0 + 0.81 $\log_2$ respectively. The fourth representative of this group had trace titers (3 log2) of antibodies to the Wuhan variant, but no antibodies to the other two variants of the pathogen.

With the official end of the pandemic in the fall of 2022 in all countries of the world, including the Republic of Kazakhstan [7], the number of anti-epidemic vaccinations against the coronavirus infection COVID-19 has sharply decreased. The regime of mandatory respiratory tract protection with masks in public places, work, educational and other groups and areas was canceled [8] For more than a year of the post-pandemic period, the intensity of the epidemic situation was low and stable. However, isolated cases of clinical manifestations of the disease with varying degrees of severity were recorded [9]. According to the Ministry of Health, by the end of 2022 and during 2023, mixed and superinfections of seasonal influenza (A and B) and coronavirus infection COVID-19 were observed among the population [10, 11, 12].

In national health care, the question of the advisability of routine vaccination in the vaccination calendar has arisen. The current and forecast state of immunity against COVID-19 in the future, in the absence of vaccine prevention and the development of an epidemic situation after the end of post-vaccination immunity, remains unknown. Since, as is known, the duration of post-vaccination and post-infectious immunity according to the dynamics of specific antibodies lasts from 6 to 9 months [13, 14, 15].

In connection with this situation, the need arose for preliminary monitoring of the immune state of people against coronavirus infection in the post-pandemic period after the completion of post-vaccination and post-infectious immunity. For such studies, a group of 20 people from one work team consisting of more than 200 employees was selected.

The studies consisted of collecting and analyzing anamnesis, consisting of information about vaccination and the timing of its receipt, the type of vaccine, coronavirus infection COVID-19, identifying and assessing the level of virus-neutralizing antibodies and their ability to neutralize different variants of the SARS-CoV-2 virus that have circulated and are circulating among the population of the Republic of Kazakhstan. According to data from the Committee for Sanitary and Epidemiological Control of Domestic Health Care, different genetic lines of the Omicron variant of the pandemic virus circulated in the country in 2022-2023 [16, 17]. The data obtained, according to the collected anamnesis, showed that the target group included subjects who received the Sputnik V vaccine (20%), RU, for 30 months, and QazCOVID-in (60%), KZ, for 18-29 months, as well as those who did not receive immunoprophylaxis (20%) with any vaccine. At the time of the study, from among those vaccinated with QazCOVID-in, one subject felt unwell, from the group of unvaccinated subjects, one subject was in a state of loss of taste and smell, and the second suffered from the disease for two weeks. There were no clinically ill participants among the participants who received the Sputnik V vaccine.

Serological testing showed that 95% of participants had virus-neutralizing antibodies to the Wuhan, Omicron and Delta variants of the SARS-CoV-2 virus. Seropositivity was observed in all participants receiving both Sputnik V (100%) and QazCOVID-in (100%) vaccines. Seronegativity was observed in one (25%) of four subjects in the nonvaccination group. Antibody titers to the SARS-CoV-2 virus in the group previously vaccinated with the Sputnik V vaccine were significantly higher against all three variants of the pandemic virus. They averaged 6.91 + 0.85 $\log_2$, 7.75 + 0.43 $\log_2$, 7.25 +0.83 $\log_2$, and 5.75+0.83 log2 against the Wuhan, Omicron and Delta variants, respectively). Antibody titers to the SARS-CoV-2 virus in the group previously vaccinated with the Sputnik V vaccine were significantly higher against all three variants of the pandemic virus. They averaged 6.91 + 0.85 $\log_2$ against the Wuhan variant, 7.75 + 0.43 $\log_2$ against the Omicron variant, and 7.25 + 0.83 $\log_2$ against the Delta variant.

In the group of subjects previously vaccinated with the QazCOVID-in vaccine, the average titer of antibodies against different variants of the virus was 5.75 + 0.71 $\log_2$, 6.25 + 1.09 $\log_2$, 6.25 + 1.09 $\log_2$, and 4.75 +2.96 $\log_2$ vs. Wuhan, Omicron, and Delta variants, respectively). In the group of subjects who had not previously received the vaccine, seropositivity was observed in 75%, in whom the average antibody titer was at the level of the antibody titer detected in people previously vaccinated with the QazCOVID-in vaccine, and averaged 5.89 + 0.62 $\log_2$, 6.33+1.25 $\log_2$, 6.33+1.25 $\log_2$, 5.0+0.81 $\log_2$ vs. Wuhan, Omicron and Delta variants, respectively).

Analysis of the level of antibody titers against each variant of the virus indicates that in all three groups the level of antibodies to the Wuhan and Delta virus variants was the highest and averaged 6.4 + 1.39 $\log_2$ and 6.2 + 1.89 $\log_2$, respectively, while for the Omicron variant the antibody titers were comparatively lower and averaged 4.75+1.84 $\log_2$. A high level of seropositivity with maximum antibody titers among the studied population long after the cessation of vaccination indicates the presence of a constantly operating immunizing factor that supports herd immunity. It must be assumed that such

a factor is the causative agent of a pandemic coronavirus infection, which is widespread among the population and, transmitted from person to person, regardless of his immunity [18, 19, 20], multiplies in the body and stimulates the formation of antibodies. However, in most cases, due to the presence of residual immunity or immune memory formed from vaccination with a vaccine or a previous infection, the virus causes a subclinical infection without clinical signs. The disease manifests itself clinically in varying degrees of severity in the absence or weakness of specific and general immunity, as well as the development of mixed and superinfections [21, 22, 23]. Based on a comparison of antibody titers, it can be assumed that previous vaccination and the type of vaccine used have a positive effect on protection from the development of clinical disease and the formation of humoral immunity factors. This is indicated by 100% seropositivity and a high level of antibodies with no and low levels of clinical disease (8.3%) in the anamnesis in subjects previously vaccinated with the Sputnik V vector vaccine and the inactivated QazCOVID-in vaccine, while in those not previously vaccinated, seropositivity was 75% with a no less low titer of antibodies, but with the development of clinical disease in 50%.

Using in parallel a neutralization reaction with three variants of the virus that circulated among the population of the republic, the protective effectiveness of existing factors of humoral immunity of subjects against these pathogens was assessed in a comparative aspect. The data obtained show that the studied blood serum samples in all three groups neutralize the variants of the pathogen "Wuhan" and "Delta" with a relatively greater activity, and the variant "Omicron" to a slightly lesser extent, indicating a probable antigenic difference between the first two variants of the pathogen and the third. The reliability of such a judgment requires confirmation by additional detailed studies. It is possible that such a difference in antibody titers is associated with the dominant spread of the "Delta" variant, which in terms of its appearance is closer to the original variant of the pathogen, before the appearance of the "Omicron" variant.

Thus, the data obtained indicate that in the studied cohort of people, regardless of previous immunoprophylaxis, there was 95% seroconversion to the SARS-CoV-2 virus with high antibody titers, reminiscent of post-infectious or post-vaccination immunity. Failure to take the vaccine for at least the last 18 months indicates a post-infectious etiology of humoral immunity factors. Additional confirmation of this are clinical cases of the disease with the isolation of the virus among the studied volunteers, previously vaccinated and not vaccinated with the vaccine. Based on this statement, it follows that the SARS-CoV-2 virus continuously circulates with widespread prevalence among people with and without the development of clinical disease.

Conclusion

1. Of the people studied, 95% were seropositive for antibodies that neutralize the SARS-CoV-2 virus of the Wuhan, Delta and Omicron variants related to coronavirus infection COVID-19. Among the volunteers, 20% were vaccinated with the Sputnik V vaccine within 30 months, 60% with the QazCOVID-in vaccine within 18-29 months, and the remaining 20% did not receive immunoprophylaxis with any vaccine.

2. Average antibody titers in people previously vaccinated with the Sputnik V vaccine were 7.75+0.43 $\log_2$ against the Wuhan variant, 7.25+0.83 log2 against the Delta variant and 5.75+0.83 $\log_2$ against the Omicron variant. Similar antibody titers in volunteers vaccinated with the QazCOVID-in vaccine were 6.25 + 1.09 $\log_2$ against the Wuhan variant, 6.25 + 1.09 $\log_2$ against the Delta variant and 4.75 + 2.96 $\log_2$ versus the Omicron option. Virus-neutralizing antibodies were also detected in three out of four unvaccinated individuals. Their average antibody titers to the Wuhan, Omicron and Delta variants were 6.33 + 1.25 $\log_2$, 6.33 + 1.25 $\log_2$ and 5.0 + 0.81 $\log_2$, respectively.

3. The presence of antibodies to the SARS-CoV-2 virus in the studied subjects in the long term after the use of vaccines, as well as seropositivity for this pathogen in people who did not take an immunoprophylactic drug, indicates that the causative agent of coronavirus infection COVID-19 is circulating among the population in the post-pandemic period without massive stimulation of clinical disease development. This circulating virus causes a sub infection, during which ongoing immunity is formed.

4. The neutralizing activity of antibodies to the three variants of the SARS-CoV-2 virus used indicates that there is a close relationship between the antigens of the virus variants and the immunity factors formed against one of them have protective effectiveness against the other two variants of the pathogen. A significant difference in antibody titers to different variants indicates a possible antigenic drift in the Omicron variant from the antigenic specificity of the previous two variants.

5. It is likely that post-vaccination immunity, depending on the type of vaccine used, has a different positive stimulus on the level of post-infectious immunity formed during subinfection, since in those vaccinated with the Sputnik V vector vaccine, antibody titers exceeded those in persons vaccinated with the inactivated QazCOVID-in vaccine by 1.0-1.5 $\log_2$, and by 0.75-1.39 $\log_2$ in unvaccinated subjects. The level of antibody immunity formed during subinfection is equivalent among those vaccinated with the whole-virion inactivated vaccine and unvaccinated individuals.

Authors' Contributions

Conceptualization, LK. Data curation, BM and LK. Formal analysis, LK. Methodology, LK, BM, MR, KZh. Investigation, GZ, TT, AT, KB. Writing original draft, LK. Writing-review and editing, BM and LK. All authors have read and agreed to the published version of the manuscript.

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 1 (009). – P. 38-47. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2025.5(009).1854
UDC 619: 579.62: 636.7 **Research article**

Clinical status in viral diseases of dogs complicated by associations of opportunistic microorganisms

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Received: 30-01-2025 Accepted: 12-03-2025 **Published:** 31-03-2025

Abstract

Background and Aim. Infectious pathologies are prevalent among canines, with the majority being of viral etiology. The most notable examples include carnivore plague and adenovirus infection. A distinctive attribute of viral infections is their tendency to occur in clusters, whereas monoinfections are observed with a lower frequency. Clinically, high mortality rates are frequently observed in viral diseases, often due to the development of secondary infections that complicate the course of the primary disease. The objective of the present study was to investigate the clinical manifestations (symptoms, haematological and biochemical blood parameters) of viral diseases (i.e. canine plague and adenovirus infection) in dogs complicated by opportunistic microorganisms, in the conditions of Kostanay city, RK.

Materials and Methods. Canines of various breeds, sexes and age groups were utilised as subjects for the study. The diagnosis of viral diseases in canines was conducted in a comprehensive manner. Dogs with confirmed viral infection were sampled for the isolation and identification of opportunistic microorganisms.

Results. In Kostanay city, from 2022 to 2024, 15 cases of plague of carnivores and 12 cases of adenovirus infection were registered, 13 isolates of opportunistic microorganisms were detected and identified. Blood analysis in monoinfection showed decreased erythrocyte count, haemoglobin and platelet levels. In adenovirus type CAV-1, there were abnormalities in total bilirubin, ALT, AST alkaline phosphotase, and albumin. Blood analysis in viral diseases complicated by associations of opportunistic microorganisms showed leukocytosis, increased neutrophils, globulins and urea indicating the development of secondary infection.

Conclusion. The combination of clinical symptoms and laboratory data allow a more accurate assessment of the severity of the animal's condition and identify lesions of various body systems. The prevalence of opportunistic microorganisms in viral pathologies aggravate the course of the main disease and cause the development of secondary infection.

Keywords: adenovirus; blood biochemistry; hematology; opportunistic microorganisms; carnivorous plague.

Introduction

Annually, diseases of infectious etiology are documented in domestic animals, constituting a substantial proportion of the total number of pathologies. Their danger lies in the fact that they are characterised by their widespread distribution, extensive coverage of a large number of livestock, ability to be transmitted from one animal to another, from animal to human, and vice versa, causing zooanthroponosis, which causes great economic damage [1].

Among the infectious pathologies registered in dogs, most of them are diseases of viral etiology. The most dangerous infectious diseases of dogs include parvovirus enteritis, plague of carnivores, adenovirus infection, rabies, infectious hepatitis, etc [2-4]. This is due to the fact that the virus causing the diseases is quite widespread in nature, as well as the difficulty and delay in diagnosis of some viral infections. Diseases continue to circulate in the canine population due to long term virus carriage in dogs. For example, dogs that have had plague are able to secrete the virus into the external environment for up to 3-4 months, while adenovirus infection - secrete the virus for up to seven years and remain viral carriers for life [5, 6]. According to researchers, viral diseases of dogs, despite the widespread use of effective vaccines, remain the most dangerous and most common pathologies, with 100% morbidity and mortality up to 91% [2, 5, 7]. Diseases lead to significant economic losses, which consist of direct losses due to high mortality of especially valuable animals, reduced performance and breeding qualities in sick and diseased individuals, as well as the costs of diagnostic, treatment and preventive measures [8].

A distinctive attribute of viral infections is their associative nature, characterised by the involvement of multiple pathogen species in the development of the disease. In contrast, mono infections are significantly less prevalent. In recent years, there has been a significant increase in the study of associative viral infections in farm animals [9, 10], while the study of individual nosologies in domestic animals, particularly dogs, has been less extensive [11, 12].

As a result of our earlier diagnostic studies of samples obtained from sick dogs, the presence of one of three types of pathogenic viruses, such as adenovirus, carnivore plague virus, and parvavirus enteritis, was determined. The prevalence and antibiotic resistance of microorganisms of the Enterobacteriaceae family such as *E. coli, Klebsiella, Proteus, Citrobacter, Enterobacter* were studied [8, 13].

The favourable course of infectious diseases requires timely diagnosis and good quality treatment of dogs, which in practice is complicated by the usually late arrival of owners at the veterinary clinic. Clinically, in viral diseases, there is often a high mortality rate in animals. This is due not only to the high virulence of viruses, but also to the development of secondary infections complicating the course of the underlying disease [14, 15].

Significant issues, such as clinical parameters in viral pathologies of dogs complicated by associations of opportunistic microorganisms, remain understudied to date.

The aim of the study was to investigate clinical manifestations (symptoms, haematological and biochemical blood parameters) in viral pathologies (plague of carnivores, adenovirus infection) of dogs complicated by associations of opportunistic microorganisms in the conditions of Kostanay city, RK.

Materials and Methods

Dogs of different breeds and sex and age groups, patients of veterinary clinics of Kostanay served as an object of research.

The diagnosis of viral diseases in canines was made in a comprehensive manner, with consideration given to epizootological data, anamnestic interviews with the owners, and clinical examinations conducted in accordance with generally accepted methods. Furthermore, general and biochemical blood tests were performed, and if deemed necessary, ultrasonography of the abdominal cavity organs and X-rays were conducted.

Blood samples were obtained from canines via the saphenous vein of the forearm. The blood studies were conducted at the Research Institute of Applied Biotechnology of KRU Ahmet Baytursynuly, in the laboratory of clinical-diagnostic, microbiological research and safety of materials of biological origin. Haematological studies were performed using a veterinary haematological analyser Exigo 17 (Spanga, Sweden), while biochemical analysis was performed on an automatic biochemical analyser BioChem FC-120 (High Technology Inc., North Attleborough, MA, USA). The data automatically displayed in the analysers were taken as norms of the dogs' blood parameters.

The final diagnosis of plague was made on the basis of the results of an immunochromatographic analysis for the detection of specific antigens of virus (QBQVET, Moscow, Russian Federation). The diagnosis of canine adenovirus was conducted through the utilisation of real-time polymerase chain reaction (PCR), employing the RealBest-Vet DNA CAdV-1 kit (Vector-Best, Novosibirsk, Russian Federation).

In canines with confirmed viral infection, biomaterial (oral and anal washes) was collected for the isolation and identification of opportunistic microorganisms.

The isolation and accumulation of pure cultures of microorganisms were performed using universal chromogenic, differential diagnostic media. The identification of *E.coli, Klebsiella, Citrobacter, Enterobacter, Proteus, Staphylococcus aureus* and *Streptococcus cultures* was performed in accordance with the approved methodological guidelines for the microbiological diagnosis of diseases caused by Enterobacteriaceae [16], as well as in accordance with Bergey's bacterial identifier.

The isolation of microorganisms from the study material was achieved through the sowing of samples on MPB, followed by incubation for a duration of 18-20 hours at a temperature of 36-37 °C. Subsequent to this, the samples were transferred to cups containing chromogenic media CHROMagar™, which were then subjected to further culturing. When well-defined colonies characteristic of the growth of *E.coli, Klebsiella, Citrobacter, Enterobacter, Proteus, Staphylococcus aureus, Streptococcus spp.* on this medium appeared, smears were prepared and subjected to Gram staining. In the event that morphologically typical bacteria were identified in the smears, their biochemical properties were subsequently studied.

Results

A total of 15 cases of carnivore plague and 12 cases of adenovirus infection were documented in veterinary clinics in Kostanay city among canines aged between two and 12 months. The occurrence of these viral infections in canines was found to be associated with the absence of vaccination.

In animals with a confirmed diagnosis of 'plague of carnivores', the initial history-taking during admission revealed the initial signs to be behavioural changes. Canines exhibited symptoms such as lethargy, inactivity, reduced appetite, and a refusal to eat, accompanied by elevated body temperatures reaching 40.5 °C. On examination, the animals exhibited signs of generalised distress, including lethargy, dullness of coat, anorexia, elevated body temperature ranging from 39.5 to 40.3 °C, and enlarged lymph nodes. The majority of animals (n=11) exhibited signs of gastrointestinal tract damage, characterised by the onset of diarrhoeal syndrome with mucous-bloody diarrhoea and frothy vomiting. In four dogs, signs of respiratory distress, such as wheezing, were observed, along with conjunctivitis, characterised by discharge, and coughing during physical activity. Consequently, the intestinal form of the disease was observed in 11 animals, while the pulmonary form of plague of carnivores was identified in 4.

A detailed examination of canines diagnosed with adenovirus infection revealed a depressed state, decreased appetite, drowsiness, enlarged lymph nodes, and elevated body temperature of up to 39-40.1 °C. Furthermore, nine animals exhibited signs of moderate respiration, dry cough and minor nasal discharge. In three animals, the manifestation of adenovirus infection was characterised by vomiting with bile, yellowing of mucous membranes, blurred corneas, and liver enlargement upon palpation. Consequently, adenovirus of the second type (CAV-2) was detected in nine animals, and in three dogs, it was found to be of the first type (CAV-1) or to be infectious canine hepatitis.

During the course of a microbiological examination of biomaterial from canines (n=27) afflicted with viral diseases, it was possible to isolate and identify opportunistic microorganisms in 40.7% (n=11). The results of this examination are presented in Table 1.

Table 1 – Species composition of microorganisms isolated from dogs

Disease / Microorganism	Carnivore plague (n=15)		Adenovirus infection (n=12)		Total (n=27)
	intestinal form (n=11)	pulmonary form (n=4)	CAV-1 (n=3)	CAV-2 (n=9)	
E. coli	2	1	0	1	4
Klebsiella	1	0	1	0	2
Citrobacter	0	0	0	0	0
Enterobacter	0	0	0	1	1
Proteus	1	0	0	0	1

Continuation of table 1

Staphylococcus aureus	0	1	0	2	3
Streptococcus spp.	1	1	0	0	2
Total	5	3	1	4	13

In the course of the study, 4 (30.7%) E. coli strains, 3 (23%) *Staphylococcus aureus* strains, *Streptococcus spp.* and *Klebsiella* strains 15.4% each, *Proteus* and *Enterobacter* strains 1 (7.7%) each were isolated. Citrobacter strains were not detected. In two animals, an associated carrier of microorganisms was detected: *E. coli* + *Staphylococcus aureus* and *Staphylococcus aureus* + *Enterobacter*.

The results of blood examination of dogs with carnivore plague are presented in Table 2.

Table 2 – Haematological and biochemical parameters of blood of dogs with plague of carnivores

Indicator, units of measurement	Normal	Carnivore plague		Plague in association with microorganisms	
		intestinal form (n=6)	pulmonary form (n=2)	intestinal form (n=5)	pulmonary form (n=2)
Erythrocytes, 1012/ l	5.5–8.5	4.7±0.38	4.85±0.49	3.82±0.45	4.7±0.98
Haemoglobin, g/l	120–180	107±4.8	103.5±4.9	104±5.1	109.5±3.5
Leucocytes, 109/ l	6-15	4.7±0.46	3.5±0.7	18.2±1.24	17.5±2.1
Lymphocytes, 10^9	1–5 × 10^9	0.93±0.17	0.8±0.28	0.84±0.31	0.9±1.4
Platelets, *109 g/l	200–500	145±17	161 ±12.7	133±19	170±14
ESR, mm/hour	2–6	10.3±0.92	12±2.8	10.7±0.8	9.5±2.1
Neutrophils, 10^9/л	3–11	5.3±1.37	4±1.4	13.8±1.19	14.5±2.1
Total protein, g/l	55–75	38.6±4.5	44±1.4	40.1±5.7	48±6.3
Albumin, g/l	25–40	19.3±1.9	23.5±0.7	23.3±1.7	20±2.8
Globulins, g/l	20–35	37.8±2.4	37±1.4	39.1±3.1	41±2.8
Total bilirubin, µmol/L	2–10	13.2±1.3	11±2.1	15.1±1.7	11.5±0.7
ALT, U/L	10–100	129.3±8.2	102.5±14.8	133.8±7.4	100±3.5
AST, U/L	10–50	64.6±5.2	51±2.8	67.8±7	49±2.8
Urea, µmol/L	2.5–9.5	12±1.7	11.15±2.6	16.1±1.9	16.5±2.1
Creatinine, µmol/L	44–159	163±4.1	164.5±4.9	169.5±5.7	167±5.6
Alkaline phosphatase, IU/L	20–150	177±6.7	151±6.36	181.3±7.8	149±7.7
Glucose, µmol/L	3.5–6.1	2.2±0.42	3.2±0.28	2.1±0.8	3.2±0.28
Potassium, µmol/L	3.5–5.5	2.03±0.35	2.75±0.35	2±0.5	2.9±0.14
Sodium, µmol/L	135–155	109.3±9.4	125±7.1	108±8.3	134.5±0.7
Chlorine, µmol/L	95–120	78.8±6.65	81.75±2.1	81±7.1	92.5±3.5

In the monoinfection course of plague in carnivores, leucopenia, lymphopenia, slightly decreased haemoglobin levels, increased ESR, increased urea and creatinine, and decreased glucose, potassium and sodium levels were observed. In the associative course of the disease, compared to monoinfection, there was lycocidosis, increased neutrophil counts, globulin, and urea. Alkaline phosphatase and total bilirubin levels increased in both cases.

The results of blood tests of dogs with adenovirus infection are presented in Table 3.

Table 3 – Haematological and biochemical blood parameters in adenovirus infection

Indicator, units of measurement	Normal	Adenovirus infection		Adenovirus in association with microorganisms	
		CAV-1 (n=2)	CAV-2 (n=6)	CAV-1 (n=1)	CAV-2 (n=3)
Erythrocytes, 1012/ l	5.5–8.5	4.8±0.14	4.3±0.52	5.3	5.1±0.21
Haemoglobin, g/l	120–180	116.5±2.1	102±3.7	118	108.3±5.4
Leucocytes, 109/ l	6-15	18±0.7	3.9±0.89	17	17.7±1.7
Lymphocytes, 10^9	1–5 × 10^9	0.85±0.2	0.87±0.26	1	0.8±0.14
Platelets, *109 g/l	200–500	173.5±10.6	134±21	168	186.6±8.4
ESR, mm/hour	2–6	9±2.8	11±1.3	8	12±1.8
Neutrophils, 10^9/л	3–11	10.5±0.7	7.1±2.1	15	14.3±1.7
Total protein, g/l	55–75	41.5±2.1	48±5.1	37	48.3±2.9
Albumin, g/l	25–40	18.5±2.1	24.5±2.3	18	44.3±2.1
Globulins, g/l	20–35	36±1.4	43.8±3.8	41	43.3±3.8
Total bilirubin, µmol/L	2–10	16.5±21	10.1±1.1	18	11.33±0.8
ALT, U/L	10–100	123.5±4.9	98±9.3	131	90.6±7.08
AST, U/L	10–50	68±5.6	48±7.2	63	47±2.5
Urea, µmol/L	2.5–9.5	10.75±1.7	15.3±1.9	12.2	13.56±0.9
Creatinine, µmol/L	44–159	163±2.8	172.1±4.7	165	161±1.4
Alkaline phosphatase, IU/L	20–150	180.5±4.9	148±9.8	183	139.6±12
Glucose, µmol/L	3.5–6.1	3.6±0.14	1.9±0.55	3.5	2.5±0.33
Potassium, µmol/L	3.5–5.5	3.7±0.14	2.4±0.47	3.7	2.9±0.63
Sodium, µmol/L	135–155	135.5±0.7	107.6±8.8	136	134.3±2.8
Chlorine, µmol/L	95–120	96.5±2.1	68.1±7.08	95	105±9.3

In the study of indicators of general blood analysis of dogs with adenovirus infection leukocytosis, lymphopenia, thrombocytopenia, decreased haemoglobin and total protein levels, increased alkaline phosphotase, creatinine and COE were revealed. However, in adenovirus complicated by associations of opportunistic microorganisms, leukocytosis, increased neutrophils, globulins and urea were detected. In both cases, in adenovirus type CAV-1 there were deviations in the indices of total bilirubin, albumin, ALT, AST, alkaline phosphotase, in a greater direction than in adenovirus CAV- 2, while the level of glucose, potassium, sodium, chlorine were within normal limits.

Discussion and Conclusion

Viral diseases of canines are highly prevalent, with some of the most common viral infections being carnivore plague and adenovirus infection [2]. In veterinary clinics in Kostanay city, 15 cases of plague of carnivores and 12 cases of adenovirus infection were recorded among dogs aged between 2 and 12 months. The absence of vaccination and the age of the animals have been identified as underlying factors contributing to viral disease infection in dogs [17, 18].

In animals with a confirmed diagnosis of 'plague of carnivores', the infection was characterised by a range of symptoms, including lethargy, reduced activity, decreased appetite or refusal of food, and elevated body temperature reaching up to 40.5 °C. In the majority of animals (73.3%), gastrointestinal tract lesions were observed. These observations are consistent with those reported in studies conducted in various countries, including Canada and Egypt, where diarrhoea with blood, vomiting and dehydration were detected in 68-83% of animals with viral pathology [18, 19]. Furthermore, a pulmonary form of plague was detected in 26.7% of dogs. Symptoms of respiratory and gastrointestinal involvement are common in cases of carnivore plague [20].

Haematological changes associated with carnivore plague included a decrease in erythrocyte count, haemoglobin levels, and platelet counts, indicating the presence of anaemia. Furthermore, biochemical analysis revealed elevated levels of alkaline phosphatase [21].

In all cases of canine adenovirus infection, physical examination revealed a depressed state, decreased appetite, drowsiness, enlarged lymph nodes, and elevated body temperature of up to 39-40.1 °C. Furthermore, in the case of adenovirus type CAV-1, the following signs were observed: vomiting with bile, yellowing of mucous membranes, blurring of the cornea of the eyes, and enlargement of the liver. These signs are indicative of infectious hepatitis, as is typical of this particular type of disease [22, 23]. In the case of adenovirus type CAV-2, the animals exhibited signs of respiratory disease, including respiration, coughing and nasal discharge [24].

Blood tests in dogs with adenovirus showed thrombocytopenia, leukopenia and anaemia, as in several studies in Brazil and India [25, 26]. In addition, adenovirus type CAV-1 showed abnormalities in total bilirubin, Alt, Ast, alkaline phosphatase, and albumin, as in other liver diseases in dogs [27, 28].

A microbiological examination of biomaterial from dogs (n=27) afflicted with viral diseases revealed the presence of opportunistic microorganisms in 40.7% of cases. Specifically, 4 (30.7%) strains of *E. coli,* 3 (23%) strains of *Staphylococcus aureus*, *Streptococcus spp*. and *Klebsiella* strains 15.4% each, *Proteus* and *Enterobacter* strains 1 (7.7%) each were isolated. Citrobacter strains were not detected. Furthermore, the presence of associated microorganisms was identified in two animals: *E. coli* + *Staphylococcus aureus* and *Staphylococcus spp. + Enterobacter.*

The present study examined the differences between haematology and blood biochemistry in viral diseases complicated by associations of opportunistic microorganisms in comparison with monoinfections. The results demonstrated that leukocytosis, increased neutrophil, globulin and urea levels were evident. It is most likely that these blood changes are due to the development of a secondary infection [29, 30].

In the city of Kostanay 15 cases of plague of carnivores and 12 cases of adenovirus infection were registered. Conditionally pathogenic microorganisms were detected and identified in 40.7% of dogs with viral diseases (30.7% of *E. coli* strains, 23% of Staphylococcus aureus strains, 15.4% of Streptococcus spp. and Klebsiella strains, 7.7% of Proteus and Enterobacter strains). Furthermore, microbial carriage was detected in two animals: *E. col*i + Staphylococcus aureus and Staphylococcus aureus + Enterobacter.

Furthermore, the haematology and blood biochemistry of dogs in monoinfection showed decreased erythrocyte count, haemoglobin and platelet levels, indicating anaemia. In the adenovirus type CAV-1, abnormalities were detected in total bilirubin, AlAt, AsAt, alkaline phosphotase, and albumin, indicating liver dysfunction.

Furthermore, the presence of leukocytosis, elevated neutrophil levels, increased globulins, and increased urea was observed in cases where viral diseases were complicated by the presence of opportunistic microorganisms, suggesting the development of a secondary infection.

Consequently, the integration of clinical manifestations and laboratory findings enables a more precise evaluation of the severity of the animal's condition, thus facilitating the identification of lesions affecting diverse bodily systems. The presence of opportunistic microorganisms in the context of viral pathologies has been demonstrated to exacerbate the progression of the primary disease, often leading to the development of secondary infections.

Authors' Contributions

RR and AN: conceptualized and designed the study, conducted a comprehensive literature search, and analyzed the collected data. YuA, GA and AZ: carried out the research implementation and analyzed the results. RR and AM: performed the final editing and proofreading of the manuscript. All authors have read, reviewed, and approved the final version of the manuscript.

Acknowledgements

The research has been undertaken as part of the research objectives of the thesis and will subsequently be presented at a public defence of the thesis.

Conflicts of Interest

There is no conflict of interest.

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 1 (009). – P. 48-62. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2025.5(009).1866
UDC 637.06; 637.07 **Review article**

Meat Product Adulteration: Modern Detection Methods and Food Safety Assurance

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Received: 13-02-2025 **Accepted:** 19-03-2025 **Published:** 31-03-2025

Abstract

Intentional adulteration of meat products through falsification can impact product safety and consumer properties. Adulteration involves the addition of low-quality or unauthorized ingredients and deviations from declared standards, posing potential health risks to consumers. Various laboratory methods, including DNA analysis, chemical, and physicochemical studies, are used to control adulteration. These methods allow for precise determination of product composition and identification of discrepancies with declared characteristics. However, there is no universal method capable of detecting all types of counterfeit. Identifying counterfeit products requires a combination of analytical approaches, each targeting specific violations. For detecting adulteration in meat products, especially those containing poultry meat, polymerase chain reaction (PCR) is the most effective method. This review examines various types of food adulteration and analytical methods for their detection. Improving detection methods to ensure food safety is a key task for protecting consumer health. It is also necessary to strengthen responsibility for compliance with laws and regulations governing the quality requirements of meat products and preventing their falsification.

Keywords: adulteration; food safety; meat products; quality control; standard.

Introduction

Food safety is one of the key issues in modern society. With the rise in living standards, meat product consumption increases, creating conditions for food adulteration to reduce costs and gain illegal profits [1]. Dishonest producers resort to refilling, substitution, and other deceptive practices, leading to a rise in counterfeit products and a decline in meat quality. The most common method of meat adulteration is replacing expensive meats (such as beef and lamb) with cheaper alternatives (such as chicken or duck) or using inedible meat (e.g., fox or mink meat) [2]. Meat adulteration violates market regulations, infringes on consumer rights, and poses risks to health and safety. Counterfeit products may contain pathogenic microorganisms or toxic substances that cause poisoning, illness, or allergic reactions [3]. Additionally, meat adulteration disrupts religious traditions in Muslim countries, where the consumption of products containing pork or donkey meat is prohibited [4].

Therefore, one of the key tasks of food quality control laboratories is species identification of raw materials used in the food industry and determining the origin of meat in finished products. Verifying

the authenticity of animal-derived food products is crucial for economic, sanitary, legal, religious, and medical reasons. Over the past decades, several incidents have occurred involving the use of unconventional meat types and wild animal meat. For example, in 1981, a major "meat substitution scandal" broke out when horse and kangaroo meat were discovered in shipments of Australian beef. In 2013, the "horsemeat scandal" emerged when horse and pig DNA were detected in beef burgers [5]. Such cases raise serious concerns about the authenticity and safety of animal-derived products, which is critically important for protecting consumer health. To prevent these risks, it is necessary to develop accurate and effective meat identification methods, enabling regulatory authorities to strengthen control measures and safeguard public health. Finding a fast, precise, convenient, and cost-effective method for determining meat composition remains a challenging task in the field of food quality control [6]. Traditional methods based on external characteristics (such as smell, color, texture, and taste), including organoleptic analysis and histological examination, do not allow for precise identification of the species composition of meat products. Protein-based analysis methods, such as ELISA and electrophoresis, also have limitations due to protein degradation during thermal processing. Unlike proteins, DNA remains stable even after food processing. Unlike proteins, DNA remains stable even after technological processing. Therefore, DNA-based methods are considered the most reliable for meat identification. These include PCR [7], multiplex PCR, fluorescent quantitative PCR, and the LAMP method, all of which provide high accuracy and reliability in determining the composition of meat products [8]. Despite the widespread use of advanced technologies such as digital PCR and CRISPR-Cas systems for food sample analysis, developing highly accurate analytical methods for detecting trace amounts remains a significant challenge. Based on the above, this research aims to analyze the current state of the adulteration of animal-derived food products, as well as the modern methods used for their accurate identification and reduction of adulteration. In accordance with the aim, the following objectives are addressed in the study:

- To provide a concise yet comprehensive overview that enables an understanding of the scale and key areas of food adulteration involving animal-derived products, thereby contributing to the more effective detection, prevention, and mitigation of such practices in the future;
- To offer readers the opportunity to analyze and critically evaluate existing approaches and methodologies used in the study of food adulteration and the techniques applied for its detection;
- To establish an informational foundation that facilitates the development of original scientific research focused on food adulteration issues, while avoiding unnecessary duplication of previously published works on this subject.

Types of Meat Product Adulteration

Food fraud has been known since ancient times, such as adding alum to bread or gypsum and starch to milk. With industrialization and market expansion, the diversity of food products has increased, leading to more complex adulteration schemes. Meat product fraud is among the most prevalent types of food adulteration. This is supported by data from 2013, which saw a peak in recorded fraud cases, coinciding with the horsemeat scandal. After 2013, the number of reported fraud cases remained consistently high over a 20-year period [9]. It can be assumed that the horse meat scandal exposed serious issues related to food fraud and highlighted the vulnerability of the global meat supply chain, particularly in raw meat, to counterfeiting [10].

A typical case of intentional meat adulteration is interspecies substitution, aimed at deceiving consumers by replacing expensive meat with cheaper alternatives, such as substituting beef with pork. Although this type of meat fraud rarely poses a health risk, it violates consumer interests and seriously hinders the development of the regional meat industry [11]. Sausages made from minced meat are particularly vulnerable to adulteration, as grinding meat hides its original morphological characteristics, making visual detection of fraud difficult. Due to price differences, cheaper meats such as pork, chicken, and buffalo meat are often used to substitute beef in meat products [12].

Studies in various countries have shown similar adulteration practices. For example, 25.6% (64 samples) of sausages containing chicken, pork, beef, and duck purchased from local markets in Sichuan, China, were adulterated. The most common form of violation in China was the addition of undeclared duck to products labeled as chicken or pork. In Italian markets, about 57% of packaged meat products, including sausages, pates, and meatballs, contained unlabeled pork, beef, and chicken ingredients. In

Turkey, it was found that sausages labeled as containing 5% beef contained no beef DNA, while a sample of 100% beef meatballs included chicken [13].

Another method of meat product adulteration is exceeding the permissible levels of food additives, particularly fiber. Increasing fiber content to 8% when the norm is 1–5% causes excessive moisture accumulation in the product and leads to loosening of the minced meat. A similar case occurred in 2017 when the Brazilian meat scandal involved various forms of adulteration, including the substitution of animal-derived ingredients, excessive addition of food additives, and water injection. Additionally, meat semi-finished products often use gelling and meat-replacing components. Partial or complete substitution of beef or lamb with cheaper offal or poultry meat is one of the most common methods of adulterating meat products. Moreover, this trend in the beef supply chain per-sists over time [14]. Numerous incidents of clenbuterol-contaminated meat products in China in 2011 threatened consumer health and undermined trust in food supplies [15].

Mislabeling of food products is also a serious form of fraud and can pose significant risks to consumer safety. An example is the mislabeling of toxic fish species, such as pufferfish, mackerel, and escolar (containing ciguatoxin), as safe species [16]. Cases have been reported where products labeled as squid turned out to be toxic pufferfish species. Such incidents not only posed serious health risks but also undermined consumer trust in the seafood industry [17]. A literature review indicates that the most common types of food fraud are mislabeling (20.7%), artificial enhancement (17.2%), and substitution (16.4%). Mislabeling is widely discussed in the literature and has been identified in 57% of processed meat products. The regions most affected by food fraud are Austral-ia (79% of mislabeled products) and South America (67%) [18].

Methods for Detecting Meat Product Adulteration

Quality control represents a system of standards aimed at ensuring food safety in accordance with consumer expectations and needs. Various parameters, including physical, chemical, microbiological, nutritional, and sensory properties, are used to maintain product safety. These quality indicators depend on factors such as taste, color, aroma, texture, and overall product perception. Chemical quality indicators depend on the content of sugars, proteins, fiber, as well as peroxide levels, free fatty acids, and enzyme activity [19]. One of the analytical methods used for food analysis is chromatography. This method is based on the interaction of analytes between the mobile and stationary phases, allowing for effective separation of substances. Methods are classified based on the type of stationary and mobile phases. The stationary phase is a solid material coated on a column through which the mobile phase is pumped. Compounds with minimal affinity for the stationary phase and short retention times elute first and are detected. However, despite the high accuracy of chromatographic methods, they are less effective for detecting adulteration in complex meat mixtures or thermally processed products. Additionally, their application is limited by the high cost of equipment and the complexity of sample preparation [20].

Gas chromatography is used for identification, authentication, and prediction of food quality characteristics. However, it requires sample derivatization due to the high boiling points and density of compounds. Liquid chromatography is used for identification, classification, and quality assessment of various food products. High-performance liquid chromatography (HPLC) allows for the analysis of polar and non-polar solutions without the need for derivatization. Chromatography is used for tocopherol analysis, amino acid determination, adulteration identification, detection of milk and cheese adulteration, and analysis of marker peptides in gelatins [21]. Chromatography, especially when combined with modern techniques such as spectrometry, remains one of the key tools for improving analysis accuracy and preventing food adulteration [22].

Mass spectrometry is a reliable and highly effective method for meat authentication, particu-larly in processed products containing meat from various mammalian and poultry species [23]. For example, pork-specific peptides can be used to differentiate pork from beef, goat, and chicken meat. Tandem mass spectrometry (MS/MS) can detect 0.55% pork in beef and 0.24% in processed beef [24]. The strategy of using peptide markers is based on proteomics, including enzymatic cleavage of specific peptide bonds in proteins. However, sample preparation for this method takes a significant amount of time: treatment with dithiothreitol to reduce disulfide bonds and iodoacetamide for thiol alkylation can take up to 24 hours. Thus, the complexity and duration of these preparation procedures limit the method's applicability for species identification in real meat products [25].

Immunochromatographic analysis (ICA) is a promising alternative and complement to traditional food analysis methods. This approach is based on the interaction of the target component with specific antibodies, followed by their separation on specialized membranes [26]. The advantages of ICA include speed, simplicity, low cost, specificity, and sensitivity. The analysis does not require complex sample preparation compared to more sophisticated methods. The main requirement for developing an ICA method aimed at assessing meat product composition is the selection of a suitable biomarker that can specifically recognize the tissue of a particular animal in the product. The suitability of a biomarker is determined by species specificity and resistance to degradation. Currently, widely used biomarkers include troponin I [27], myoglobin [28], hemoglobin [29], and immunoglobulin [30].

Food authenticity has become an important aspect of ensuring food safety and quality amid the increasing number of fraud cases and unethical practices reported worldwide in recent years. Various analytical methods of chromatography and spectroscopy, as well as molecular and immunological methods, have been developed to detect meat product adulteration [31-35]. Among molecular methods, special attention is given to PCR and real-time PCR, which are used for target DNA detection. In some countries, reverse transcription PCR (RT-PCR) is accepted as a standard method for meat authentication [36-38]. The scheme of one-step or two-step RT-PCR is shown in Figure 1.

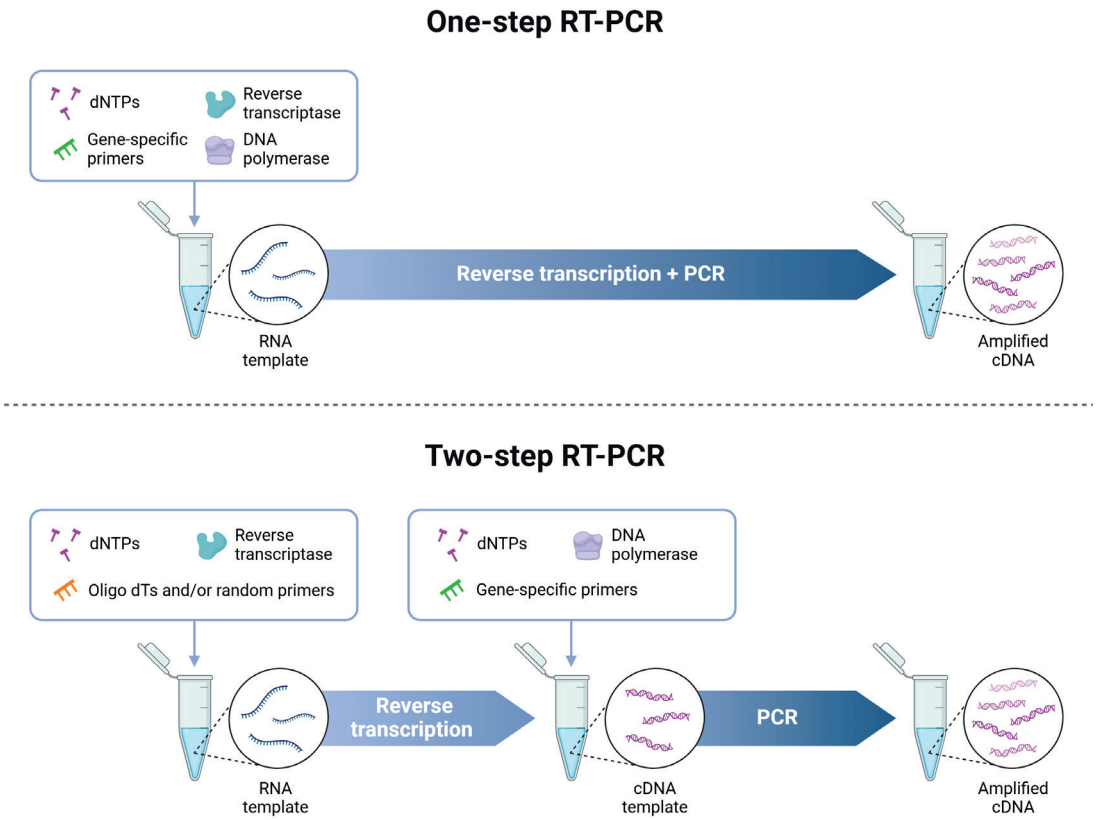

Figure 1 – Scheme of one-step and two-step RT-PCR

Multiplex PCR (mPCR) methods are convenient tools for simultaneous detection of multiple targets on a single platform. Due to their simplicity and low cost, enabled by standard agarose gel analysis, they have become widely used in the food industry [39]. However, as the number of pri-mers and reaction complexity increases, the likelihood of mutual interference between components, such as templates and primers, increases, which can reduce amplification efficiency or cause complete failure [2]. Currently, most mPCR methods are limited to detecting 8 to 12 animal sources on a single platform in one reaction. Only one method is known to identify 12 meat ingredients using universal primers [40]. Primer specificity is a key condition for successful mPCR. To ensure accurate species identification, primers

must demonstrate strict specificity to target species and significant mismatch with non-target species [41]. Even a single base pair mismatch at the 3' end of primers can significantly reduce amplification efficiency [42]. Therefore, there is an urgent need to develop rapid, sensitive, and reliable analytical methods for identifying various meat species in meat products [43].

Innovative Technologies in Combating Adulteration

In recent years, with the improvement of video surveillance systems and increased computational capabilities, imaging methods have demonstrated potential for non-contact detection of food adulteration [44]. Imaging is a non-contact method based on optical principles that provides complete information about objects, including their chemical properties and sensory characteristics. This plays a key role in assessing the external properties of food products. Among modern imaging methods used for meat quality and safety analysis, hyperspectral imaging (HSI), X-ray imaging (RI), and thermal imaging (TI) can be highlighted [45].

Compared to traditional chemical analysis methods, near-infrared spectroscopy (NIRS) offers advantages such as high speed, non-destructive nature, and low cost. This method is widely used for chemical composition analysis, food quality assessment, and adulteration detection [46, 47] Their results showed a high degree of correlation with data obtained by ICA methods [48, 49].

The application of Raman spectroscopy for meat quality and safety assessment covers aspects such as spoilage, adulteration, and other issues. The use of the similarity index (SI) in meat spectros-copy has proven effective as an alternative tool for classification, allowing differentiation of meat samples from the same animal species but different origins. The main advantage of SI is its simplicity and accessibility for non-specialists, enabling the use of this method in the industry by employees with varying levels of training to track meat origin [50]. Terahertz spectroscopy (THz spectroscopy) has the ability to identify meat from different tissues, grades, or even brands of the same grade, creating a theoretical and experimental basis for verifying meat authenticity and detecting adulteration in practical applications [45].

The combination of thermal imaging and a high-precision convolutional neural network enables the detection of lamb mince adulteration with pork and the addition of flavoring essence to lamb [51]. Additionally, thermal imaging can be used to predict chicken meat temperature after cooking. Combined with multilayer neural networks, this technology demonstrates significant potential in assessing beef quality [52]. The integration of mathematical processing of chemical data with instruments such as an infrared spectroscopy source and metal-oxide-semiconductor sensors ("electronic nose"), the schematic of which is shown in Figure 2, has great potential for detecting meat adulteration.

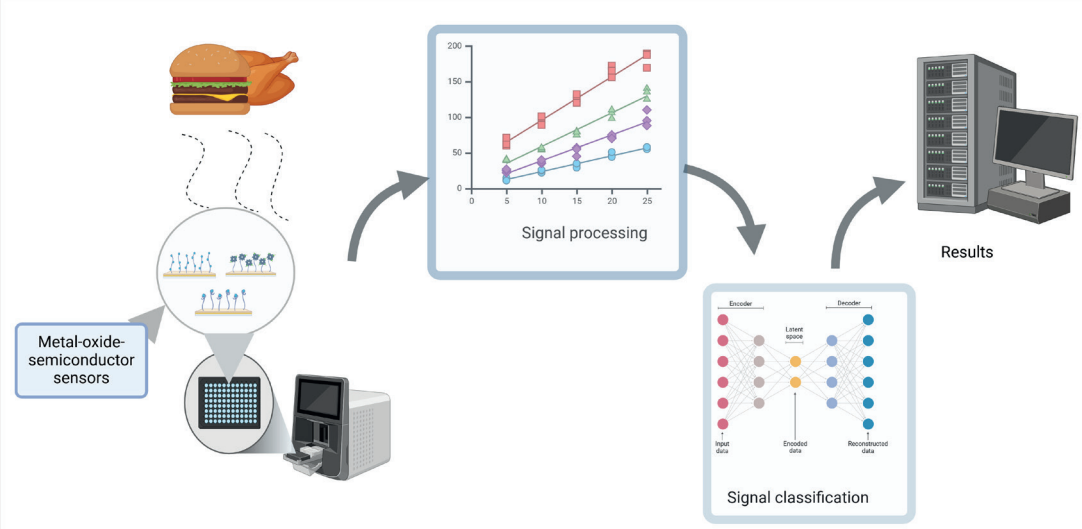

Figure 2 – Scheme of the "Electronic Nose" System

However, both approaches have their limitations, and their combined use remains relatively rare. Therefore, the development of more accurate, rapid, cost-effective, and universal technologies for

detecting meat adulteration is one of the priority areas for future research [53]. Other studies have demonstrated the effectiveness of using an "electronic nose" for analyzing chicken and beef seasonings, as well as predicting their sensory attributes. The method proved effective for identification processing and storage violations in sausages and can extract flavor "fingerprints" of products [54].

Currently, for freshness assessment, adulteration detection, odor analysis, and detection of specific compounds in meat products, there is a widely used non-destructive detection technology (NDDT) [55]. This technology plays a key role in ensuring meat quality and safety, becoming one of the most important tools in food control. Compared to traditional methods, NDDT has clear advantages, such as higher efficiency and no impact on the test object, making it promising for wide-spread use in meat product control [56]. Despite the rapid development of spectroscopic and imaging methods, they face several limitations. Spectroscopic methods require a significant amount of physicochemical experiments before the modeling stage, and models need to be regularly updated depending on sample classes and conditions [57].

Regulatory and Legal Framework

Meat product adulteration is regulated by international standards and national legislation in various countries. The key aspects focus on ensuring product quality, food safety, and consumer protection. The primary international standard is the Codex Alimentarius, which sets global food safety standards, including those for meat and meat products. This code regulates labeling, species identification, the use of additives, and analytical methods for detecting adulteration [58]. Another international standard is ISO 22000 (Food Safety Management Systems), which sets requirements for producers in identifying and preventing adulteration. ISO 22000 is intended to replace HACCP (Hazard Analysis Critical Control Points) in food safety matters. The main difference is that in ISO 22000, systems such as Good Manufacturing Practice and Good Hygiene Practice are prerequisites, leading to fewer critical control points [59]. The international standard ISO 17025 establishes requirements for laboratories engaged in product testing, including the detection of meat adulteration.

Among national laws and standards, European legislation is noteworthy. The European Union is known for its strict food safety standards, which are among the highest in the world. The EU has developed comprehensive legislation aimed at protecting consumers and ensuring food and feed safety. The key regulatory document is the General Food Law Regulation (EC No 178/2002), which establishes the basic principles, objectives, and requirements for food safety. According to this regulation, the European Food Safety Authority (EFSA) was established, beginning operations on January 1, 2002, and located in Parma, Italy. The agency serves as an independent body providing scientific advice to EU policymakers on food chain safety. Regulation (EU) No 1169/2011 on food labeling requires the indication of meat species, country of origin, and composition, while Regulation (EU) No 853/2004 regulates sanitary standards for production and control [60].

In the United States, the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) plays a key role in regulating meat product safety. FSIS oversees for meat quality, poultry, and eggs, including their processing and labeling. FSIS programs regulate the safety and quality of meat and poultry products intended for public consumption, as well as proper labeling for interstate or international markets [61, 62].

In Kazakhstan, the regulation of meat product falsification is based on national legislation, as well as international standards adopted within the framework of the Eurasian Economic Union (EAEU). The main aspects of control include safety, quality, and transparency of information about product composition. Technical Regulations (TR) of the Customs Union (CU) TR CU 034/2013 "On the Safety of Meat and Meat Products" establishes mandatory safety requirements, including control over the content of harmful substances, identification of meat and its origin. It prohibits the use of substitutes (e.g., soy or starch) without indication on the label and describes methods for analyzing the composition of meat products. TR CU 022/2011 "Food Products in Terms of Labeling" requires manufacturers to specify product composition, meat type, ingredient proportions, and country of origin. It also prohibits misleading consumers (e.g., claiming one type of meat is used when another is actually present). In addition to EAEU standards, Kazakhstan also applies ISO 22000 and ISO 17025 standards [60].

From the national legislation of Kazakhstan, it is important to note the Code of the Republic of Kazakhstan "On Public Health and the Healthcare System". This regulation aims to prevent the

falsification of food products, including meat, and contains requirements for the quality and safety of food products, as well as their sanitary and epidemiological examination. The Law of the Republic of Kazakhstan "On Consumer Rights Protection" obliges the provision of accurate information about the composition and quality of products and provides for liability for violations of consumer rights in case of falsification. The requirement for product compliance with technical regulations is governed by the Law of the Republic of Kazakhstan "On Technical Regulation" and National Standards ST RK. These documents contain requirements for the quality of meat and meat products, harmonized with international standards [60].

Conclusion

The falsification of meat and meat products remains one of the most significant problems in the food industry, affecting economic, social, and environmental aspects. This phenomenon under-mines consumer trust, harms honest producers, and creates health risks due to the use of undeclared, low-quality, or hazardous ingredients. The main forms of falsification include substituting one type of meat for another (e.g., using pork instead of beef), adding unacceptable ingredients (water, starch, soy proteins) without indication on the packaging, and selling expired products as fresh. Such violations complicate the identification of product origin and may threaten consumer health due to the potential presence of allergens, microbial contaminants, or chemical substances.

To effectively address the problem of falsification, a systematic approach is required, including laws establishing clear requirements for labeling, quality, and safety of meat. Examples of successful practices can be found in EU regulations, such as EC No 178/2002, which ensure a high level of consumer protection. In Kazakhstan and EAEU countries, technical regulations are in place, but their constant adaptation to modern challenges is necessary.

The use of advanced technologies, such as DNA analysis to determine the species composition of meat, physicochemical studies to identify additives, and high-precision methods for detecting microbial and chemical hazards, significantly enhances control effectiveness. Laboratories certified to international standards (e.g., ISO 17025) play a key role in identifying falsifications. The use of technologies such as blockchain can help create transparent supply chain tracking systems, where each stage of production and processing is recorded and protected from falsification.

The global problem of falsification requires the exchange of experience and data between countries. An example is the activities of the EFSA, which coordinates the work of national agencies and scientific centers. Such exchanges allow for the unification of standards and the development of joint methods to prevent violations. Producers must bear strict responsibility for non-compliance with standards. The introduction of significant fines, product recalls, and criminal penalties for falsification can be effective tools in combating violations. Additionally, it is important to ensure production transparency, up to tracking the origin of raw materials, so that consumers can be confident in the quality and safety of the products they purchase. Raising public awareness about the signs of quality products and potential risks of falsification also plays a significant role. Informed consumers can recognize violations, choose more reliable products, and support honest producers.

Thus, only a comprehensive approach combining the efforts of government authorities, producers, the scientific community, and consumers themselves can effectively combat meat falsification. Solving this problem will not only improve public health and strengthen trust in the food industry but also contribute to the creation of a more sustainable, transparent, and fair system of meat production and trade.

Authors' Contributions

SB; KM and AB: Designed and supervised the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. KT: Statistical and bioinformatic analysis, and drafted the manuscript. SS: Conducted study and bioinformatic analysis. All authors have read, reviewed, and approved the final manuscript

Acknowledgements

The study was conducted within the framework of the scientific and technical program No. IRN BR22885795 "Improving food safety" for the period 2024-2026.

Conflicts of Interest

The authors declare that they have no competing interests.

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 1 (009). – P. 63-71. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2025.5(009).1873
UDC 636.087(045). 68.39.15 **Research article**

Determination of the residual content of antibiotics in feed on a Randox analyzer

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Received: 19-02-2025 **Accepted:** 20-21-2025 **Published:** 31-03-2025

Abstract

Background and Aim. Antibiotics are widely used in animal husbandry to prevent diseases and stimulate growth, but their residual content in animal feed and products poses a significant risk of antibiotic resistance. The purpose of this article is to substantiate the need to use organic feed, free of antibiotics, to reduce the risks of antibiotic resistance and improve the quality of livestock products.

Materials and Methods. In this work, the content of antibiotics in various types of feed (succulent, roughage and concentrated) was analyzed using the Randox method. The study showed that in many samples, the antibiotic content exceeds detection limits, especially for streptomycin (86.43 parts per billion), quinolones (35.56 parts per billion) and tetracyclines (17.56 parts per billion). In coarse feeds, concentrations of ceftifur (29.28 parts per billion) and quinolones (11.92 parts per billion) also exceed detection limits, while in concentrated feeds, levels of streptomycin (24.25 parts per billion) and quinolones (20.14 parts per billion) remain significant.

Results. Statistical analysis revealed significant differences between the feed groups for tamfenicol ($p=0.002$), tylosin ($p=0.006$) and tetracycline ($p=0.02$), which confirms the need for enhanced control and monitoring of antibiotic residues. The absence of official maximum permissible levels (MPL) for antibiotics in feed in Kazakhstan requires the development and implementation of regulatory regulations.

Conclusion. The transition to the use of organic feed and alternative methods of preventing animal diseases seems to be a promising solution to reduce the risk of antibiotic resistance and improve the safety of livestock products.

Keywords: antibiotic resistance; antibiotics; detection limit; feed; organic feed; Randox.

Introduction

Organic animal husbandry is a system designed to ensure a comfortable and stress-free life for animals in accordance with their natural needs, which encourages the use of certified organic and biodegradable materials from the environment in terms of nutrition, animal health, animal husbandry and intentionally avoids the use of synthetic materials such as medicines, feed additives and genetically engineered materials for breeding [1].

In organic agriculture, special attention is paid to organic feeds that are nutritious and natural. Feeds should not contain any substances that artificially stimulate growth, synthetic amino acids or genetically modified organisms (GMOs). The feed must be organic, produced by certified organic farmers, which are beneficial not only for growth and production, but also for their health and wellbeing [2].

One of the ways to solve the problem of providing meat products to the population is to accelerate the growth of animals and poultry. For this purpose, various growth stimulators are used in animal husbandry and poultry farming, which significantly reduce the cost of final products [3].

Antibiotics are widely used in animal husbandry to treat diseases, stimulate growth and increase animal productivity [4]. However, according to research, their excessive use can lead to the formation of resistant bacterial strains, which poses a threat to human health [5]. Strict restrictions on the use of antibiotics in feed have been introduced in the EU and the USA, which has become an incentive for the development of organic animal husbandry [6].

According to the World Health Organization (WHO), more than 70% of all antibiotics in the world are used in agriculture, which poses a serious threat to human health. Studies by *Smith* et al. (2022) show that residual amounts of antibiotics in feed can have a negative effect on the intestinal microflora of animals, reducing their immunity [7].

The resistance of zoonotic bacteria of the genera Salmonella and Campylobacter associated with food infections is undoubtedly associated with the use of antibiotics in farm animals; food infections caused by such resistant bacteria have been repeatedly documented in humans. Resistance to so-called "critically important antibiotics" used in medicine is of particular concern. In diseases caused by polyresistant strains of Salmonella Typhimurium with resistance to quinolones, treatment failures, a higher frequency of hospitalization and a higher risk of death were recorded. In cases of human diseases caused by macrolide-resistant compylobacteria, there was a higher incidence of severe forms of infection and deaths [8].

Studies show that prolonged consumption of feed containing antibiotics leads to an imbalance of the intestinal microflora in animals, which can reduce their natural immunity [9]. According to the World Health Organization (WHO), antibiotic resistance is one of the main threats to global health [10].

The Randox method is one of the most modern and accurate ways to detect antibiotic residues in feed. Its high sensitivity makes it possible to detect even minimal concentrations of substances, which makes it an indispensable tool in feed quality control [11]. The use of Randox reduces the risk of errors and makes it possible to detect even trace amounts of antibiotics that can accumulate in the body of animals and enter products [12].

In Kazakhstan, control of residual antibiotics in feed is carried out in accordance with the Technical Regulations of the Customs Union TR CU 021/2011 "On Food Safety" and TR CU 034/2013 "On the safety of meat and meat products" [13]. These regulations regulate the maximum permissible levels of antibiotics in animal feed and products.

There is also a Sanitary and epidemiological conclusion of the Ministry of Health of Kazakhstan, which defines the hygienic standards of residual antibiotics in animal products [14]. Nevertheless, monitoring of antibiotic residues requires improvement, especially in the context of increasing global food safety requirements [15].

The demand for organic animal products (meat, milk and eggs) is growing day by day. The use of antibiotics, growth stimulants, and steroids is strictly prohibited in the organic farming system. Since no harmful drugs are used in organic farming, animal health and product quality are improved [16].

Unlike traditional animal husbandry, organic production eliminates the use of synthetic antibiotics. Studies have shown that animals raised on organic feed have a healthier intestinal microflora and a lower susceptibility to infections [17]. Organic animal husbandry is actively developing in Europe and the USA, which is confirmed by data from the International Federation of Organic Agriculture [18].

Economic analysis shows that the transition to organic animal husbandry can be beneficial if there is government support and demand for environmentally friendly products [19].

The purpose of this article is to substantiate the need to use organic feed, free of antibiotics, to reduce the risks of antibiotic resistance and improve the quality of livestock products.

Materials and Methods

According to the task of developing criteria for assessing feed safety, work was carried out on sampling feed of all types, mainly feed additives of various origins and formulations, imported and domestic manufacturers, as well as coarse, concentrated feed of vegetable origin, haylage and silage.

Sampling was carried out in accordance with the requirements of ND and GOST. Sampling is the production of a small proportion of feed from a batch by repeated sampling of spot samples from various locations in the batch. These point samples were combined by mixing, and a combined sample was formed, from which the required amount of laboratory samples was prepared and weighed by division.

Of the selected 150 samples of feed:hay of various types in quantity - 18 samples, corn silage - 14, haylage - 15, compound feed, including granular feed - 17, extruded feed - 9, meal, cake (rapeseed, flax, sunflower), sunflower husk - 29, monofilament for cattle - 8, concentrated feed - 34, feed additives, Russian production - 2, fish feed - 3 samples, of which 2 are produced in Denmark and China, mixed feed for chickens, Russian production - 1.

Hay and straw were sampled from at least 10 different locations over the entire area and thickness of the layer, taking bundles weighing 100-120 g so that the crumbled parts of plants were also included in the sample according to GOST 27262-87 "Feed of plant origin. Methods of sampling". The resulting average sample was packed in a dry plastic bag, the appropriate documents were attached and delivered to the laboratory within 2-3 days.

A sample of haylage and silage was taken from the center of the trench at a distance of 0.5-1.0 m from the wall. The average sample of silage and haylage was placed in a bag and sent to the laboratory within a day from the moment of sampling.

Samples from concentrated feeds and feeds of plant origin, feed additives of various origins and recipes were selected according to the generally accepted method GOST 13586.3–83 "Grain. Acceptance rules and sampling methods"and were sent to the laboratory within 2-3 days.

In the laboratory, the samples were prepared for further study by drying the sample in a dry-fire cabinet and further grinding, in accordance with the requirements of regulatory documentation. An ordinal sample registration number was used for each plastic bag containing the selected sample and recorded in the sample registration log. Then the feed was prepared for sample preparation and homogenized in a mill

Then, they were sieved through a sieve and weighed 1 gram for extraction of residual organic substances using methanol. The work on sample preparation was carried out at the National Center of Biotechnology

The Anti-MicrobialArrayII (AMII) kit quantifies quinolones, ceftiofur, thiamphenicol/florfenicol, streptomycin/dihydrostreptomycin (DHS), tylosin/tilmycosin, and tetracyclines simultaneously. Each set includes 6 carriers, 9 calibration points, buffers necessary for recovery, and other reagents (conjugate, chemiluminescent solution). Each carrier consisted of 9 microchips.

The SPSS 25.0 software was used to obtain descriptive statistics.

Results

The detection limit (LOD) is the minimum concentration of a substance that an analytical method is able to detect with a high degree of confidence. If the concentration of the antibiotic is below this threshold, the method will not be able to reliably determine its presence, even if trace amounts are present in the sample.

Table 1 – Limit of detection of antibiotics for feed

Sample number	Types of antibiotics					
	Quinolones (QNL)	Ceftifur (CEF)	Tamphenicol (TAF)	Streptomycin (STR)	Tylosin (TIL)	Tetracycline (TCN)
Detection limit (parts per billion)	*10*	*15*	*15*	*80*	*10*	*10*

In this work, the Randox method was used to detect residual amounts of antibiotics in feed. The LOD for various antibiotics was:

1. Quinolones (HNL) – 10 parts per billion
2. Ceftifur (CEFT) – 15 parts per billion
3. Tamphenicol (TAF) – 15 parts per billion
4. Streptomycin (PP) – 80 parts per billion
5. Tylosin (TIL) – 10 parts per billion
6. Tetracycline (TCN) – 10 parts per billion.

Using a method with a low detection limit makes it possible to detect even minimal concentrations of antibiotics, which is important for assessing their residual content and potential risk to animal and human health.

Table 2 shows a statistical analysis of data on the content of residual antibiotics in succulent, roughage, and concentrated feeds.

Table 2 – The content of antibiotics in various types of feed

Antibiotics		N	+	Average	δ	95% confidence interval for the mean value		min	max	P-value
						Lower limit	Upper limit			
CEF	Succulent	26	19	32.23±4.93	21.48	21.88	42.59	2.53	87.13	0.17
	Roughage	31	21	29.28±4.30	19.68	20.32	38.24	1.45	68.94	
	Concentrated	78	20	18.52±6.51	29.13	4.88	32.15	1.63	133.12	
QNL	Succulent	26	20	35.56±15.30	68.44	3.53	67.58	1.40	316.00	0.368
	Roughage	31	22	11.92±1.51	7.09	8.78	15.06	2.07	28.12	
	Concentrated	78	23	20.14±13.55	64.98	-7.96	48.24	1.17	316.00	
TAF	Succulent	26	19	8.62+1.19	5.18	6.13	11.12	0.55	18.83	0.002
	Roughage	31	22	6.35±0.88	4.12	4.52	8.17	0.63	14.73	
	Concentrated	78	23	3.66±0.71	3.39	2.19	5.12	0.40	16.02	
STR	Succulent	26	19	86.43±52.03	226.77	-22.87	195.73	1.82	984.00	0.402
	Roughage	31	18	41.90±21.43	90.93	-3.32	87.12	0.00	374.42	
	Concentrated	78	18	24.25±3.38	14.32	17,12	31.37	1.06	48.14	
TIL	Succulent	26	19	4.96±0.52	2.28	3.86	6.06	0.90	8.46	0.006
	Roughage	31	22	4.46±0.57	2.66	3.28	5.64	1.08	12.83	
	Concentrated	78	23	2.70±0.42	2.03	1.83	3.58	0.40	8.62	
TCN	Succulent	26	19	17.56±1.74	7.60	13.89	21.22	6.78	36.64	0.02
	Roughage	31	18	12.73±1.24	5.25	10.12	15.34	0.00	21.45	
	Concentrated	78	20	11.56±1.60	7.14	8.21	14.90	3.76	36.14	

Among succulent feeds, the highest concentrations were recorded for streptomycin (86.43±52.03 ppm), which exceeds the detection limit of 80 ppm. A significant content was also noted in quinolones (35.56±15.30) and ceftifur (32.23±4.93), which indicates the possible use of these antibiotics in the feed production process or their ingestion from the environment. The p-value values for tamphenicol (0.002), tylosin (0.006), and tetracycline (0.02) indicate statistically significant differences between feed types.

Roughage feeds showed lower levels of residual antibiotics compared to succulent feeds. The average concentration of ceftifur was 29.28±4.30 parts per billion, and quinolones – 11.92±1.51, which remains above the detection limit. The lowest levels were recorded in tetracyclines (12.73±1.24) and tylosin (4.46±0.57). Despite the relatively low values, the difference between feeds remains statistically significant for some groups of antibiotics.

Concentrated feeds showed the lowest levels of antibiotics among all groups. The average concentration of streptomycin was 24.25±3.38, which is below the detection limit (80 ppm), but its presence was nevertheless detected. Quinolones (20.14±13.55) and ceftifur (18.52±6.51) also exceed the sensitivity threshold of the method. Tamphenicol (3.66±0.71) and tylosin (2.70±0.42) were detected in low concentrations, indicating their minimal use in this group of feeds.

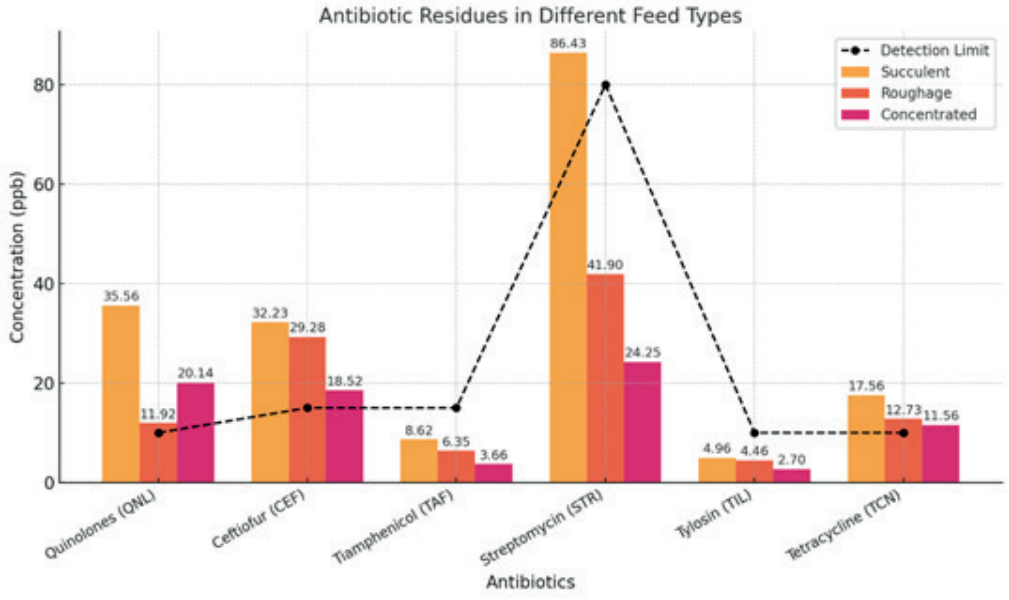

Figure 1 – Comparison of the actual antibiotic concentrations with the detection limit

The diagram shows differences in the average content of antibiotics in feeds of various types (succulent, roughage, concentrated) in comparison with the detection limits. The visual representation of the data confirms that:

In succulent feeds, concentrations of antibiotics significantly exceed detection limits, especially for streptomycin (86.43 ppm), quinolones (35.56 ppm) and ceftifur (32.23 ppm). This may be due to the storage and fermentation characteristics of these feeds.

The content of antibiotics in coarse feeds varies, but on average remains lower than in succulent feeds. However, ceftifur (29.28 ppm) and quinolones (11.92 ppm) also exceed detection limits.

Concentrated feeds have the lowest levels of antibiotics, but they are not completely free of these substances. Streptomycin (24.25 ppm) and quinolones (20.14 ppm) are still found in significant concentrations.

The concentration of antibiotics in feed significantly exceeds the established detection limits, especially for quinolones (CNL) and cephalosporins (CEFT), which indicates potential risks to human health and the need for enhanced monitoring. The largest excess was recorded in succulent feeds, where the level of HCL was higher than normal by 255.6%, and the level of CEFT – by 114.9%. In coarse feed, the excess of HCL was 19.2%, and CEFT was 95.2%. In concentrated feeds, the excess was lower, but still significant: for HNL – 101.4%, and for CEFT – 23.5%.

Discussion and Conclusion

The results of this study show that the content of antibiotics in feed exceeds the detection limits, which indicates the possible use of these substances in agriculture. Similar results were obtained in a study by *Zhang* et al., where residual concentrations of quinolones and cephalosporins were found in the range of 30-40 ppm, which is comparable to our data (35.56 ppm in succulent feeds) [20].

The study by *Ahmed* et al., confirms that streptomycin residues in feed can reach 85-95 ppm, which coincides with our results (86.43 ppm in succulent feed). This confirms that this antibiotic continues to be actively used in agriculture [21].

Chen et al. have found that high concentrations of antibiotics in feed contribute to the development of resistance in pathogenic microorganisms, which can later be transmitted through the human food chain. Our data revealed significant levels of tetracyclines (17.56 ppm in succulent feed), which indicates the risk of the formation of resistant strains [22].

According to *Kim* et al., exceeding the detection limits of tylosin in feed can negatively affect the microflora of animals and reduce their immunity. In our study, the concentration of tylosin reached 4.96 ppm, which requires additional monitoring and control [23].

The absence of official maximum permissible level (MPL) for antibiotics in feed in Kazakhstan creates difficulties in their regulation. According to *Yerubayev*, the introduction of national standards for the control of antibiotic residues in feed is an important step to reduce their impact on livestock products. The introduction of such standards in Kazakhstan will reduce the risks of antibiotic resistance and improve the quality of livestock products [24].

Our data confirm the importance of enhanced control and the transition to alternative methods of preventing animal diseases without the use of antibiotics.

The analysis of the antibiotic content in the feed showed that their concentrations vary significantly depending on the type of feed. Succulent feeds have the highest levels of antibiotics, especially quinolones (35.56 parts per billion), streptomycin (86.43 parts per billion) and tetracyclines (17.56 parts per billion). In coarse and concentrated feeds, the antibiotic content is lower, but still exceeds the detection limits, which indicates their residual presence.

The concentration of antibiotics in feed significantly exceeds the established detection limits, especially for quinolones (CNL) and cephalosporins (CEFT), which indicates potential risks to human health and the need for enhanced monitoring. The largest excess was recorded in succulent feeds, where the level of HCL was higher than normal by 255.6%, and the level of CEFT – by 114.9%. In coarse feed, the excess of HCL was 19.2%, and CEFT was 95.2%. In concentrated feeds, the excess was lower, but still significant: for HNL – 101.4%, and for CEFT – 23.5%.

A comparison of the data obtained with the detection limits showed that in many samples, antibiotic concentrations exceed these values, especially for streptomycin, which is more than twice as high as the detection limit in succulent feeds. Statistical analysis confirmed significant differences between feed groups for antibiotics such as tamphenicol, tylosin, and tetracycline ($p < 0.05$), indicating a heterogeneous distribution of antibiotics and possible factors of their accumulation in different types of feed.

The results obtained confirm the need for strict monitoring of antibiotic residues in feed, as well as the development of alternative methods of animal husbandry, including the use of organic feed, probiotics and phytopreparations. The introduction of restrictions on the use of antibiotics in feed can reduce the risk of the formation of antibiotic-resistant bacterial strains and improve the safety of animal products.

In Kazakhstan, there are no officially approved maximum permissible level (MPL) of antibiotics in feed, which makes the control of residues of these substances a particularly urgent task. In the context of growing food safety requirements, it is necessary to strengthen monitoring of the content of antibiotics in feed and introduce stricter control regulations.

Authors' Contribution

ZhS: Designed and supervised the study and drafted the manuscript. KSh: Statistical analysis and drafted the manuscript. ShG: Designed and conducted the study. OA: Conducted the study and drafted the manuscript. ZhA: Conducted the study and bioinformatic analysis. All authors have read, reviewed, and approved the final manuscript.

Acknowledgements

This study was carried out under the scientific and technical program BR21882327 "Development of new technologies for organic production and processing of agricultural products" for 2023-2025.

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 1 (009). – P. 72-84. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2025.5(009).1874
UDC 576.8 **Research article**

Analysis of fatp1 and px-domain genes to investigate the possibility of using them in species-specific diagnosis of *Trichinella nativa* infection

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Received: 26-02-2025 **Accepted:** 17-03-2025 **Published:** 31-03-2025

Abstract

Background and Aim. Trichinellosis, caused by nematodes of the genus *Trichinella*, is a zooanthroponotic infection which importance is steadily increasing due to its widespread distribution and significant impact on human and animal health. This study focuses on the species of *Trichinella nativa*, one of the most resistant species adapted to cold conditions, which frequently infects various wild mammalian species.

Materials and Methods. Recent molecular studies have helped to clarify the genetic structure of *T. nativa,* but significant gaps remain in understanding its immunogenic profile and biological characteristics. A comprehensive bioinformatics analysis using the BepiPred 3.0 tool of two key *T. nativa* proteins, the long-chain fatty acid transport protein (FATP1) and the phox-homology (PX) domain, to identify potential B-cell epitopes and understand their interaction with the host immune system has identified several significant immunogenic regions in both proteins. A more detailed study of these proteins and their analysis may be relevant for the development of diagnostic and therapeutic agents.

Results. Our results emphasize the need for further research on the immunobiology of *T. nativa*, especially in the context of increasing cases associated with climate and environmental changes.

Conclusion. This study provides important insights that can contribute to the development of specific diagnostic methods and effective control strategies for trichinellosis, improving public health in the affected Kazakhstan regions.

Keywords: bioinformatic analysis; diagnosis; FATP1; protein; PX-domain gene; trichinellosis.

Introduction

Trichinella nativa is one of the most cold-resistant species within the genus *Trichinella*, which causes its distribution in the Arctic and subarctic regions of the world, including Kazakhstan [1]. This species has adapted to harsh climatic conditions and is found in a variety of wild animals including bears, foxes and wolves, indicating its wide ecological range [2].

The study of *Trichinella nativa* faces several significant challenges, among which the limited data on its biological and immunological characteristics stand out. Although studies in recent years have shed light on general aspects of the morphology and genetics of *Trichinella* species, the lack of data on its immunoactive proteins remains one of the main obstacles in understanding the pathogenesis and host-parasite interactions [3, 4]. Modern omics technologies, such as proteomics and genomics, are gradually beginning to resolve this gap, revealing the molecular mechanisms underlying the host immune response to *T. nativa* infection [5].

The study of *Trichinella nativa* immunoactive proteins is a key element for the development of effective diagnostic tests that will allow more accurate infection presence identification at early stages. One promising area is research on serine proteases, which have shown to be useful in the context of early diagnosis of trichinellosis at the intestinal larval development stage [6]. While data on these markers for *Trichinella spiralis* are already beginning to be integrated into serologic tests, information on *T. nativa* is still scarce [7].

The development of specific antigens for serological diagnosis could greatly accelerate the detection of infection and minimize the risk of disease spreading in the population [7]. Although exosecretory products of muscle larvae show promising results, more research is needed to adapt and utilize them in the diagnosis of T. nativa [8].

In the present study, analysis of bioinformatic resources highlights two key proteins of high potential relevance in the context of the *T. nativa* immune response. Long-chain fatty acid transport protein 1 (FATP1) plays an important role in lipid metabolism, as highlighted in studies on *T. spiralis*, where this protein was detected in the serum of infected mice and showed potential as a secreted protein [9]. Other studies also indicate the importance of this class of proteins in linking to immune responses by participating in phosphoinositide signaling and modifying immune cell functions [10].

Moreover, the phox-homology (PX) domains have been shown in various studies to play a significant role in biological signaling and interactions with cell membranes, allowing them to participate in immunomodulation and interactions with immunomodulatory proteins in parasites [11, 12].

The choice of these proteins for analysis is based not only on their presumed functional significance in the life cycle of *T. nativa*, but also on the limited study of their role in the immune response. It is the unique function of these proteins in cellular pathways that led to the hypothesis that they may be determinants in *T. nativa* immune activation.

Thus, the aim of the present study is to perform bioinformatic analysis of potential epitopes of these proteins and to evaluate their interactions with components of the immune system. This approach can increase the understanding of the molecular mechanisms of *Trichinella nativa* pathogenesis and provide new opportunities for the development of more accurate diagnostic and therapeutic tools.

Materials and Methods

Ethical approval.

The study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine and Animal Husbandry Technology, S. Seifullin Kazakh Agrotechnical Research University (KATRU) and was performed in accordance with the “Guidelines for Animal Housing and Care: Species Specific Provisions for Laboratory Rodents and Rabbits” (Interstate Standard, GOST 33216-2014) [13]. All protocols were performed in accordance with the “International Guidelines for Biomedical Research Using Animals” [14].

Experiments were conducted at the Research Platform of Agricultural Biotechnology, KATRU from August 2024 to February 2025. Four male Soviet Chinchilla rabbits aged 7-8 months with a live weight of 4100-4600 g were used in the study. Rabbits were kept in proper hygienic conditions in the vivarium of KATRU, Astana, Kazakhstan.

Larvae isolation. T. nativa larvae were obtained by isolating them from muscle tissue samples of spontaneously infected wild animals. The isolation from samples of muscle tissue of animals was carried out by the method of compressor trichinoscopy and digestion in artificial gastric juice (AGJ) in accordance with the methods of Methodological Guidelines 4.2.2747-10 “Methods of sanitary-parasitological examination of meat and meat products”. Detected and isolated helminthological material was preserved in 70% ethanol solution [15].

RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was measured using NanoDrop 2000 (Thermo Scientific, USA). Total RNA was reverse transcribed into first strand cDNA using ProtoScript II First Strand cDNA Synthesis Kit (New England BioLabs, England).

Setting up PCR. The reaction was performed on a VerityPro amplifier (Applied Biosystems, USA):

The composition of the reaction mixture included:

Compounds	Volume, µl
DreamTaq Buffer	2
MgCl2	1
dNTPs	2
Primers (F, R)	2
DreamTaq polymerase	0.25
DNA	2 (100 ng)
MilliQ (H2O)	Up to 25

Procedure PCR:

Steps	Temperature (°C)	Time	Number of cycles
Initialization	95	5 min	1
Denaturation	95	30 sec	30
Primer annealing	58	30 sec	30
Elongation	72	60 sec	30
Final elongation	72	5 min	1

Sequencing. DNA sequencing was performed using BigDye Terminator v3.1 kit (Thermo Fisher, USA) according to the manufacturer's protocol. SeqStudio genetic analyzer (Thermo Fisher, Applied Biosystems, USA) was used to analyze amplified fragments.

Bioinformatic analysis. Epitope analysis. The BepiPred 3.0 tool was used to predict potential B-cell epitopes of PX-domain and FATP1 proteins. This method is based on a machine learning algorithm that predicts linear epitopes based on amino acid sequence. The input data consisted of amino acid sequences of proteins obtained from the NCBI database. The results of the analysis were presented graphically, where regions with high probability value (>0.5) were considered as potential epitopes.

Three-dimensional structure modeling. The PHYRE 2.2 program was used to build three-dimensional models of the PX-domain and FATP1 proteins. The methodology included amino acid sequence analysis using a homology modeling approach. The modeling results were evaluated for amino acid sequence coverage and model validity. Visualization of 3D structures was performed in PyMOL software for further interpretation of protein structural features.

Phylogenetic analysis was performed to study the evolutionary relationships of the PX-domain and FATP1 proteins *of T. nativa*. Amino acid sequences were aligned using the MAFFT program. Phylogenetic tree construction was performed using the IQ-TREE program using the maximum likelihood method with automatic model selection. The tree was visualized using the FigTree program version 1.4.3. The obtained data allowed us to estimate the evolutionary position of proteins in comparison with their homologs from other *Trichinella species.*

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7.0 and Microsoft Excel 2010. Values of $p<0.05$ were considered statistically significant.

Results

During the initial stages of research, 1520 larvae were isolated from the iris of foxes captured in the territory of Akmola region. Morphological and molecular analysis confirmed their belonging to the species *T. nativa.*

During the experiment, a total RNA with a concentration of 150-200 ng/µl was isolated, after which cDNA synthesis on the matrix of isolated RNA was performed. Specific primer pairs were selected for amplification of target genes encoding FATP1 and PX domain using Primer BLAST tool:

For FATP1:

T.n. FATP1_PrF: CGTCATGGGTTGATTGTTTT

T.n. FATP1_PrR: GTCTTTGTACTTCAGTGCGTCA

For PX domain:

T.n. PXdp_PrF: GTTATTGGCGAAGGCAGCAGTG

T.n. PXdp_PrR: TTTGTTCGCGGGAAGGCTAG

PCR analysis successfully detected the target fragments, which was confirmed by agarose gel electrophoresis. The detected bands coincided with the expected sizes, indicating the presence of the desired sequences in the samples (Figure 1).

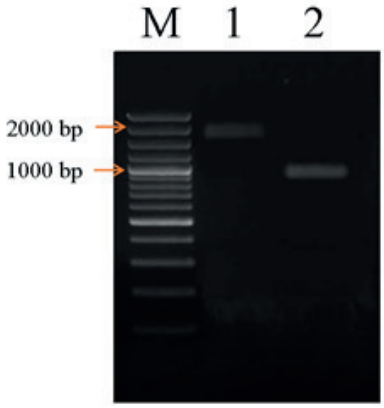


Figure 1 – Electrophoresis of PCR products amplified using specific primers for fatty acid transport protein 1 (FATP1) and PX domain genes (Note: M – molecular marker (Thermo Scientific GeneRuler 100 bp Plus DNA Ladder, ready-to-use), 1 – FATP1 amplification product, 2 – PX do-main amplification product)

After PCR reaction, the amplified fragments were subjected to sequencing for further analyses.

The sequencing of the FATP1 gene (Long-chain fatty acid transport protein 1) with a length of 1920 nucleotide pairs was performed by the Sanger method. Since this method allows us to read a sequence of up to 1000 nucleotide pairs in length, we used three-step sequencing with three short primers for complete analysis. The sequencing scheme is presented in figure 2, which shows the amplification sites, and the location of the primers used (PrF1, PrR1, PrF2, PrR2, PrF3, PrR3) that ensure sequential reading of the entire nucleotide sequence.

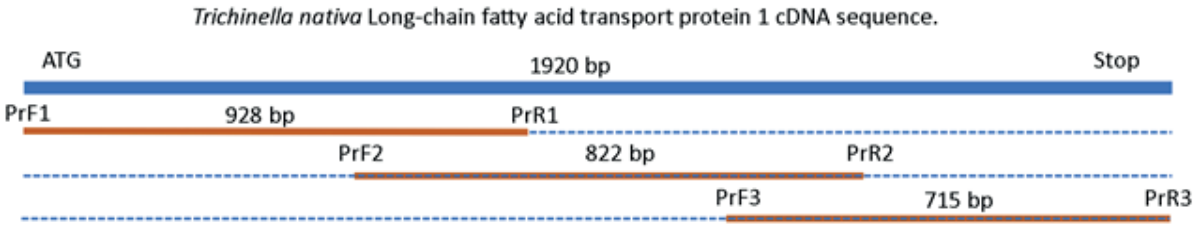


Figure 2 – Sequencing scheme of the FATP1 (Long-chain fatty acid transport protein1) gene of *T. nativa*

At the same time, the size of the PCR product of the PX domain was 969 nucleotide pairs, which allowed its sequencing using the standard method without additional fragmentation. For further bioinformatic analysis of the proteins, the obtained nucleotide sequences were converted into amino acid sequences using a standard genetic code.

The bioinformatic study of these proteins began with the analysis of epitope regions (Figure 3), which play a key role in understanding their immunogenicity and potential use in diagnostics and vaccine development.

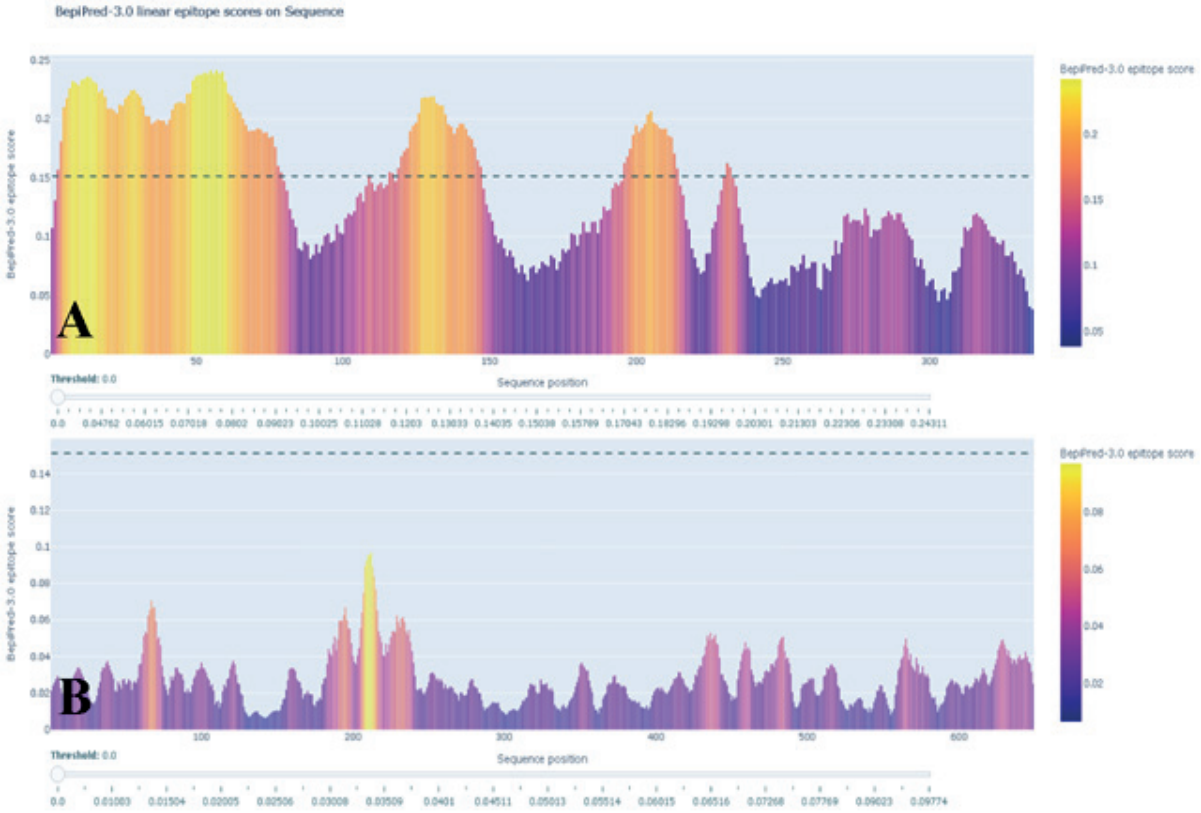


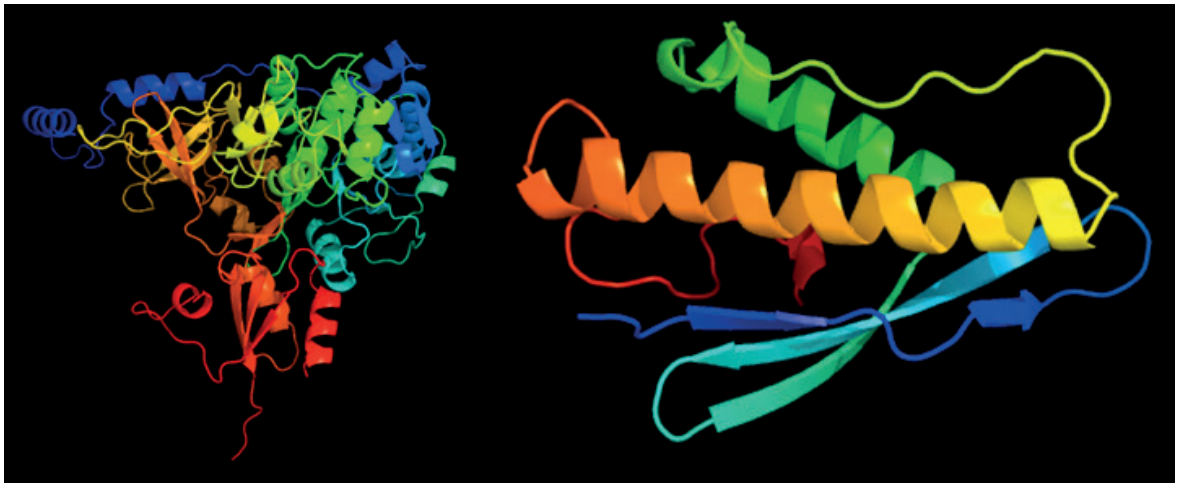
Figure 3 – Linear B-cell epitope analysis of the PX domain (A) and FATP1 (B) of *T. nativa* using BepiPred-3.0 (Threshold-0.1512)

Linear epitope analysis of PX domain protein using BepiPred-3.0 revealed several key immunogenic regions highlighting its significant role in the immune response. Epitope areas above the threshold value (0.1512) are clearly identified in the graph. The first significant region, spanning positions 30-60, shows pronounced peaks with a maximum value of about 0.23. This region is probably critical for recognition by the immune system because it is in a domain that is involved in interactions with other proteins or lipids. The second region, located between positions 120-160, is also characterized by high immunogenic activity. The consistently high epitope score confirms the importance of this region as a promising antibody target. The third region (positions 200-240) shows similar immunogenicity, which strengthens the arguments in favor of a significant role of PX do-main protein in the pathogenesis of *T. nativa.*

Compared to PX domain protein, Long-chain fatty acid transport protein 1 showed less pronounced immunoactive results by explicit epitope analysis. However, they are also of diagnostic interest. Longchain fatty acid transport protein 1 showed moderate immunogenicity, emphasizing its importance in the body's immune response. Using BepiPred-3.0, we were able to identify several regions with potential epitope potential. The first region, covering positions 90-110, shows a stable exceeding of the threshold value (0.09774), indicating the presence of a linear epitope capable of interacting with antibodies. Of particular interest is the second region (positions 180-200), where a peak epitope score of ~0.14 is observed. This site may be functionally significant and involved in immune interactions. The third region, located between positions 450-470, although characterized by a more moderate epitope score, may complement the overall immune picture of the protein.

After performing epitope region analysis of proteins, it becomes clear that it is necessary to study their three-dimensional structure to better understand the biological functions. Epitope analysis provides important information about potentially immunoactive regions. However, to more accurately interpret

protein interactions with other molecules and to develop more effective diagnostic and therapeutic strategies, further investigation of their spatial organization is needed. This will provide a more complete understanding of their functional mechanisms and role in biological processes (Figure 4).



FATP1 protein PX domain

Figure 4 – Predicted three-dimensional structure of *T. nativa* proteins

FATP1 has been significantly studied due to its important role in the transport of long-chain fatty acids across cell membranes. The protein covers a substantial portion of its amino acid sequence with high model accuracy (81% coverage, 100% confidence). Its structural organization includes both alpha-helices and beta-sheets, which allows FATP1 to function not only as a transporter but also as an aquilizing fatty acid linker. Accordingly, its complex configuration makes it a promising target for diagnostic methods and antigenic domain studies [16, 17].

In contrast to FATP1, the structure of the PX-domain has a coverage of only 34% amino acids, but it retains full model fidelity. The PX-domain is a membrane-binding region involved in the insolation of proteins to cell membranes through binding to phosphoinositide. Despite its compact structure, understanding its functional aspects requires further investigation. This limitation in studying structural configuration makes epitope analysis more challenging [18]. FATP1 has demonstrated a more complete and detailed structural characterization, which makes it more suitable for diagnosis and therapeutic interventions at the molecular level. In contrast, the PX-domain, with less coverage, is still the subject of further research.

Both proteins show significant differences in their functions; FATP1 is involved in fatty acid transport, whereas the PX-domain is involved in membrane binding. These functional differences determine their biological relevance and directions in research developments. Precise knowledge of the structure of FATP1 opens opportunities to study antigenic determinants, which may improve the efficiency of development based on it. For the PX domain, further studies are essential to identify and detail its functional utilization. These protein patterning results demonstrated the relevance and need for detailed studies of FATP1 and PX domain. Further advances in understanding their structures and functions will facilitate the development of novel treatment and diagnostic strategies at the molecular level.

Next, the reference sequences of this protein were analyzed among different *Trichinella* species. For this purpose, amino acid sequences were aligned to identify similarities and differences among the species studied. This alignment process helps to identify conserved and variable regions of the protein that may be important for its functional activity or interaction with other molecules (Figure 5).

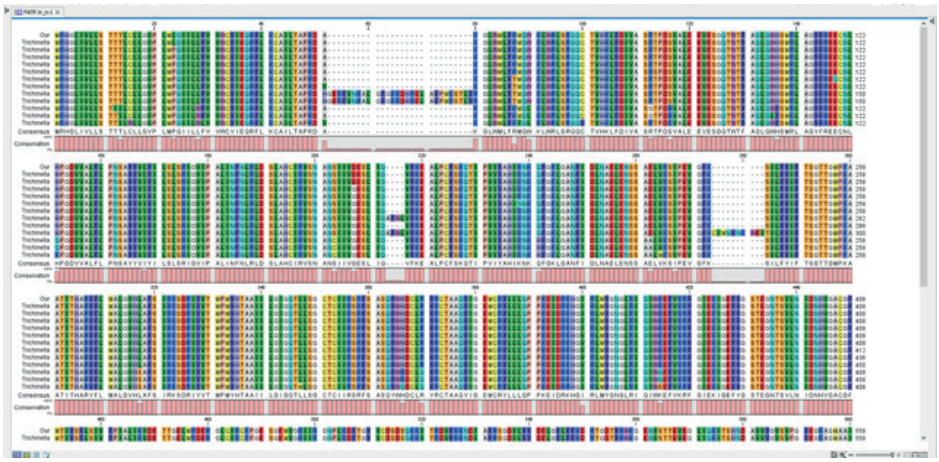


Figure 5 – Alignment of the amino acid sequence of Long-chain fatty acid transport protein 1 (FATP1) of *Trichinella spp*

The alignment showed that the amino acid sequence in different *Trichinella* species is highly conserved. However, sufficiently variable regions (90.3%) were identified and showed differences between species, which may indicate specificities. Based on the amino acid sequence data obtained, a phylogenetic tree was constructed. This allowed visualizing the evolutionary relationships between different *Trichinella* species and assessing the degree of their genetic difference (Figure 6).

Figure 6 – Phylogenetic tree of the Long-chain fatty acid transport protein 1 (FATP1) protein in *Trichinella spp.* (Bootstrap = 10,000)

The phylogenetic tree was constructed with 10,000 bootstrap replications to assess the reliability of clustering. The amino acid sequences of FATP1 obtained from the NCBI database were used in the analysis. The results of phylogenetic analysis demonstrate a clear separation of the studied sequences into evolutionarily valid clusters corresponding to known species of the genus *Trichinella*. High bootstrap support values confirm the reliability of the obtained topology. In particular *T. spiralis, T. nativa,* and *T. murrelli* form separate groups, indicating the divergent evolution of the FATP1 protein in different ecological niches and geographic ranges.

Of particular interest is the phylogenetic position of *T. patagoniensis* and *T. nelsoni*, which form distinct, well-supported clusters. This may indicate specific adaptive changes in the structure and function

of FATP1 in these species. The identification of *T. pseudospiralis* as a distinct group is consistent with previously published data on its unique biological features and the absence of cuticles in adults.

Analysis of the obtained data indicates that the key motifs of the FATP1 protein are conserved among *Trichinella* representatives, which is confirmed by their high phylogenetic similarities. However, certain species-specific differences are also observed, which may indicate differences in the mechanisms of fatty acid transport caused by adaptation to different conditions of existence. This study deepens the understanding of the evolutionary history of the FATP1 protein in *Trichinella spp*. and lays the foundation for further functional studies aimed at identifying the molecular mechanisms of parasitism and metabolic adaptation in this nematode genus.

Similar studies were performed with the PX domain (Figure 7).

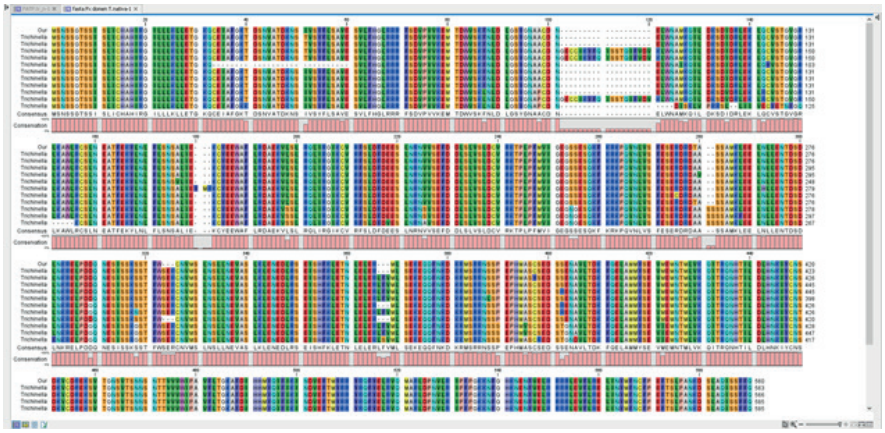


Figure 7 – Amino acid sequence alignment of the Peroxisomal targeting signal receptor (PX) domain in *Trichinella spp*

PX domain alignment, as for FATP1, showed high conservation (89.3%). However, in contrast to the previous protein, slightly more variable sites were found in the PX domain, indicating more pronounced differences between species in this region (Figure 8).

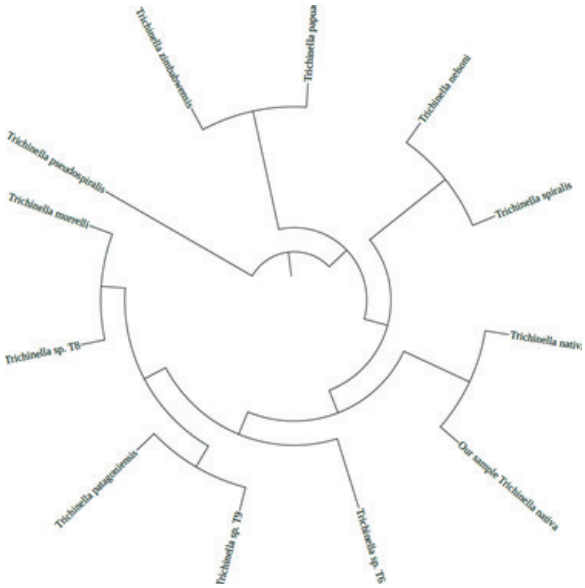


Figure 8 – Phylogenetic tree of the Peroxisomal targeting signal receptor (PX) domain in *Trichinella spp.* (Bootstrap = 10,000)

The amino acid sequences of the Peroxisomal targeting signal receptor domain (PX) obtained from the NCBI database were used in the analysis. The PX domain plays a key role in peroxisomal protein transport, which is critical for maintaining cellular metabolism and adaptation in parasitic nematodes.

The results of phylogenetic analysis demonstrate a clear divergence of *Trichinella* species, reflecting their evolutionary differences. *T. spiralis* forms a distinct cluster, confirming its phylogenetic isolation within the genus. *T. nativa, T. nelsoni,* and *T. murrelli* group into close clusters, indicating that conserved structural features of the PX domain are conserved in North American and Arctic isolates. This analysis emphasizes the importance of the PX domain in the physiology of *Trichinella spp.* and points to species-specific adaptations associated with the evolution of this parasitic genus.

The results obtained may be useful for further studies in parasite molecular biology, including the development of novel therapeutic targets based on peroxisomal transport proteins.

Discussion and Conclusion

Nematodes of the genus *Trichinella* are parasites capable of infecting a wide range of mammals, including humans, through the consumption of infected meat, especially pork and game that has not been adequately heat-treated [19]. According to recent epidemiologic studies, Trichinellosis is prevalent on all continents, including both industrialized regions and developing countries, exhibiting unique patterns of transmission and showing significant differences in clinical symptoms [20]. Such differences underscore the importance of in-depth research to investigate Trichinella natural focal areas and develop comprehensive trichinellosis control and prevention strategies that will be effective on a global scale [21].

Recent molecular studies have helped to further understand the genetic structure of *T. nativa*, allowing the identification of unique genetic markers that distinguish it from other *Trichinella* species [22]. These discoveries have important implications for epidemiologic monitoring and the development of species-specific diagnostic methods. Moreover, recent studies show that climate change may influence the expansion of *T. nativa's* range, which requires continuous surveillance and revision of existing control strategies to prevent new outbreaks of Trichinellosis in vulnerable regions [23].

The results of our study confirm that the FATP1 gene and the PX domain are promising molecules for further study in the context of diagnosis of *T. nativa* infestation. Sequencing and amino acid sequence analysis revealed high conservation between different parasite species, indicating the importance of these proteins in biological functions and their possible role in diagnosis [9, 24].

Initial work with RNA and successful sequencing demonstrated the activity and stability of these sequences, which is critical for further studies at the molecular level. The unique immunogenic regions identified, particularly in the PX domain, provide comprehensive information for the development of specific diagnostic tools and vaccines. It should be noted that despite the less pronounced immunogenic characteristics of FATP1, its role in fatty acid metabolism and transport is no less significant and requires further study [9, 16, 17, 22, 25].

Phylogenetic analyses highlighting evolutionary relationships between different *Trichinella* species emphasize the divergent evolution of FATP1 and the PX domain. Conserved regions in these proteins indicate their importance, while sequence variability may open new avenues for understanding the mechanisms of parasite adaptation to different ecological niches [26].

Despite significant progress in the development of Trichinellosis diagnostics, the problem of creating test systems specific to *T. nativa* remains unsolved. Currently, the most available serologic diagnostic methods are focused on *T. spiralis*, the most common trichinella species, which reduces their effectiveness in areas where *T. nativa* predominates [27]. These methods do not consider the unique antigenic structures of *T. nativa*, resulting in low specificity and sensitivity of the tests in arctic and subarctic regions where this species is most prevalent [28].

Recent studies emphasize the need to develop new antigenic panels that can detect *T. nativa* specific immunological responses [29].

Given the data obtained, it can be assumed that further studies of the structural organization and functional activity of FATP1 and the PX-domain may lead to the creation of new biomarkers for diagnosis and therapeutic methods, which will significantly increase the effectiveness of trichinellosis control. Research in this area is highly relevant and promising, given the importance of investigating the mechanisms of pathogenesis, impact on the host organism and the development of new antigenic targets for vaccine development [6].

This study provides significant insights into the molecular characteristics of *T. nativa* genes, particularly focusing on the immunogenic potential of FATP1 and PX-domain proteins. The bioin-

formatics analysis identified several B-cell epitopes in both proteins, suggesting their potential as diagnostic targets. Structural modeling and phylogenetic analysis further highlighted the evolutionary relationships and functional significance of these proteins within the *Trichinella* genus. The findings emphasize the need for further research to validate these molecular markers through experimental approaches, including serological testing and functional assays. Given the expanding geographical distribution of *T. nativa* due to climate change, developing specific diagnostic tools is crucial for effective surveillance and control of trichinellosis. The results of this study contribute to a better understanding of the parasite's immune interactions and provide a foundation for future advancements in diagnostic and therapeutic strategies.

Authors' Contributions

AZh: Investigation and drafted the manuscript. NG, FT: Investigation (bioinformatic analysis). NA: Investigation (isolation of trichinella larvae). AA: Conceptualized the study. AG: Data analysis. ZS: Investigation and edited the manuscript. OA: Conceptualized the study, investigation, data analysis, and drafted the manuscript. All authors have read, reviewed, and approved the final manuscript.

Acknowledgements

The study was funded by the Ministry of Science and Higher Education of the Republic of Kazakhstan to frame the Grant Financing for 2024-2026 the project No. AP23489156 "Identification of alternative genes of *Trichinella nativa* immunospecific proteins for latex diagnosticum producing".

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 1 (009). – P. 85-91. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2025.5(009).1881
UDC 636.084:636.088.31 **Research article**

Assessment of the milk produced by mastitis-affected cows using an extruded feed supplement containing phytogenics

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Received: 24-03-2025 **Accepted:** 18-03-2025 **Published:** 31-03-2025

Abstract

Background and Aim. For farmers and dairy producers, producing safe and high-quality milk is a top priority. Despite the enormous number of animals, there are several causes for low milk yield, such as the prevalence of livestock-borne diseases, widespread livestock farming techniques, and a low realization of the genetic potential of animals. Mastitis is a common infectious condition in dairy production that is the primary source of antibiotic residues in milk and has a substantial financial impact. The study aimed to investigate the effects of an extruded polyfunctional feed supplement called «BioFeed-P» which contains bitter wormwood extract on the physico-chemical and organoleptic characteristics of mastitic cow milk.

Materials and Methods. The research material included samples of cow's milk and feed additives made of extruded components that were created by barothermal treatment of grain forage, specifically barley, and oats, with the addition of the extruded BioFeed-P additive and wormwood bitter extract. The additive was created and manufactured at the NCJSC «S.Seifullin Kazakh Agrotechnical Research University» in the manufacturing and testing workshop of «NFT-KATU» LLP. Standard organoleptic and physicochemical techniques were used to study milk.

Results. It was demonstrated that feed additives improved the physicochemical characteristics of milk. There was a 3.1% increase in protein content and a 10% increase in milk fat content. Following the application of extruded additives with phytogenics, a 68% reduction in the number of somatic cells in the milk of mastitis-affected cows was noted.

Conclusion. When combined with bitter wormwood extract, the extruded BioFeed-P additive improves physicochemical parameters and dramatically lowers the number of somatic cells in milk from mastitis-affected cows.

Keywords: extrusion; phytogenics; BioFeed-P; bitter wormwood extract; cow mastitis; veterinary and sanitary expertise.

Introduction

Numerous factors influence the quality and hygienic standards of cow's milk as both a food product and a raw material. These factors include the individual traits of the animals [1], the characteristics of their feed ration [2, 3, 4, 5], and the farm’s adherence to primary milk processing technology and cleanliness.

Statistically, non-communicable udder disorders are among the most common pathologies that cause significant economic harm to dairy farming. These disorders result in both quantitative and qualitative losses in raw milk. Moreover, milk from cows affected by mastitis not only loses quality but also becomes

contaminated with microorganisms, including pathogens that can cause food-borne illnesses in humans. Therefore, it is essential to have veterinary and sanitary expertise, as well as to conduct scientifically based sanitary assessments of milk from cows with mastitis, due to the potential risks to human health.

Antibiotics are commonly used to treat mastitis, but their unwarranted use raises concerns about the development of resistant microorganisms. This resistance can lead to the transfer of resistant bacteria from animals to humans [6]. Some farmers have found ways to circumvent regulatory controls by using antibiotic formulations that are not listed in the official guidelines. As stated by *Stein,* and *Chirilă* [7], we are currently facing a medical and public health crisis known as the post-antibiotic era. All antimicrobial medications used in clinical practice can lead to microbial resistance, often occurring almost immediately after new antibiotics are introduced. The extensive use of antibiotics in agriculture, including aquaculture, livestock, and poultry farming, as well as targeted contamination during food manufacturing, particularly in meat and sausage products, is a major contributor to the rise of antimicrobial-resistant bacteria.

In response to these concerns, Sweden became the first country to ban the use of certain antibiotics in animal feed in 1986 [8]. Following this, in 2006, European Union member states enacted Regulation No. 1831/2003, which prohibited the use of all growth stimulants and antibiotics in animal feed.

Alternatives to antibiotics, such as plant-based remedies, are gaining attention for preventing various types of mastitis. Recently, many researchers focusing on farm animals have investigated biologically active compounds known as probiotics and phytogenics [9]. These compounds, derived from plants, include tannins, flavonoids, essential oils, phytoncides, and other beneficial substances [10]. Studies have shown [11, 12] that phytobiotics can enhance immune function, significantly benefit animal health, and improve productivity. It is worth noting that phytobiotics are used in phytogenetics but typically for animals that are fed solely on these compounds.

Sultanayeva et.al. [13] examined the effects of the developed the extruded BioFeed-Padditive, on the quality and safety of goat milk. This additive contains various components, including resins, essential oils, glycosides such as salicin and populin, and flavonoids like pinostrobin. Additionally, it comprises phenolic carboxylic acids, phenolic glycosides, chalcones, leucoanthocyanins, organic acids (such as benzoic, ascorbic, and malic acids), and fatty oils essential for animal health. The aim of this study was to evaluate the effect of several feed additives on the milk productivity of Saanen goats.

Materials and Methods

The extruded polyfunctional feed additive was produced at the feed room «NFT-KATU» LLP based in the NCJSC «S.Seifullin Kazakh Agrotechnical Research University», Faculty of Veterinary and Livestock Technologies. The extrusion line at the production and testing shop includes the following machinery made in the Kostanay region (Republic of Kazakhstan). Kostanay (LLP «Agrotekhservice-12»): PD-2000 pneumatic hammer crusher, SG-800 kg mixer with load cells and cyclone, frequency-controlled feed hopper, PE-350 extruder, and PG-600 flat matrix pelletizer. Under the circumstances of LLP «Agrofirm Rodina» in the Akmola region, a scientific and financial study was carried out to determine the efficacy of using extruded polyfunctional feed additives in feeding dairy cows.

The study included 60 Holstein dairy cows with high yields, both black and brown. In total, 120 milk samples from mastitis-afflicted animals were collected. Cows in the mastitis group were given the extruded BioFeed-P additive for 15 days during treatment. Dairy production, as well as milk quality and safety, were examined and assessed.

Organoleptic properties of milk were determined by me and the student according to the standard Republic of Kazakhstan 1732-2007 «Milk and dairy products. Organoleptic method of determining quality indicators».

Dairy milk yield was studied by control milking for 15 days. Milk sampling was performed into sterile disposable bottles before the addition of the extruded BioFeed-P additive and during the subsequent 15-day experiment. The qualitative parameters of milk were determined using a milk analyzer «Expert Super Plem Kombo» (percentage of fat, protein, skimmed milk powder and lactose, acidity, density, temperature, freezing point, conductivity), the number of somatic cells on the analyzer «Ekomilk Scan».

Latent mastitis of animals was preliminarily determined using the California test in LLP «Agrofirm Rodina» in the Akmola region.

The animal experiments were approved by the local ethical committee of the Faculty of Veterinary Science and Technology of Animal Husbandry of NCJSC «S.Seifullin Kazakh Agrotechnical Research University», protocol №6 from 28.03.2022.

Statistical analyses were performed with SAS, version 9.4 (SAS Institute, Cary, NC). Normality of distribution of measured variables was tested with the Kolmogorov–Smirnov test. Since the observed values showed significant deviation from normal distribution, a logarithmic transformation was applied to all variables, expect for freezing point. After transformation, the differences between pre- and post-treatment were evaluated with the General Linear Model (GLM), with treatment as a fixed, and individual animal as a random factor to account for between-animal variability.

Results

Organoleptic tests of milk

Organoleptic examination of milk from cows with mastitis showed that the samples met the established standards both before and after using the extruded BioFeed-Padditive (Table 1). The taste was not determined because the milk was obtained from cows with mastitis. Most of the animals had latent mastitis, which was pre-determined by the California test.

Table 1 – Organoleptic indicators of milk before and after using the extruded BioFeed-P additive

Indicator	Observed characteristics (n=120)	
	pre-treatment	post-treatment
consistency	uniform, non-sticky	homogeneous, not thick, non-sticky
bloom	Milky white to creamy, without impurities	milky white to creamy, free of foreign matter
smell	typical for cow's milk	characteristic of cow's milk, without extraneous odors, including those of applied phytopreparations

The results of physicochemical tests of milk samples collected from mastitic cows before and after using the extruded BioFeed-P additive are summarized in Table 2.

Table 2 – Physico-chemical parameters of milk of mastitic cows using the extruded BioFeed-P additive

Indicator	Norms*	Measured values (n=15)		P-value
		pre-treatment	post-treatment	
Fat, %	≤2.8	4.2±0.49	4.56±0.37	0.440
SMP, %	≤ 8.2	8.75±0.09	8.73±0.12	0.975
Density, °A	≤ 27	29.6±0.69	29.24±0.71	0.780
Lactose, %	4.0-5.5	4.78±0.05	4.77±0.07	0.956
Salts, %	0.6-0.9	0.73±0.01	0.73±0.01	0.954
Protein, %	≤ 2.8	3.22±0.01	3.21±0.03	0.954
Temperature, °C	-	21.01±0.09	22.92±0.28	<0.001
Freezing point, °C	≤ -0.505	-0.56±0.01	-0.56±0.01	0.478
Added water, %	0	0	0	
pH	6.4-6.7	7.31±0.03	7.35±0.02	0.361
Somatic cell counts in 1 cm^3	>750 000**	402.11±106.68	127.0±6.36	0.017

Notes: * CU TR 033/2013 «On safety of milk and dairy products».
** are not in force in the Republic of Kazakhstan. The applicable standard for top-grade milk is 200,000 units.
Data are presented as the Microsoft Excel 2021 program for calculations.

It was shown that latent mastitis has no significant effect on the physico-chemical parameters of milk in the context of averaged parameters for the group, except for an increase in the content of somatic cells in milk. It is considered that milk with somatic cell content of 500 000 units in 1 ml and higher is obtained from cows with mastitis. At present, the norms for Kazakhstan are 200,000 somatic cells in 1 ml for top-grade milk and 1,000,000 cells for first and second-grade milk, as set out in CU TR 033/2013 «On the safety of milk and dairy products» in the edition before the changes of 31.12.2019 (CU TR 033/2013 «On the safety of milk and dairy products», approved by Decision of the Commission of the CU from 28.05.2010 №299).

The results showed no significant changes in majority indicators between pre-treatment and post-treatment values. SMP remained nearly constant with pre-treatment at 8.75 ± 0.09 and post-treatment at 8.73 ± 0.12, both close to the norm of ≤8.2%. Similarly, density showed no significant change, with pre-treatment at 29.6 ± 0.69 and post-treatment at 29.24 ± 0.71, both slightly above the norm of ≤ 27°. Lactose and salt levels also remained stable, with pre-treatment values of 4.78 ± 0.05 and 0.73 ± 0.01, respectively, and post-treatment values of 4.77 ± 0.07 and 0.73 ± 0.01, showing no significant differences. Temperature, however, increased significantly from 21.01 ± 0.09 °C pre-treatment to 22.92 ± 0.28 °C post-treatment ($P<0.001$). Other indicators, including protein, freezing point, pH, and added water, also showed no significant differences, while somatic cell count decreased significantly.

After application of the extruded BioFeed-P additive, the number of somatic cells in the experimental group of cows decreased by 68%, so the average values before the experiment were 402.11±106.68 units/ml, after the treatment measures – 127.0 ± 6.36 units/ml, which corresponds to the required standards for milk of the highest grade. These changes indicate a decrease in the level of udder inflammation and improved milk safety, which in turn proves the effectiveness of phytogenics application in mastitis of dairy cows. The decrease in the number of somatic cells is the main proof that the developed feed additives containing phytogenics are effective in the treatment of mastitis, as somatic cells are an indicator of udder inflammation in animals. The decrease in their number indicates that the treatment was effective and reduced the inflammatory process in the udder of the experimental group of animals.

Addition of BioFeed-P additive also resulted in slight, but not significant changes in the fat index by 10% from 4.2 ± 0.49 to 4.56 ± 0.37, and in protein index by 3.1%, from 3.21 ± 0.03 to 3.22 ± 0.01%.

Discussion and Conclusion

Milk obtained from cows suffering from mastitis does not meet the organoleptic, physicochemical, and sanitary standards outlined in regulatory documentation, rendering the product unsuitable for food purposes. At the production facility of «Agrofirma Rodina» LLP, significant attention is dedicated to ensuring the quality and safety of milk. Daily, during the milking process, specialists perform individual checks on the animals for signs of mastitis and carry out the California mastitis test, which helps identify the latent form of the disease. If a positive result is obtained, affected cows are separated into distinct groups for treatment, and their milk is collected in a separate tank.

Our experiments demonstrated that after two weeks of administering the extruded BioFeed-Padditive and an extract of bitter wormwood to the cows with mastitis, the organoleptic and physicochemical parameters of the milk improved significantly across almost all key indicators.

Previously, we examined the effectiveness of the phytobiotic feed additive BioFeed-P in enhancing animal productivity. This preparation is a source of biologically active compounds, primarily flavonoids, which include pinostrobin, tectochrysin, pinocembrin, and chrysin. Flavonoids have a broad spectrum of effects on the body, including anti-inflammatory, analgesic, and anti-allergic properties. They are also characterized by hepatoprotective, cytostatic, apoptotic, estrogenic, and anti-estrogenic effects. Following the application of BioFeed-P to boost productivity, the animals exhibited increased immunity, improved metabolic processes, and enhanced quality indicators in goat milk (protein levels increased by 1.3%, lactose by 3.32%, and somatic cell count decreased by up to 40%). The average daily weight gain of fattening steers in the experimental group surpassed that of the control group by 36%.

Enriching feed with phytobiotic supplements further enhances the immune status of animals, which contributes to increased milk production. In earlier studies, Balji and Knicky used balsamic poplar bud extract to enhance the meat productivity of young steers. The effects of various phytobiotics on animal performance were reviewed.

Another phytogenic used in our experiments was an extract of bitter wormwood. According to international scholars, bitter wormwood contains various biologically active substances that produce specific physiological effects when introduced into the body. These substances include bitter glycosides, essential oils, flavonoids, phytoncides, alkaloids, organic acids, vitamins, tannins, and resins. Mugwort notably contains the essential oil absintol, which ranges from 0.12% to 2%.

The substance includes oxygen derivatives of bicyclic terpenes, sesquiterpenes, and monocyclic terpenes, featuring 10-25% thujol (C6H18O) and up to 10% thujone (C10H14O). Other components include pinene (C10H16), pellandrene, cineole, borneol, β-caryophyllene, β-sepinene, bisabolene, and hamazulene. Among the bicyclic sesquiterpenes is cadinene (C15H24), along with monocyclic catolactones such as ketopelanolide A, ketopelanolide B, and oxipelanolide. The bitter glycosides are represented by ten sesquiterpene lactones: absintin, anabsintin, guaianopides, artabsin, arborescin, and others, which contribute to the herb's distinctive bitter flavor. The aglycone of the glycosides, artaboin, can be processed to yield hamazulene. Additionally, the flavonoid artemetin (artemisinin) has been isolated from the herb, while inulin (a phytoncide) has been identified in the roots. The organic acids present include succinic and malic acids, along with esters of thujol alcohol with acetic, isovaleric, and palmitic acids, as well as vitamins C, K, B6, and provitamin A [14, 15].

Mugwort is recognized for its antimicrobial properties, demonstrating the ability to inhibit the growth of pathogens associated with mastitis, including Escherichia coli and Staphylococcus aureus. This effect is attributed to the essential oils, which disrupt bacterial metabolism and cell wall integrity, thereby hindering reproduction [16, 17]. Moreover, wormwood bitter enhances feed intake, subsequently improving the overall condition of the animal.

BioFeed-P phytobiotic comprises various compounds, including salicylates known for their potent anti-inflammatory properties. These compounds aid in reducing inflammation within the mammary gland and help prevent further tissue damage.

Overall, both BioFeed-P phytobiotic and wormwood extract possess a rich chemical composition, and their combined properties make them effective auxiliary agents in treating latent mastitis in cows. The use of extruded granules as a carrier for phytogenics minimizes the digestive burden and enhances metabolic processes during the animal's illness, positively influencing the recovery of cows. The use of the phytobiotic preparation «BioFeed-P» and wormwood extract in extruded granule form has been suggested to enhance the health, milk quality, and productivity of cows suffering from mastitis. Furthermore, these additives facilitate a swift recovery and boost immunity following illness.

Dairy farms, as well as private subsidiary farms, instead of antibiotic drugs, it is desirable to use grain fodder extruded and containing phytobiotic feed additives, such as BioFeed-P, extract of bitter wormwood or others. Phytobiotics used in our experiments contribute not only to the improvement of qualitative and quantitative indicators of milk, but also have therapeutic and preventive properties in mastitis of cows.

Authors' Contribution

GA: conducted laboratory research, prepared the initial text of the article; YuB: developed the aim and objectives of the work, as well as determined the methodology of the experiment, carried out the production of phytogenics; LS: participated in the experimental part of the work, responsible for the final revision of the text and design of the article in accordance with the requirements of the publication; GZ: performed statistical analysis and reviewed the manuscript. All authors read, reviewed and approved the final manuscript revision.

Acknowledgments

We express our gratitude to «NFT-KATU» LLP for providing the material and technical base for the production of extruded additives, to «S. Seifullin Kazakh Agrotechnical Research University» NJSC for providing the opportunity to participate in the research work, and to «Agrofirma Rodina» LLP for providing animals for the experimental work. Financial support for this study was provided by the Ministry of Science and Higher Education of the Republic of Kazakhstan within the framework of project AP23484620 «Development and implementation phytogenics and phytobiotics for prevention and treatment of animal mastitis with assessment of quality and safety milk».

Conflicts of Interest

The authors declare no conflicts of interest.

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Submitted for printing 19.01.2025, Signed for printing 31.03.2025,
Format 60×84 $^{1}/_{8}$, Conditionally printed sheets 6,25, Journal circulation 300,
Order № 25017

Printed in the Publishing House of S. Seifullin Kazakh
Agrotechnical Research University
010011, Zhenis avenue 62a, Astana, Kazakhstan
Phone: +7-7172-31-02-75