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Advances and challenges in the development of Crimean-Congo Hemorrhagic Fever vaccines: from traditional approaches to modern technologies

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Abstract

Crimean-Congo Hemorrhagic Fever (CCHF) is a high-mortality, tick-borne viral illness endemic to Africa, Asia, the Middle East, and certain regions of Europe. Notwithstanding its peril to public health, there is presently no licensed vaccination available. This study highlights important progress and challenges in developing CCHF vaccines, including traditional methods (inactivated and live-attenuated vaccines) and new approaches (subunit, DNA, mRNA, and viral vectors). We also investigate the functions of adjuvants and delivery technologies – such as nanoparticles and viral vectors – in enhancing immunogenicity and safety. Insights from historical endeavors and recent advancements underscore the pressing necessity for safe, effective, and scalable vaccinations, especially in endemic areas. Future initiatives must emphasize cross-protective formulations, thermal stability, and international cooperation.

Keywords: Crimean-Congo Hemorrhagic Fever Virus; Live-Attenuated Vaccines; mRNA Vaccines; Vaccine Development.

Introduction

Crimean-Congo Hemorrhagic Fever (CCHF) is a serious zoonotic illness caused by a *Nairovirus* of the *Bunyaviridae* family. CCHF, largely transmitted by *Hyalomma* ticks and through contact with infected animal or human blood, has a case fatality rate of 10% to 40%, contingent upon healthcare accessibility. The virus sustains a natural cycle between ticks and vertebrates, primarily livestock, with humans acting as inadvertent hosts [1, 2, 3].

CCHF is endemic in regions of Africa, Asia, the Middle East, and Eastern Europe, with recorded instances in more than 30 countries Europe [4, 5, 6, 7]. Figure 1 illustrates that the geographic distribution of endemic regions encompasses Kazakhstan, specifically the southern provinces of Turkestan and Zhambyl, where environmental circumstances promote tick circulation and human exposure. The extensive dispersion highlights the critical necessity for region-specific vaccination measures and global cooperation [8, 9].

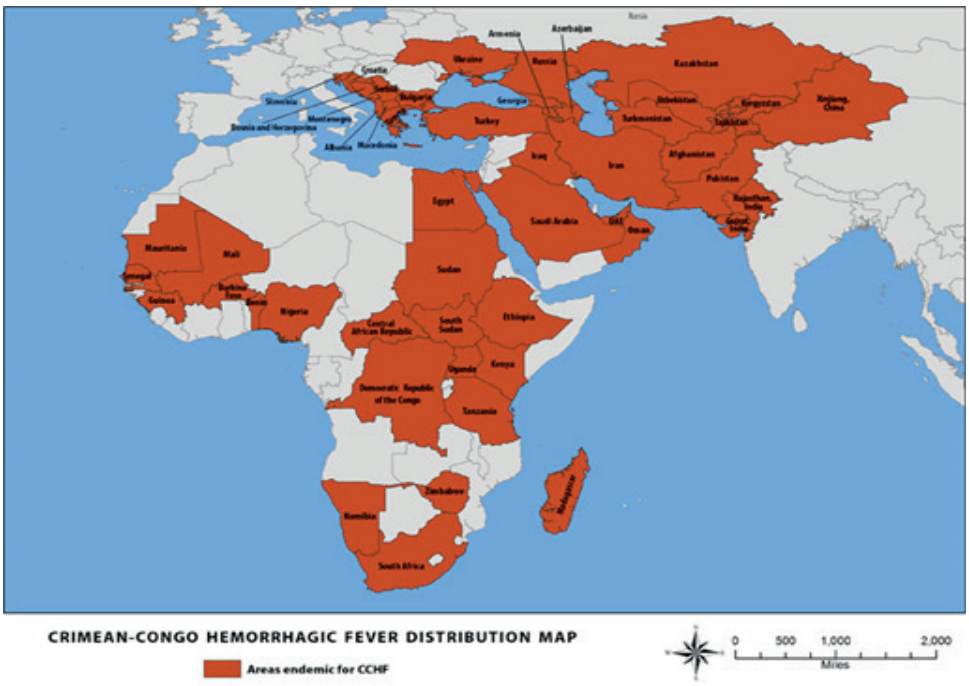

Figure 1 – Global distribution of Crimean-Congo Hemorrhagic Fever Virus (CCHFV) endemic regions. Countries marked in red have reported CCHFV presence or outbreaks. Data reflect surveillance records compiled by international health organizations [10]

The disease presents a significant concern owing to its epidemic potential and multiple modes of transmission (vector-borne and person-to-person). This disease is designated as a Risk Group 4 agent and a possible bioterrorism threat, highlighting the critical necessity for a viable vaccination [11, 12].

The development of vaccines has been impeded by the virus's genetic variability, segmented RNA genome, and biosafety concerns. Traditional vaccine methods, like inactivated and live-attenuated vaccines, have safety problems and limited effectiveness, while modern approaches face technical and immune system challenges [13].

This study outlines the current status of CCHF vaccine development, including traditional methods and new approaches, while highlighting advancements in antigen formulation, delivery methods, and adjuvant technology. It also addresses the primary obstacles to clinical translation and suggests priorities for future advancements.

Historical Development of CCHF Vaccines

The sole vaccine administered to people for Crimean-Congo Hemorrhagic Fever (CCHF) was created in the Soviet Union in 1970 and implemented in Bulgaria starting in 1974 [13, 14, 15]. This vaccine was made by using a virus that was killed with formalin and grown in the brains of baby mice, then mixed with aluminum hydroxide $Al(OH)_3$ to help boost the immune response. The immunization regimen comprised two subcutaneous doses administered 30 days apart, succeeded by a booster at one year and additional boosters every five years thereafter. It was authorized for adults aged 16 and older and largely distributed to high-risk populations, including military personnel, healthcare workers, agricultural laborers, and inhabitants of endemic areas [15, 16].

Epidemiological data from Bulgaria indicate a notable reduction in reported occurrences of CCHF subsequent to the vaccine's use. Between 1953 and 1974, 1,105 cases were documented, exhibiting a 17% case fatality rate. From 1975 to 1996, merely 279 instances were documented, with a death rate dropping to 11.4%. No illnesses were recorded among immunized military or laboratory workers. Nonetheless, it is probable that enhancements in tick management, diagnostic capabilities, and overall awareness probably played a role in the noted reduction [17].

The vaccine was never authorized outside Bulgaria due to concerns over its safety and efficacy. Vaccines made from mouse brain tissue pose a risk of allergic encephalomyelitis and further autoimmune problems. Moreover, research indicated that while the vaccination might provoke T-cell responses to the CCHF nucleoprotein, neutralizing antibody titers were typically low, necessitating frequent boosts for prolonged protection [18, 19].

The aforementioned restrictions, along with the requirement for biosafety level 4 (BSL-4) facilities for vaccine manufacture, have hindered worldwide adoption. Recent research endeavors have transitioned toward recombinant subunits, viral vectors, and nucleic acid-based vaccine platforms. Nonetheless, the Bulgarian vaccination serves as a significant historical reference and exemplifies a rare instance of practical implementation of a CCHF vaccine.

After the original vaccine, successive decades experienced disjointed and regionally separated research initiatives. Multiple experimental vaccines – comprising recombinant subunit, modified vaccinia Ankara-based vectors, viral vector vaccines, and nucleic acid-based platforms – have demonstrated encouraging immunogenicity in animal models but have not yet attained licensure [13, 14].

The lack of broadly recognized animal models, along with biosafety constraints and the significant genetic heterogeneity of the virus, has consistently hindered vaccine development. Recent global teamwork efforts, like the CCHF Vaccine [20] collaboration and clinical studies started by the University of Oxford in 2023 [21], show a renewed worldwide interest and joint efforts to create a vaccine for CCHF that works for everyone.

Live-Attenuated and Inactivated Vaccines

Live-Attenuated Vaccines

Even though live-attenuated vaccines for the CCHF virus could offer strong immune responses, they haven't moved forward much because of serious safety concerns. The possible risks – especially viral reversion to virulence or genetic reassortment – have hindered clinical advancement. As a result, existing research and clinical studies demonstrate limited engagement or enthusiasm in the advancement of live-attenuated vaccines for CCHF [13].

Inactivated Vaccines

Recent studies have concentrated significantly on inactivated vaccines derived from safer cell culture techniques. A formalin-inactivated, cell culture-based vaccination (CCVax) has demonstrated encouraging preclinical outcomes. CCVax given to BALB/c mice produced strong CCHF-specific IgG antibodies that lasted for at least 12 months after vaccination, which was much better than the results from mouse brain-derived vaccines (MBVax). Also, a study with mice that had temporary immune suppression found that CCVax provided complete protection (100%) against a deadly virus, and it led to more neutralizing antibodies and T-cell responses compared to MBVax [22, 23].

Despite these promising early results, increasing production is held back by safety issues related to handling the live virus, as well as logistical problems such as the need for strict temperature control to keep the vaccine effective in areas with limited resources.

Subunit Vaccines and Protein-Based Approaches

Subunit vaccines, which employ isolated viral proteins to elicit immune responses, are regarded as one of the safest vaccination platforms. The main parts studied for Crimean-Congo Hemorrhagic Fever (CCHF) vaccines are the viral proteins Gn and Gc, the nucleoprotein (NP), and the non-structural protein GP38. These components are generally generated through recombinant expression systems and combined with adjuvants to augment immunogenicity.

A detailed study of glycoprotein subunits was conducted using the outer parts of Gn and Gc from the CCHFV strain IbAr10200. Antigens were made in *Drosophila* insect cells and given to STAT1-knockout mice in two doses of 1.4 μg, spaced three weeks apart, using the Sigma Adjuvant System. Even though the Gc-e vaccine triggered an average antibody level of 1:333, all the vaccinated mice showed signs of illness and eventually died from the virus after being exposed to a serious challenge. A similar outcome was observed with Gn-e, suggesting that high antibody levels alone might not protect against the virus without a strong cellular response [24].

Conversely, vaccinations utilizing the nucleoprotein (NP) have exhibited more favorable outcomes. A modified adenovirus type 5 that makes NP (Ad-N) provided some protection in mice lacking STAT1, with just one shot given in the muscle leading to 33% survival after being exposed to the virus. The protective effect is probably due to the strong $CD8^+$ T-cell responses triggered by the NP antigen, which is mostly similar in many CCHFV strains [25].

A contemporary method entails the amalgamation of several antigens. A 2024 study involved giving mice shots under their skin with either recombinant NP, GP38, or both, using AddaVax and monophosphoryl lipid A to boost their immune response. When faced with a different strain of CCHFV, all the mice that were vaccinated only with NP survived, while 83% of the mice that received both NP and GP38 survived. The dual-antigen group demonstrated fewer clinical symptoms and weight loss, indicating additional protective effects from the combination of humoral and cellular targets [26].

Although subunit vaccines are safe and versatile, they frequently necessitate enhanced delivery mechanisms and adjuvants to elicit strong protection. Furthermore, immunogenicity does not consistently correlate with protection, as evidenced by the glycoprotein investigations. Future improvements should focus on using multiple antigens, better ways to present antigens (like virus-like particles or nanoparticle platforms), and testing in relevant animal models, including non-human primates.

Nucleic Acid-Based Vaccines

Nucleic acid vaccines – DNA and RNA – are very promising options for Crimean-Congo Hemorrhagic Fever (CCHF) because they can be designed quickly, are safe, and can activate both parts of the immune response. However, their efficacy is heavily contingent upon the chosen antigen, delivery method, and the immunological pathways they engage.

DNA vaccines aimed at the viral glycoprotein precursor (GPC) have demonstrated limited efficacy in protection. A study injected a plasmid that contains the full GPC of the IbAr10200 strain into the muscles of mice in three doses of 50 μg each, using electroporation. The vaccinated mice produced neutralizing antibody titers and attained over 60% survival after a fatal challenge. The result validates the protective efficacy of DNA vaccines while underscoring the necessity for enhanced delivery and boosting protocols to ensure consistent protection [27].

Importantly, better results were achieved with a DNA vaccine targeting GP38, which is a non-structural protein released from the M segment. After receiving three identical doses, animals vaccinated with GP38 showed an 80% survival rate after being challenged. Following three identical dosages, GP38-vaccinated animals exhibited an 80% survival rate after challenge. Even though there wasn't strong neutralizing action, the protection was still steady, suggesting that Fc-mediated effector functions or increased T-cell activation might be involved. These findings establish GP38 as a formidable and hitherto undervalued immunogen [28].

RNA-based platforms, especially self-replicating RNA (repRNA), seem increasingly promising. A single dose of repRNA that produces NP or GPC, combined with a LION™ nanocarrier, provided full protection for mice. In contrast to DNA vaccines, these designs necessitated minimal boosting and generated strong T-cell responses and IgG binding titers; however, they exhibited low or negligible neutralizing antibodies. This study highlights the significance of cellular immunity in CCHFV protection and the efficacy of repRNA in inducing it [29].

Traditional mRNA vaccines encased in lipid nanoparticles have exhibited immunogenicity. Mice vaccinated with glycoprotein-expressing mRNA generated antigen-specific IgG and IFN-γ+ T lymphocytes. Formulations that included the NSm protein showed weaker immune responses, which might be due to changes in the immune system or competition between different parts of the antigen, highlighting the importance of carefully choosing the right antigens [30, 31].

RNA vaccines, especially the replicon-based types, offer better protection with fewer doses than DNA vaccines and more effectively activate the T-cell responses needed for CCHFV immunity. In several studies, GP38 and NP are more effective than GPC as antigens, challenging the usual focus on neutralizing antibodies. These data underscore that effective CCHFV vaccinations may depend more on the breadth and quality of T-cell immunity than only on elevated antibody titers.

Viral Vector-Based Vaccines

Viral vector-based vaccines constitute an innovative and swiftly evolving platform for CCHFV vaccination. These vaccines use modified viruses to deliver CCHFV proteins directly into host cells, which boosts both antibody and cell-based immune responses. Various vector systems have been examined, demonstrating differing efficacy based on the vector type and antigen employed.

The ChAdOx2 CCHF vaccine, which uses a harmless chimpanzee virus to deliver the complete glycoprotein precursor (GPC) of CCHFV, is one of the leading options. In early tests, mice given the ChAdOx2-CCHF vaccine showed strong antibody and T-cell responses, leading to 100% survival after being exposed to a deadly virus. The protection was even better when this vaccine was given first, followed by a booster shot of Modified Vaccinia Ankara (MVA) CCHF. A detailed tissue examination confirmed complete protection, showing no signs of the virus or any changes in the tissues [32, 33].

A different adenoviral method used a human Ad5 vector that carries the nucleoprotein (NP) of CCHFV. In IFNAR−/− mice, one dose gave 30% protection, while a prime-boost method increased protection to 78%. In IFNAR−/− mice, a single administration provided 30% protection, whereas a prime-boost strategy enhanced it to 78%. Even though there were only a few neutralizing antibodies, strong NP-specific IgG and T-cell responses were found, showing that cell-mediated immunity played

an important role in protection [13, 32, 34]. These findings highlight the importance of choosing the right antigens: while GPC-based vaccines offered full protection, NP-based ones only gave partial protection.

Besides adenoviruses, other different viral vectors have been investigated:

• Bovine Herpesvirus Type 4 (BoHV-4) has surfaced as a viable candidate owing to its minimal human toxicity and robust immunogenicity. A modified version of BoHV-4 that makes CCHFV NP (called BoHV4-ΔTK-CCHFV-N) triggered strong immune responses andspecific antibodies in both BALB/c and IFNα/β/γR−/− mice. Even though there were no detectable neutralizing antibodies, the vaccine completely protected against serious infections with the Ank-2 strain. Tests showed that giving antibodies and checking T-cells resulted in 75% protection, confirming that both antibody and T-cell responses are involved [35, 36].

• Vesicular Stomatitis Virus (VSV) vectors have demonstrated significant efficacy. A recombinant VSV expressing the CCHFV glycoprotein was evaluated in STAT1−/− mice. The vaccinated mice produced strong anti-GP IgG and neutralizing antibodies, but all of them died after being exposed to a dangerous strain of CCHFV. The VSV platform's rapid replication and elevated antigen expression may enhance its robust immunogenicity [37].

Adenoviral vectors are the most advanced regarding translational potential and continuing clinical study; yet, these other systems have significant advantages. BoHV-4 can hold large genetic changes and triggers a variety of immune responses, while VSV allows for effective single doses and higher levels of expression. However, there are still limitations, like the need for cold storage, the chance of existing immunity (especially for Ad5), and the lack of information about how long the effects last in large animals or humans.

In summary, vaccines that use viral vectors, like adenoviruses, BoHV-4, and VSV, show great promise for treating CCHFV. Their effectiveness will depend on improving how we choose antigens, the amount given, and how they are delivered, as well as comparing different options to find the best ones for use in humans. Their success will hinge on the ongoing optimization of antigen selection, dosage regimens, and administration methods, along with comparative assessments across platforms to identify the most dependable candidates for human application.

Adjuvants and Delivery Systems

The efficacy and nature of the immune response generated by CCHFV vaccines are contingent upon both the antigen and the method of its delivery and processing. Diverse platforms activate unique pathways − some promote antibody responses, while others enhance T-cell activation. Figure 2 summarizes these pathways, illustrating how inactivated, subunit, nucleic acid, and viral vector vaccines activate the immune system.

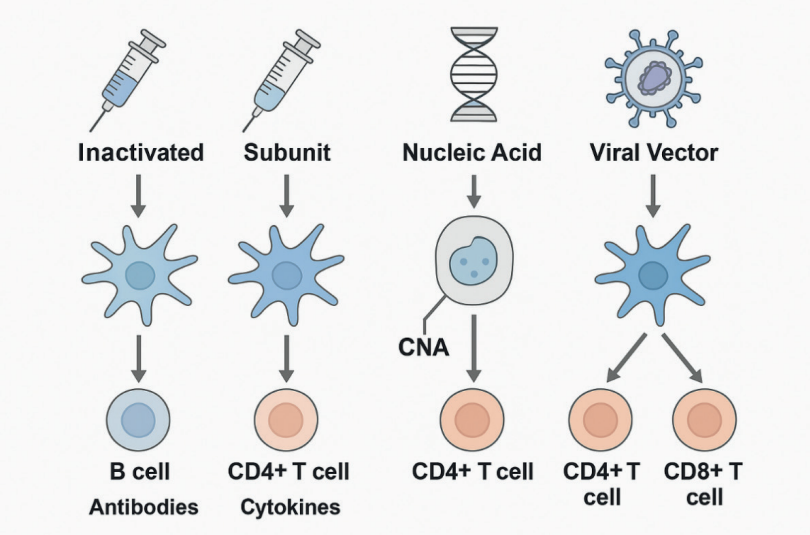

Figure 2 – Schematic depiction of immune response pathways elicited by several CCHFV vaccination platforms. Inactivated and subunit vaccinations predominantly stimulate antibody synthesis, frequently necessitating adjuvants. DNA, RNA, and viral vector platforms induce more extensive responses by activating both helper and cytotoxic T cells. Comprehending these distinctions is crucial for the selection and optimization of vaccination candidates

The effectiveness of CCHFV vaccine candidates, especially those using subunit and nucleic acid methods, often needs to be improved with adjuvants and better delivery systems. These components improve how strong and effective the immune response is, which is especially important for viruses like CCHFV that need both antibody and cell-based immunity for good protection.

Adjuvants

Adjuvants are agents that enhance the immunological response to an antigen. Various adjuvants have been assessed for CCHFV vaccines:

• Aluminum-based adjuvants (alum) continue to be the predominant choice in inactivated CCHFV vaccinations. In IFNAR−/− mice, alum-adjuvanted cell culture-derived vaccinations induced specific IgG responses and conferred partial protection after viral challenge. However, their ability to create strong cellular immunity is limited, which may explain the moderate level of protection seen in some models [34, 38].

• To address this, more effective adjuvant compositions have been investigated. A good example is the combination of Montanide ISA 201VG with Poly (I:C), which is a man-made version of double-stranded RNA that activates TLR3. This two-adjuvant method was used to create glycoprotein subunit vaccines, which produced a balanced immune response shown by higher levels of IFN-γ and antigen-specific IgG in BALB/c mice. The formulation additionally facilitated cross-presentation, crucial for effective cytotoxic T-cell activation [39].

Delivery Systems

Antigen delivery strategies are essential in influencing the immunological result. Numerous advanced techniques have been created to enhance antigen stability, absorption, and presentation:

• Gram-Positive Enhancer Matrix (GEM) particles, originating from Lactococcus lactis, have been utilized for the surface display of CCHFV glycoproteins through a protein anchor system (PA). The GEM-PA platform facilitates high-density antigen presentation and rapid purification without requiring adjuvants. In mouse studies, vaccines made with GEM-displayed Gn and Gc parts triggered strong immune responses, including specific IgG and IFN-γ, showing that this system could be useful for both mucosal and overall vaccination methods [40].

• Zera® Protein Fusion Technology employs plant-derived fusion tags to facilitate the self-assembly of antigens into protein bodies. This method enhanced protein expression, aggregation, and immunogenicity when applied to CCHFV Gn and NP antigens. Mice that received Zera-fused nanoparticles showed strong IgG responses and produced IFN-γ from splenocytes, suggesting that this technology could improve the effectiveness of vaccines made from both DNA and protein subunits [40, 41].

Together, these adjuvants and delivery methods help overcome important challenges in developing CCHFV vaccines, like the weak immune response from purified proteins, the need to lower doses, and the activation of cellular immunity. As vaccination methods improve, carefully mixing antigens with effective adjuvants and smart delivery systems will be key to achieving long-lasting and broad protection.

Key Insights and Future Directions

Recent advancements in CCHFV vaccine research indicate a distinct trend: cellular immunity, especially T-cell responses, seems more vital for protection than only neutralizing antibodies. Subunit vaccines targeting GP38 or NP, even though they don't produce strong neutralizing antibodies, often perform better than glycoprotein-based vaccines in tests. This view shifts the focus from just measuring antibody levels to looking at the overall immune response when evaluating how well a vaccine works.

Replicating RNA (repRNA) and viral vector vaccines have shown the best effectiveness (up to 100%) in early tests, often needing just one dose, highlighting their ability to be given quickly and with fewer doses. In contrast, DNA vaccines need multiple doses and electroporation to reach similar effectiveness, which limits how they can be used in practice. Conversely, DNA vaccines necessitate numerous administrations and electroporation to achieve similar efficacy, thereby constraining their practical application.

Adjuvants and delivery methods are essential components. Technologies like GEM particles, Zera® fusion, and effective combinations of adjuvants (like Montanide with Poly I:C) have significantly

improved how well subunit vaccines work. These technologies enhance antigen presentation and provide practical benefits such as simplified formulation and needle-free alternatives.

Notwithstanding this advancement, obstacles persist. Most research concentrates on select virus strains, and the mechanisms of cross-genotype protection remain inadequately comprehended. The absence of standardized animal models and established immunological correlates of protection impedes cross-platform comparisons. Significantly, no vaccine has progressed to advanced human trials; nevertheless, the ChAdOx2 CCHF candidate represents a crucial advancement.

Future research should emphasize broad-spectrum antigens, multi-antigen formulations, and scalable delivery systems appropriate for endemic environments. Coordinated worldwide investment could expedite the development of a safe, effective, and accessible CCHFV vaccine.

Conclusion

Despite extensive study over several decades, there is presently no licensed vaccination available for Crimean-Congo Hemorrhagic Fever. Recent advancements – especially in RNA replication, viral vector technology, and antigen-specific subunit platforms – have substantially progressed the area. The focus has shifted from just targeting antibodies to also building strong cellular immunity, improving delivery methods, and creating widely protective antigens. The creation of a vaccine is both a scientific advancement and a public health imperative in endemic nations like Kazakhstan, where the circulation of CCHFV is thoroughly recorded in southern areas. Connecting preclinical achievement with clinical application is essential for ensuring protection for high-risk populations in Kazakhstan and elsewhere.

Authors' Contributions

AT, BM and LK: Conceptualization, formal analysis, designed the study, writing - original draft. AT GZh, TT, KB: Conducted an extensive literature review and analyzed the data. AT, BM and LK: Data Curation, Writing Review & Editing. MR, KZh and BM: Supervision. All authors have read, reviewed, and approved the final manuscript.

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Episotological monitoring of brucellosis of large and small cattle in the Pavlodar Region of the Republic of Kazakhstan

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Abstract

Background and Aim. Despite the efforts to eliminate brucellosis in the Pavlodar region, its local epizootics remain a huge concern. The aim of this work was to conduct epizootic surveillance and analyze the epizootiological situation with animal brucellosis in the Pavlodar region in 2019-2023.

Materials and Methods. The materials used in this study include the official reports of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan (CVCS of MoA of RK), the Republican Anti-epizootic Unit (RSI RAU), the regional branch of the Republican Veterinary Laboratory (RVL), the Republican State Enterprise on the rights of economic management "Scientific and Practical Centre for Sanitary and Epidemiological Expertise and Monitoring" of the Ministry of Public Health of the Republic of Kazakhstan (RSE SPC SEEaM of MoPH of RK), and the results of our own epizootiological research.

The research methods used in the study are in full compliance with the official guidelines for diagnosing brucellosis in animals.

Results. We have established the leading role of cattle and small ruminants in the epizootiology of brucellosis, identified the most significant factors promoting brucellosis persistence in livestock and ascertained the occurrence of the infection in the human population in every district of the region.

Using the epizootic surveillance data acquired over the past 5 years, we have identified areas with high, moderate and low incidence of animal brucellosis, as well as disease-free zones, and constructed an epizootic map that can be used to implement adequate interventions.

Conclusion. Epizootic surveillance, coupled with the analysis of dynamics of brucellosis spread to new sites, its incidence in livestock and the results of screening tests, will facilitate epizootic control and help to elaborate a methodologically sound strategy for implementing adequate interventions.

Keywords: brucellosis; diagnostic tests; epizootic map; epizootic surveillance; morbidity.

Introduction

Brucellosis is a socially and economically significant disease widely spread across the Republic of Kazakhstan [1].

Brucellosis is a zoonotic, predominantly chronic infection of humans and animals caused by the pathogenic microorganisms of the genus Brucella. Insufficient control, diagnostics and prevention and the threat the disease poses for human health dictate a need for effective strategies that could be effectively adopted by livestock farming and address the diversity of business models [2, 3].

Brucellosis of cattle and small ruminants contributes significantly to infectious morbidity, reduces livestock population and negatively affects the economy of Kazakhstan [4].

Despite the efforts to eliminate brucellosis in the Pavlodar region, its local epizootics remain a huge concern.

Epizootic surveillance plays a key role in the prevention and control of brucellosis in animals. It involves collecting, analysing and interpreting data on the spread of the infection among animals in a specific area. Surveillance allows brucellosis outbreaks to be timely detected, facilitating a rapid response to the threat and preventing its spread.

Long-term surveillance provides invaluable information about the epizootiology of brucellosis in the area, helping to estimate the risk of this infection. This information is crucial for developing an effective system of brucellosis prevention and control [5, 6].

Materials and Methods

The materials used in this study include the official reports of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan (CVCS of the MoA of the RK), the Republican Anti-epizootic Unit (RSI RAU), the regional branch of the Republican Veterinary Laboratory (RVL), the Republican State Enterprise on the rights of economic management "Scientific and Practical Centre for Sanitary and Epidemiological Expertise and Monitoring" of the Ministry of Public Health of the Republic of Kazakhstan (RSE SPC SEEaM of the MoPH of the RK), and the results of our own epizootiological research.

The research methods used in the study are described in the official guidelines for diagnosing brucellosis in animals [7].

The following data has been analyzed to study the epizootic activity of brucellosis:

- results of the epizootic and serological surveillance of animal brucellosis across the region, implemented by the Laboratory of Brucellosis;
- statistical reviews and official reports on animal brucellosis prepared by the veterinary inspectors of the Pavlodar region, RSI RAU and RVL.

The acquired information was summarized using the official statistical reports from 2023 prepared by the CVCS of the Ministry of Agriculture of the Republic of Kazakhstan [8]. The epizootic situation was analyzed by the methods described by *S.A. Dudnikov* [9].

The retrospective data on the spread of brucellosis and its incidence among animals in 2019-2023 was used to identify areas with high, moderate and low incidence of the disease. The epizootic maps were constructed using GIS-based technology and the methods of risk assessment for disease emergence and spread, considering WHOA's recommendations.

Results and Discussion

As part of this study, we have conducted our own field and laboratory research at livestock farms of the Pavlodar region, at the Regional Veterinary Laboratory and the Laboratory of Brucellosis of Kazakh Scientific Research Veterinary Institute.

In 2019 through 2023, we were monitoring and analyzing the epizootic activity of animal brucellosis in the Pavlodar region.

The acquired data is presented in the tables below.

Table 1 – The number of epizootic sites (ES) of infectious animal diseases and animal brucellosis in the Pavlodar region in 2019-2023

Epizootic indicators	2019	2020	2021	2022	2023	Total for 5 years	Average for 5 years
Total number of ES of infectious diseases	8	50	22	18	17	115	23
Number of ES of bovine brucellosis	1	24	22	0	0	47	9.4

Continuation of table 1

Number of ES of small ruminant brucellosis	0	0	0	0	1	1	0.2
ES of animal brucellosis, % of total	12.5	48.0	100.0	0	5.8	41.7	8.3

Table 1 shows that 115 ES of infectious diseases, including 48 (41.7%) brucellosis sites, were reported in the Pavlodar region during the 5 years of surveillance. This suggests that brucellosis is a leading animal infection in the region. Table 2 shows the number of ES of cattle and small ruminant brucellosis for each district of the Pavlodar region.

Table 2 – The number of ES of cattle and small ruminant brucellosis in the districts and cities of the Pavlodar region during 2019-2023

Districts	2019	2020	2021	2022	2023	Total for 5 years	Average for 5 years
	cattle/ small ruminants	cattle/ small ruminants	cattle/ small ruminants	cattle/ small ruminants	cattle/ small ruminants	cattle/ small ruminants	cattle/ small ruminants
Uspensky	0	10\0	3\0	0	0	13\0	2.6\0
Pavlodarsky	0	3\0	3\0	0	0	6\0	1.2\0
Irtyshsky	0	4\0	1\0	0	0	5\0	1\0
City of Aksu	0	2\0	3\0	0	0\1	5\1	1\0.2
Akkuli	0	1\0	3\0	0	0	4\0	0.8\0
Mayskiy	0	1\0	2\0	0	0	3\0	0.6\0
Shcherbaktinsky	1\0	0	2\0	0	0	3\0	0.6\0
City of Ekibastuz	0	2\0	1\0	0	0	3\0	0.6\0
Zhelezinsky	0	0	2\0	0	0	2\0	0.4\0
Bayanaulsky	0	1\0	0	0	0	1\0	0.2\0
Terenkol	0	0	1\0	0	0	1\0	0.2\0
City of Pavlodar	0	0	1\0	0	0	1\0	0.2\0
Aktogaysky	0\0	0\0	0\0	0\0	0\0	0\0	0\0
Entire region	1\0	24\ 0	22\0	0 \0	0\1	47\1	9.4\0.2

Table 2 shows that there was only 1 ES of brucellosis in 2019 and 1 ES of brucellosis in 2023 (in cattle and small ruminants, respectively) reported in the Pavlodar region. In 2019-2022, there were no known ES of small ruminant brucellosis. However, a significant number of bovine brucellosis sites were reported in 2020 and 2021 (24 and 22, respectively), mainly in the Uspensky, Pavlodarsky and Irtyshsky districts and in the city of Aksu. In 2022, no brucellosis sites were detected in the region. The only area free from the infection throughout the analyzed period was the Aktogaysky district.

In Kazakhstan, mass serological testing of farm animals is routinely conducted by RVL to ensure timely detection of brucellosis.

The results of serological testing conducted by the Pavlodar branch of RVL in 2019–2023 are provided in Tables 3-8.

Table 3 – Results of serological testing for bovine brucellosis conducted in the Pavlodar region in 2019-2023

Year	Number of tested animals	Number of positive serological tests			Confirmed number of seropositive animals	% of infection
		RBT	CFT	AT		
2019	495.615	3.540	3.395	3.390	3.395	0.68
2020	504.462	2.666	2.243	2.239	2.243	0.44
2021	682.166	3.040	2.664	2.660	2.664	0.40
2022	627.264	1.982	1.874	1.868	1.874	0.30
2023	610.279	3.011	2.863	2.858	2.863	0.47
Total, 5 years	2.919.786	14.239	13.039	13.020	13.039	0.45
Average, 5 years	583.957	2.847	2.608	2.603	2.608	0.45

Note. RBT – Rose Bengal test; CFT – complement fixation test; AT – agglutination test.

As seen from Table 3, there were 2.608 seropositive bovines detected in the Pavlodar region in 2019-2023; the average incidence rate was 0.45%. The Rose Bengal test returned the highest number of seropositive results (2.847), followed by the complement fixation test (2.608) and the agglutination test (2.603). The confirmed number of brucellosis-positive animals was 2,608.

Table 4 – Results of serological testing for bovine brucellosis for each district of the Pavlodar region in 2019-2023

Districts and cities	Number of brucellosis cases and morbidity rate (%)										Total for 5 years		
	2019		2020		2021		2022		2023		Number of infected animals, total	Average values	
	Qty	%	Qty	%	Qty	%	Qty	%	Qty	%		abs. number	incidence, %
Bayanaulsky	782	0.91	999	1.21	1237	1.33	818	0.87	1.535	1.68	5.371	1.074	1.2
Pavlodar city	97	1.32	68	0.85	71	0.83	31	0.37	94	1.02	361	72	0.9
Ekibastuz city	506	1.17	239	0.66	431	0.72	331	0.59	333	0.61	1.840	368	0.8
Akkuli	764	1.89	73	0.17	79	0.14	26	0.05	22	0.05	964	193	0.5
Pavlodarsky	333	0.64	161	0.31	255	0.44	238	0.43	245	0.38	1.232	246	0.5
Aksu city	149	0.44	122	0.32	129	0.20	205	0.33	363	0.75	968	194	0.4
Mayskiy	161	0.42	89	0.21	81	0.17	77	0.16	108	0.23	516	103	0.3
Terenkol	145	0.37	32	0.09	87	0.21	68	0.16	88	0.21	420	84	0.3
Zhelezinsky	9	0.03	133	0.43	159	0.44	14	0.05	28	0.10	343	69	0.3
Irtyshsky	140	0.46	44	0.12	47	0.10	6	0.01	22	0.05	259	52	0.2
Uspensky	132	0.44	76	0.25	50	0.16	18	0.07	17	0.07	293	59	0.2
Shcherbaktinsky	120	0.33	206	0.57	36	0.06	0	0.00	8	0.01	370	74	0.2
Aktogaysky	57	0.17	1	0.00	2	0.00	42	0.09	0		102	20	0.1
Total	3.395	0.68	2.243	0.44	2664	0.40	1.874	0.30	2.863	0.47	13.039	2.608	0.45

Table 4 shows that the incidence rate of bovine brucellosis was decreasing gradually from 0.68% to 0.30% in 2019–2022, but then rose to 0.47% in 2023. The average incidence rate of the disease calculated for the 5-year surveillance period was 0.45%. Using the data from Table 4, we ranked the

districts of the Pavlodar region by the incidence of the disease (high incidence rate: ≥0.45%; moderate and low incidence: <0.45%). There were no epizootically safe districts in the region (Table 5).

Table 5 – Districts of the Pavlodar region ranked by the incidence of bovine brucellosis in 2019-2023

№	Incidence of bovine brucellosis	Number of districts and cities and their contribution to incidence (%)	Average incidence by districts and cities for 5 years, %
1	High, ≥0.45%	5 (38.5%)	Bayanaul district: 1.2 Pavlodar city: 0.9 City of Ekibastuz: 0.8 Akkulinsky district: 0.5 Pavlodarsky district:0.5
2	Moderate, 0.21-0.45%	4 (30.7%)	Aksu city: 0.4 Mayskiy district: 0.3 Zhelezinsk district: 0.3 Terenkol district: 0.3
3	Low, ≤ 0.20%	4 (30.7%)	Uspensky district: 0.2 Shcherbaktinsky district: 0.2 Irtysh district: 0.2 Aktogay district: 0.1
4	Epizootically safe zone, 0.0%	No	

Table 5 shows that the highest incidence of bovine brucellosis in 2019–2023 was observed in 5 districts and cities, which make up 38.5% of the region's territory; moderate incidence was observed in in 4 districts (30.7% of the territory) and low incidence, in 4 districts (30.7% of the territory). There were no epizootically safe districts.

A similar analysis was conducted for small ruminant brucellosis. Its results are provided in Tables 6-8.

Table 6 – Results of serological testing for small ruminant brucellosis conducted in the Pavlodar region in 2019-2023

Year	Number of tested animals	Number of positive serological tests			Confirmed number of seropositive animals	% of infection
		RBT	CFT	AT		
2019	590.747	53	53	51	53	0.01
2020	606.651	64	62	60	62	0.01
2021	774.460	79	53	51	53	0.01
2022	703.121	249	127	122	127	0.02
2023	685.466	313	245	241	245	0.04
Total, 5 years	3.360.445	758	540	525	540	0.02
Average, 5 years	672.089	152	108	105	108	0.01

Note. RBT – Rose Bengal test; CFT – complement fixation test; AT – agglutination test.

According to Table 6, there were 108 head of small ruminants infected with brucellosis in the Pavlodar region in 2019–2023; the average incidence rate was 0.01%. RBT returned the highest number of seropositive results (152), followed by CFT (108) and AT (105). The confirmed number of brucellosis-positive animals was 108.

Table 7 – Results of serological testing for small ruminant brucellosis conducted in the Pavlodar region in 2019-2023

Districts and cities	Number of brucellosis cases and morbidity rate (%)										Total for 5 years		
	2019		2020		2021		2022		2023		Number of infected animals, total	Average values	
	Number	%	Number	%	Number	%	Number	%	Number	%		abs. number (head)	incidence, %
Pavlodarsky	2	0.00	50	0.12	0	0	57	0.13	19	0.03	128	26	0.09
Zhelezinsky	0	0	0	0	43	0.07	62	0.15	56	0.14	161	32	0.07
Pavlodar city	0	0	11	0.08	3	0.01	4	0.03	9	0.05	27	5	0.04
Aksu city	0	0	0	0	0	0	0	0	63	0.08	63	13	0.02
Akkuli	40	0.08	0	0	0	0	0	0	10	0.02	50	10	0.02
Bayanaulsky	1	0.01	0	0	0	0	0	0	52	0.05	53	11	0.01
Mayskiy	8	0.01	1	0.00	2	0.00	4	0.01	15	0.02	30	6	0.01
Irtyshsky	0	0	0	0	0	0	0	0	21	0.04	21	4	0.01
Ekibastuz city	2	0.00	0	0	5	0.01	0	0	0	0	7	1	0.001
Aktogaysky	0	0	0	0	0	0	0	0	0	0	0	0	0
Terenkol	0	0	0	0	0	0	0	0	0	0	0	0	0
Uspensky	0	0	0	0	0	0	0	0	0	0	0	0	0
Shcherbaktinsky	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	53	0.01	62	0.01	53	0.01	127	0.02	245	0.04	540	108	0.02

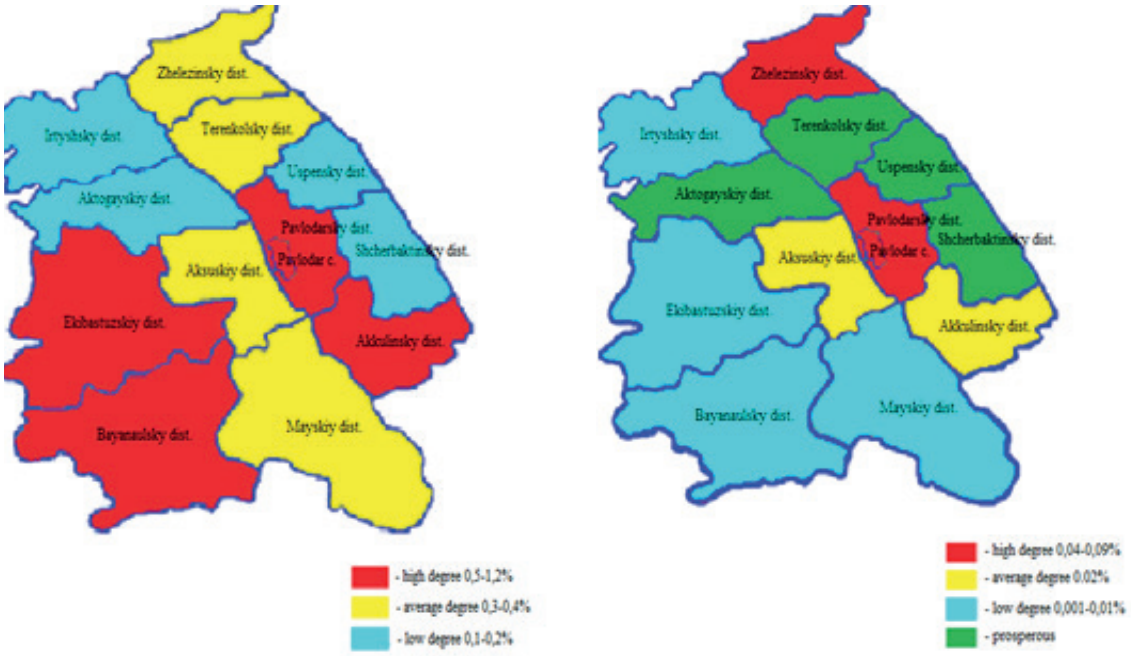
Table 7 demonstrates no dynamics in the incidence rate of small ruminant brucellosis in 2019–2021 (0.01%). However, in 2022, it grew to 0.02% and increased almost twofold to 0.04% in 2023. The relative incidence rate of small ruminant brucellosis calculated for the 5-year surveillance period was 0.02% on average. Using the data from Table 7, we ranked the districts of the Pavlodar region by the incidence of the disease (high incidence: ≥0.02%; moderate and low incidence: <0.02%). Districts with no detected cases of small ruminant brucellosis were considered epizootically safe (Table 8).

Table 5 – Districts of the Pavlodar region ranked by the incidence of bovine brucellosis in 2019-2023

№	Incidence of small ruminant brucellosis	Number of districts and cities and their contribution to incidence (%)	Five-year average incidence rates by districts and cities, %
1	High, >0.02%	3 (23.1%)	Pavlodarsky district: 0.09 Zhelezinsky district: 0.07 City of Pavlodar: 0.04
2	Moderate, 0.02-0.01%	2 (15.4%)	Akkuli district: 0.02 City of Aksu: 0.02%
3	Low, < 0.01%	4 (30.7%)	Bayanaulsky district: 0.01 Irtyshsky district: 0.01 Mayskiy district: 0.01 City of Ekibastuz: 0.001
4	Epizootically safe zone, 0.0%	4 (30.7%)	Aktogaysky district: 0 Terenkol district: 0 Uspensky district: 0 Shcherbaktinsky district: 0

High incidence of the disease was observed in 3 districts, which make up 23.1% of the region's territory, moderate, in 2 districts (15.4% of the territory), and low, in 4 districts (30.7% of the territory). Four districts (30.7% of the territory) were classified as epizootically safe.

The results of the analysis are presented as maps showing the incidence of brucellosis among cattle and small ruminants across the Pavlodar region in 2019-2023 (Figure 1).



А Б

Figure 1 – The burden of bovine (A) and small ruminant (B) brucellosis in the Pavlodar region in 2019-2023

It is clearly visible that bovine brucellosis and small ruminant brucellosis occur in almost the same parts of the region. In 2019-2023, the disease was widespread in the Akkulinsky, Zhelezinsky and Pavlodarsky districts and in the cities of Ekibastuz, Pavlodar and Aksu. The Aktogai, Terenkol, Uspensky and Shcherbaktinsky districts were epizootically safe in terms of small ruminant brucellosis, but bovine brucellosis was found everywhere across the region.

The epizootic maps illustrate the geographical distribution of bovine and small ruminant brucellosis across the region and the potential risks of its expansion. They can provide support in implementing veterinary surveillance in different areas with different epizootiological status and can be used to develop a methodologically sound plan of interventions aimed at preventing and controlling the disease.

The spread of brucellosis among livestock animals directly affects the epidemiological status of the human population. As part of this study, we collected and analyzed data on the morbidity of brucellosis in the human population of the Pavlodar region in 2019-2023 (Table 9).

Table 9 – The incidence rates of human brucellosis in the Pavlodar region in 2019-2023

Districts and cities	2019		2020		2021		2022		2023		Overall, 5 years	
	absolute number	per 100,000 population	absolute number	per 100,000 population	absolute number	per 100,000 population	absolute number	per 100,000 population	absolute number	per 100,000 population	absolute number	per 100,000 population
Uspensky	0	0	1	7.5	4	30.18	5	37.7	0	0	10	
Mayskiy	3	23.8	1	7.9	3	23.81	2	15.9	0	0	9	
Bayanaulsky	1	3.5	7	24.7	6	21.20	3	10.6	1	3.5	18	
Akkulinsky	2	13.7	1	6.9	0	0	5	34.3	1	6.9	9	
Zhelezinsky	0	0	0	0	3	16.81	4	22.4	1	5.6	8	
Terenkolsky	1	4.5	1	4.5	2	9.01	3	13.5	3	13.5	10	
Shcherbaktinsky	4	18.3	0	0	0	0	0	0.0	5	22.9	9	
Irtyshsky	0	0	3	14.4	1	4.80	1	4.8	2	9.6	7	
Pavlodarsky	2	6.9	0	0	1	3.47	1	3.5	2	6.9	6	
City of Aksu	1	1.5	1	1.5	3	4.43	3	4.4	6	8.9	14	
Aktogayskiy	0	0	0	0	0	0	2	13.2	0	0	2	
City of Ekibastuz	1	0.7	0	0	5	3.51	5	3.5	2	1.4	13	
City of Pavlodar	3	0.9	0	0	4	1.19	3	0.9	8	2.4	18	
Total	18	2.4	15	2.0	32	4.31	37	5.0	31	4.2	133	

Table 9 shows that the incidence rate of human brucellosis per 100,000 population was almost twice as high in 2021-2023 (4.2-5.0) than in 2019 and 2020 (2.4 and 2.0, respectively). Notably, there was an increase in the incidence rate of bovine (0.40%; 0.30%; 0.47%) and small ruminant (0.01%; 0.02%; 0.04%) brucellosis on livestock farms across Pavlodar region in 2021-2023. From 2019 to 2023, 133 persons contracted the infection; the average incidence rate per 100,000 population was 3.6. High morbidity rates were reported in the Uspensky, Mayskiy, Bayanaulsky and Akkulinsky districts. The absolute number of the infected individuals was the greatest in the Bayanaulsky district (18) and the city of Pavlodar (18), followed by the cities of Aksu (14) and Ekibastuz (13). The results of the comparative analysis demonstrate that human brucellosis occurred in every district and every big city of the Pavlodar region where bovine or small ruminant brucellosis were reported. This confirms the role of animals as the source of brucellosis in humans.

Brucellosis is common in many countries, especially in the areas with developed livestock production and insufficiently strict sanitary control. Its highest incidence is reported in the countries of the Mediterranean, Middle East, Central Asia, Africa and Latin America [10-14]. The World Health Organization (WHO) has included brucellosis in the list of zoonotic diseases that have serious implications for public health. According to WHO, this infection has been found in more than 170 countries in the past decade, with up to 500.000 confirmed cases of human brucellosis per year [15].

Today, the outbreaks of human brucellosis are most often reported in Central Asia, including the Republic of Kazakhstan [16-19]. New sites of brucellosis are emerging continuously, and more animals and humans are contracting the disease. Therefore, research of its sources and transmission routes should be a priority for human and veterinary medicine in Kazakhstan.

Kazakhstan is among the twenty-five countries with the highest incidence of brucellosis in the human population [20]. The high incidence of this infection is also reported by Kazakhstan's neighbors, including Iraq, Tajikistan, Saudi Arabia, Iran, and Kyrgyzstan [21].

Brucellosis transmission to humans largely occurs through the alimentary and airborne routes. In most countries, human brucellosis is contracted through the consumption of undercooked meat and unpasteurized dairy products. Extensive development of pastoral farming, inadequate approaches to sanitation and hygiene and poor food safety practices at smallholder livestock farms and markets promote the disease.

The problem of animal brucellosis in Kazakhstan has been vastly addressed by Kazakhstani scientists [22-27].

The incidence of bovine brucellosis is growing in West Kazakhstan, the Karaganda and Pavlodar regions [28]. There is an increase in the incidence of small ruminant brucellosis in the Kostanay, Zhambyl and Almaty regions. The lowest incidence rate is observed in the Mangistau region. The causative agent of bovine brucellosis B. abortus has been isolated in more than 90% of the samples collected in the northern regions of Kazakhstan, whereas the causative agent of small ruminant brucellosis B. melitensis has been found in the southeast of the country [29].

Despite the efforts to eliminate brucellosis in the past 80 years, there is a lack of comprehensive epizootic surveillance studies. The scarcity of data hinders the understanding of the dynamics of brucellosis incidence among both animals and humans.

The aim of this paper was to conduct the epizootiological surveillance of animal brucellosis, analyze the results of mass serological testing and the risks of spread of the disease, thereby contributing to the elaboration of scientifically sound anti-epizootic measures that ensure a rapid response to the threat and prevent its spread.

We have identified 5 districts and cities, which make up 38.5% of the region's territory, with high incidence of bovine brucellosis in 2019–2023, 4 districts with moderate incidence (30.7% of the territory) and 4 districts with low incidence (30.7% of the territory). Not a single district of the Pavlodar region was free from bovine brucellosis during the entire five-year surveillance period.

Three districts that comprise 23.1% of the region's territory have been identified as having high incidence of small ruminant brucellosis, 2 districts (15.4% of the territory) as having moderate incidence and 4 districts (30.7% of the territory) as having low incidence of the disease. Four districts (30.7% of the territory) represented an epizootically safe zone.

The analysis of associations between the incidence of brucellosis among humans and its incidence in livestock in 2019–2023 reveals that there were no reports of epizootic sites of bovine or small ruminant brucellosis in the region in 2022. However, serological testing conducted at the region's veterinary laboratories detected 1.874 seropositive head of cattle and 127 seropositive head of small ruminants. In 2022, 37 persons contracted the infection; its incidence per 100. 000 human population was as high as 5.0.

In 2019-2022, no epizootic sites of small ruminant brucellosis were reported in the Pavlodar region (Table 1), and yet routine serological testing conducted by RVL detected 10,176 seropositive head of cattle (Table 4) and 295 seropositive head of small ruminants (Table 7). The average incidence rate of brucellosis among humans during that period ranged from 2.0 to 5.0 per 100.000 population; there were 102 new cases of human brucellosis, which accounts for 76.7% of all cases (133) in the human population during the five-year surveillance period.

This suggests that sites where seropositive animals are detected are not always reported officially, so farms with infected animals are falsely considered safe. Confusion and inconsistency with the results of epizootic surveillance obscure the real situation in the region.

The analysis of data acquired through the epizootic surveillance of bovine and small ruminant brucellosis in rural areas suggests that the most significant factors contributing to the persistence of brucellosis are:

- incomplete screening coverage of livestock population;
- non-compliance with the guidelines on the isolation of seropositive animals: delayed separation from the herd and delayed transportation to a slaughter facility;
- promotion of interspecies contact through co-housing;
- poor control over animal movement and migration within farms and districts;
- restrictions are not always imposed on the affected farms in spite of the substantial number of seropositive animals;
- unwillingness to report abortions and stillbirths in the herd and contact veterinary laboratories for further diagnostics to determine the underlying cause;
- lack of administrative control of immunization programs and poor record keeping of vaccinations
- inadequate implementation of administrative, sanitary and veterinary containment measures at the sites of brucellosis outbreaks, etc.

These and other factors provide a conducive environment to brucellosis persistence on many livestock farms and obstruct the effective elimination of the disease.

Thus, considering the current situation with brucellosis in Kazakhstan, epizootic surveillance should be continued to estimate the spread of brucellosis and the intensity of the infectious process and assess the risks and factors promoting its spread. Future research should focus on the analysis of brucellosis control and prevention measures and their effectiveness.

Conclusion

Brucellosis is a zoonotic infection that frequently occurs across the Pavlodar region. In 2019–2023, 115 ES were reported there, including 48 sites (47%) of brucellosis, which suggests that brucellosis is a leading zoonotic infection in the region.

In 2019–2023, 13.039 infected head of cattle and 540 infected head of small ruminants were detected in the region. High cattle morbidity was observed in the Bayanaulsky, Akkulinsky, Pavlodarsky and Zhelezinsky districts, in the cities of Pavlodar and Ekibastuz.

The comparative analysis demonstrates that human brucellosis occurs in every district and big city of the region where bovine or small ruminant brucellosis is registered. This suggests the role of farm animals as a source of brucellosis infection in the human population.

The most significant factors contributing to the persistence of brucellosis among animals in the Pavlodar region are: inadequate implementation of administrative, sanitary and veterinary containment measures at the sites of brucellosis outbreaks; deliberate unreporting of abortions and stillbirths in the herd that, therefore, cannot be further investigated by veterinary laboratories to determine their underlying cause; restrictions are not always imposed on the affected farms in spite of the substantial number of seropositive animals; non-compliance with the guidelines on the isolation of seropositive animals, delayed separation of sick animals from the herd and delayed transportation to a slaughter facility, etc. These and other factors provide a conducive environment to brucellosis persistence on many livestock farms and obstruct the effective elimination of the disease.

The epizootic surveillance carried out in the past 5 years allowed us to identify epizootically safe zones in the Pavlodar region, as well as areas with high, moderate and low brucellosis incidence where appropriate anti-epizootic measures will be implemented in the future.

The study shows that timely epizootic surveillance of animal brucellosis and the analysis of the acquired data, including the results of diagnostic tests and the spread of the disease, facilitate effective control of the infection in areas with different epizootic status and can be used to elaborate a methodologically sound strategy for implementing adequate interventions.

Authors' Contributions

AA, GB and AB: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. YSh, AA, GK and BO: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript".

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UDC 619. 591.69.616.99 **Research article**

On the distribution of botfly diseases of horses and camels in the Atyrau and Kyzylorda regions of the Republic of Kazakhstan

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Abstract

Background and Aim. Botfly larvae parasitize horses and camels, adversely affects animal health and productivity, and can cause death. This raises a need for systematic control of some botfly species. Botfly parasitism causes significant economic damage in Western Kazakhstan, where camel and horse breeding are prominent.

Materials and Methods. The study covered the Kazaly and Aral districts (Kyzylorda region), and the Makhambet, Isatay, Kurmangazy districts (Atyrau region). Field research was conducted in the Kyzylorda region in May and September, 2024, and in the Atyrau region in June, July and September. During the study, a total of 25 camels and 23 horses were examined in the Kyzylorda region; 41 camels and 37 horses were examined in the Atyrau region. The ante-mortem diagnoses of camel *cephalopinosis* and horse *rhinoestrosis* were established endoscopically.

Results. Botfly larvae were found in the nasal passages of the examined camels and horses and in the stomachs of the horses. In the Kyzylorda region, no *cephalopinosis* cases were detected among camels in the spring; however, the prevalence increased to 36% in the autumn. In the Atyrau region, there were no cases of *cephalopinosis* in July, but its prevalence rose to 17.1% in the autumn.

No *rhinoestrosis* cases were detected in the Kyzylorda region. In the Atyrau region, there were no occurrences of the disease in June; however, 9.5% of horses already had *rhinoestrosis* in September.

The prevalence of *gasterophilosis* among horses of the Kyzylorda region remained at 33.3% in the spring and autumn. In the Atyrau region, the prevalence of *gasterophilosis* was 20% in the spring, rising to 100% in the autumn.

Conclusion. *Cephalopina titillator* larvae were found in camels in September. Rhinoestrus sp. larvae were detected in horses in September, while *Gasterophilus intestinalis* was observed in May, June and September. Adult botflies were active in September.

Keywords: botflies; parasites; *Equidae; Camelidae; gasterophilosis; cephalopinosis; rhinoestrosis.*

Introduction

Parasitic infections caused by botflies hinder the development of livestock production in the Republic of Kazakhstan and remain a serious concern. Despite all efforts, new cases of botfly infections among livestock are reported every year in many countries, including those that share a border with Kazakhstan and those that are geographically distant. This causes substantial economic damage to the agricultural sector [1, 2].

As long as there is a persisting threat to the biosecurity of the country and complete eradication of parasitic infections is not achieved, it is crucial to develop innovative methods for the identification of invasive pathogens, study their biological and phenological development in different regions and climate zones, and elaborate effective measures of prevention and control.

The most common parasitic infections affecting large livestock are *rhinoestrosis* and *gasterophilosis* of horses and *cephalopinosis* of camels [3-7].

Botflies belong to the infraorder Muscomorpha within the order Diptera. According to the literature, there are 30 genera and 176 species of botflies [8]. Most of these parasites are associated with mammals. There are 3 major botfly families: *Hypodermatidae, Gasterophilidae* and *Oestridae.* The larvae of *Hypodermatidae* infest the subcutaneous tissue of rodents, lagomorphs and ungulates; *Gasterophilidae* inhabit the digestive tract of equids and rhinoceroses. *Oestridae* infest the upper respiratory tract and frontal sinuses of even-toed and odd-toed ungulates. Botfly myiasis can occasionally occur in livestock handlers [9]. All of the three botfly families are common in Kazakhstan, where livestock production is historically widespread and farming is currently on the rise; they pose a significant problem to the industry that needs to be addressed comprehensively to improve the existing measures of prevention and control. Considering that botfly populations can increase rapidly under conducive conditions, e.g. favorable weather and climate or the absence of preventive livestock treatments, complete eradication of livestock diseases caused by botflies is not feasible. However, timely prevention, including treatments of the farm premises with insecticides, body checks etc., can significantly reduce the parasitic burden in domesticated animals.

Cephalopinosis is a parasitic disease of camels caused by *Cephalopina titillator* (*Clark*, 1816) from the *Oestrinae* subfamily of *Oestridae*. Its larvae infest the nasal cavity, nasopharynx, ethmoidal labyrinths, and laryngeal walls. The infestation presents as rhinitis and laryngotracheitis. The products of *C. titillator* larvae metabolism and secondary infections often exacerbate the condition of the affected animals; in severe cases, asphyxiation and death can ensue [10]. Studies have confirmed the high prevalence of this infection among camels across Asia, highlighting the importance of its prevention and control for modern camel breeding [11, 12]. The disease is most frequently reported in CIS countries, China, and Iraq [13, 14, 15].

Rhinoestrosis is a chronic disease of horses caused by the invasion of nasopharyngeal botfly larvae into the nasal cavity and the adjacent structures. The causative agents of *rhinoestrosis* are 3 species of the nasopharyngeal botflies from the genus *Rhinoestrus* that represent the *Oestrinae* subfamily of *Oestridae: Rh. purpureus, Rh. latifrons* and *Rh. usbekistanicus.* The larvae migrate to the ethmoidal labyrinths and frontal sinuses, feeding on the inflammatory products of the mucous membranes [10].

Horses infested with *Rhinoestrus spp.* suffer from persistent rhinitis; their nasal discharge often contains traces of blood. The nasal mucosa appears scarred and ulcerated. The animals look emaciated, have labored breathing, develop a neurological disorder and can die in severe cases. Necropsy typically reveals mucosal ulceration and redness at the affected sites, with pus buildup and larvae deposits at the lesion's base [16, 17].

Gastrophilosis is a parasitic disease of equids (*Equidae*) caused by the larvae of stomach botflies (*Gastrophilidae*). It typically occurs during the summer grazing period when botflies are active. Coinfection caused by other species of this parasite is common in horses (*Equus ferus caballus*) and donkeys (*Equus asinus*). After a three-week developmental period in the mouth, bot fly larvae migrate and attach themselves to the mucus lining of the horse's stomach and remain there during the winter. After about 10 months, they detach from the lining and are passed out of the body through the feces. The larvae burrow into the ground and mature. Depending on the conditions, adults emerge in three to 10 weeks. Adult females deposit eggs on the horse's legs, shoulders, chin, throat and lips. Depending on geographic location, the life cycle of bot flies is not fixed to only certain times of the year, and bot larvae can be active in horses in warm periods of the year.

In severe cases, infestation disrupts the motor and secretory functions of the equine gastrointestinal tract. Damage to the oral cavity is also common: the larvae feed on the oral mucosa and submucosa, which leads to the ulceration of the inner cheek, soft palate, and tongue. Infestation of the stomach and the duodenum causes tissue damage, swelling and mucosal inflammation, which may lead to stomach or intestinal wall rupture. The products of larval metabolism enter the bloodstream, causing intoxication,

weakness and digestive tract disorders. The causative agents of gastrophilosis are *Gastrophilus intestinalis, G. veterinus, G. haemorhoidalis, G. pecorum, G. inermis, G. nigricornis, G. magnicornis,* and *G. flavipes,* which mainly parasitizes donkeys [18].

In Eurasia, the greatest diversity of *Gasterophilus* species is observed in China, where seven species are known. Among them, G. pecorum is the most common, followed by *G. noselis, G. nigricornis, G. intestinalis, G. haemorrhoidalis,* and *G. inermis* [19]. The most common *Gasterophilus* species that parasitizes horses is *G. intestinalis* [20].

The zone of greatest distribution of camels and horses in Kazakhstan is Mangystau, Turkistan, Kyzylorda and Atyrau regions. According to the data for 2024, the largest number of camels inhabits Atyrau and Kyzylorda regions. After the dissolution of the Soviet Union, the first study of botfly myiases in Central Asia was conducted by *B.Ibraev* et al. in Kazakhstan; he reported the high prevalence of *G. intestinalis, G. nasalis* and *G. pecorum i*n the northern and central parts of the country, in Kostanay, Akmola and Karagandy regions. Their bots cause a variety of pathologic conditions in horses, including gastritis and digestive disorders [21]. *G. inermis, G. intestinalis* and *G. noselis* have been reported in Iran [22]; *G. intestinalis, G. haemorrhoidalis* and *G. noselis* occur in Turkey [23]. In other Asian countries, the diversity of botfly species is lower. So far, only *G. intestinalis* and *G. noselis* have been reported in Belarus [24] and *G. intestinalis* in Yakutia [25]. In light of the above, in the western regions of Kazakhstan, where infestations by botflies inflicts significant damage to camel and horse breeding, there is an urgent need to develop a scientifically grounded system of measures to control botflies. To prevent the substantial economic losses they cause, it is essential to determine the species composition of botflies in the studied region. The aim of our research is to investigate the biological and ecological characteristics of botflies whose larvae cause infestations in camels and horses in the Atyrau and Kyzylorda regions.

Materials and Methods

Ethical approvals

The study was conducted with written consent from the animal owners, in accordance with local regulations for the keeping of farm animals. All procedures complied with EU Directive 2010/63/EU on animal experimentation. Protocols of parasitological research design and standard operating procedures were approved by the Bioethics Commission of "Kazakh Scientific Research Veterinary Institute" LLP (conclusion dated 30.01.2021).

The study was conducted in the Aral and Kazaly districts of the Kyzylorda region in May through September, 2024, and in the Makhambet, Kurmangazy and Isatay districts of the Atyrau region in June and July, 2024. A total of 48 animals (25 camels and 23 horses) from 5 livestock farms of the Kyzylorda region and 78 animals (41 camels and 37 horses) from the Atyrau region were examined. The presence of botfly larvae was determined by the visual examination of the head, nodules, nose, nasal passages); prior to the examination, the animals were immobilized. Subsequently, the frequency index of the infection (percentage of the infected animals) and its abundance index (the average number of larvae per host) were calculated. The nasopharynx and the gastrointestinal tract were examined using an endoscopy system with a real-time video camera. The acquired image was displayed on the screen of a mobile phone with an installed Endoscope Finder application.

When examining the nasal passages of live animals using an endoscope, it was not possible to accurately count the number of detected larvae.

Adult botflies flying near the animals were collected for further analysis using a sweep net.

The collected adult botflies were placed in a killing jar containing a cotton pad soaked with ethyl acetate [26].

The presence of *Gastrophilus* was assessed by the physical examination of the animals, including their coat and skin. Egg clusters were collected by clipping horse hairs with the attached eggs.

To assess the severity of botfly infestation, horse stomachs were collected at the Atyrau Et Ortalygy slaughterhouse of Atyrau, the Kurmangazy service and procurement center in the village of Zhumeken (the Kurmangazy district), a slaughterhouse in the Makhambet district, and a meat processing plant in Aral city. Additionally, the mucosa of the nasal cavities and frontal sinuses was examined for the presence of botfly larvae (Figure 1 D). Specimens collected from each animal were placed in separate screw-cap tubes, labeled and stored in special containers until further transportation to the laboratory.

GPS coordinates of the collection sites were recorded using GPS navigators; GIS maps were created using the ArcMap software [28]. All collected specimens of eggs, larvae, pupae, and adult botflies were studied in the laboratory. Species of the collected botflies were identified using identification keys [9, 10, 18, 27]. Species identification was conducted using a stereomicroscope at the Parasitology Laboratory of "Kazakh Scientific Research Veterinary Institute" LLP.

Results and Discussion

Camels and horses of the Kyzylorda and Atyrau regions have been examined for the infestation of botfly larvae (Figure 1).

Figure 1 – Map showing examination and sampling locations in the Atyrau and Kyzylorda regions

Cephalopinosis. Ten camels were examined for cephalopinosis in the Aral and Kazaly districts of the Kyzylorda region in the second half of May; no botfly larvae were detected in the examined animals (Table 1).

Table 1 – Results of ante-mortem examination of camels for the presence of botfly larvae in the Atyrau and Kyzylorda regions

Study area	Month of research	Number of examined animals	Number of infested animals	Occurrence index, %	Type of parasite
Kyzylorda region	May	10	-	-	-
	September	15	9	60	*C. titillator*
Total		25	9	36	
Atyrau region	June- July	25	-	-	-
	September	16	7	43.75	*C. titillator*
Total		41	7	17.07	

In the second half of September, another 10 camels were examined. The live larvae of *C. titillator* were detected endoscopically in 4 camels in the village of Bogen in the Aral district; the prevalence index was 40% (Figure 2A). Another 5 *C. titillator* larvae were detected in 5 camels in the Kazaly district and the Zholdybai area of the Kumzhiek rural district; the prevalence index was 100% (Figure 2B).

In the Atyrau region, animal body checks were conducted in the Makhambet, Kurmangazy, and Isatay districts of the Atyrau region in the third decade of June and the first decade of July; no signs of botfly infestation were observed. In the first and second decades of September, 5 camels were examined. During the endoscopic examination, live larvae of *C. titillator* were detected in one animal (prevalence

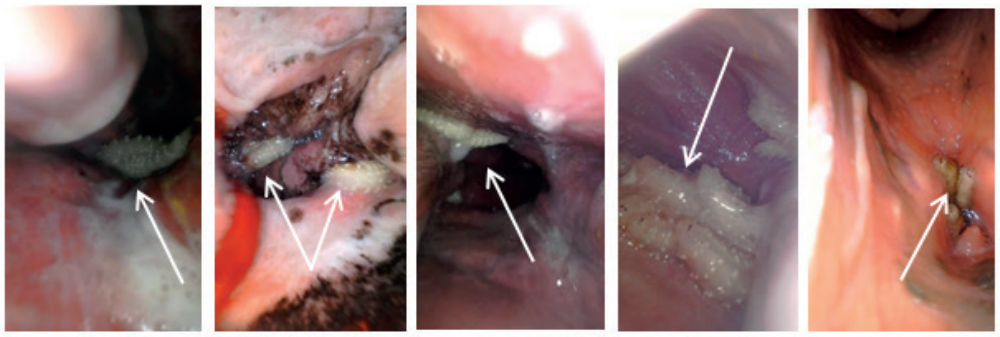
index: 20%) in the Zhanbay village of the Isatay district (Figure 2C). In the Zhylandy village of the Kurmangazy district, *C. titillator* larvae were found in 6 out of 11 examined camels (prevalence index: 54.5%) (Figure 2 D).

Rhinoestrosis. Ten horses were examined endoscopically for rhinoestrosis in the Aral and Kazaly districts of the Kyzylorda region in the second half of May; another 4 horses were examined in the second half of September. No botfly larvae were detected (Table 2).

Table 2 – Results of ante-mortem examination of horses for the presence of botfly larvae in Atyrau and Kyzylorda regions

Study area	Month of research	Number of examined animals	Number of infested animals	Occurrence index, %	Type of parasite
Kyzylorda region	May	10	-	-	-
	September	4	-	-	-
Total		14	-	-	-
Atyrau region	June- July	2	-		*Rhinoestrosis sp.*
	September	19	2		*Rhinoestrosis sp.*
Total		21	2	9,5	*Rhinoestrosis sp.*

Between the first decade of June and the second decade of July, 2 horses were examined for rhinoestrosis in the Atyrau region. In the first half of September, 5 horses were examined endoscopically at the meat processing plant in the city of Atyrau. No botfly larvae were detected in the nasal passages of the animals. In the Zhylandy village of the Kurmangazy district, 14 horses were examined for rhinoestrosis; live *Rhinoestrosis sp.* larvae were found in 2 animals (prevalence index: 14.3%) (Figure 2 E).



A-D – *C. titillator* larvae in the nasal passages of camels,
E – *C. titillator* larvae in the nasal passages of horses
Figure 2 – Nasopharyngeal botfly larvae in the nasal passages of camels and horses

Gastrophilosis. Examinations were conducted in the Aral and Kazaly districts of the Kyzylorda region and in the Isatay, Kurmangazy, and Makhambet districts of the Atyrau region.

Table 3 – Results of post-mortem examination of horses for the presence of botfly larvae in Atyrau and Kyzylorda regions

Study area	Month of research	Number of examined animals	Number of infested animals	Occurrence index, %	Abundance index, spec.	Type of parasite
Kyzylorda region	May	6	2	33.3	778	*Gastrophilus sp.*
	September	3	1	33.3	253	*Gastrophilus sp.*
Total		9	3	33,3	1031	*Gastrophilus sp.*

Continuation of table 3

Atyrau region	June- July	10	2	20	1780	*Gastrophilus sp.*
	September	6	6	100	367	*Gastrophilus sp.*
Total		16	8	50	2147	*Gastrophilus sp.*

In the second half of May, 5 horses were examined in the Aral and Kazaly districts of the Kyzylorda region. Sixteen specimens of third-instar larvae of *Gastrophilus sp.* were found in the pharynx of one horse. Thus, the intensity index was 3.2, and prevalence index was 20%.

To assess the potential degree of infestation in the areas included in the study, horse stomachs were collected at the meat processing plant of Aral city. A total of 762 specimens of *Gastrophilus sp. l*arvae were collected from the stomach of one horse. This suggests that infestation by botfly larvae might be quite significant in some areas. In the second half of September, two horses were examined; no bots were found in their pharynx. However, during the dissection of a horse's stomach at the Aral meat processing plant, 253 gastric botfly larvae were detected.

In the Atyrau region, 10 horses were examined at the Makhambet slaughterhouse starting from the first decade of June to the second decade of July. Infestation with *Gastrophilus sp.* was detected in 2 horses. A total of 1,708 2nd and 3rd instar larvae were collected (Figure 3). The intensity index was 178 specimens, and the prevalence index was 20%. In the first half of September, 5 horses were examined at the meat processing plant of Atyrau city. In all examined stomachs of 5 horses, 311 larvae of *Gastrophilus sp*. were found, with an AI of 62.2 specimens. The stomach of another horse transported to the Kurmangazy slaughter from the Zhumeken village of the Kurmangazy district in the second half of September contained 56 Gastrophilus larvae.

A B

A – before larvae removal, B – after larvae removal

Figure 3 – Infestation of a horse stomach by *Gastrophilus sp.* Larvae

In September, we observed congregates of botflies around horse pastures. Female botflies oviposit eggs in the mane, on the medial surface of forelegs, the chest, and the flanks. Twelve adult botflies were collected in the village of Zhanbay in the Isatay district using a sweep net, and another two were collected in the village of Aktogay (the Moinak area) in the Makhambet district.

In the Kazaly district, Kumzhiek, Zholdybai area, 1 botfly was collected. The collection procedure was carried out near the head, neck, and back of the horse, i.e. areas most susceptible to botfly attacks. The number of eggs per horse exceeded 300. The eggs were found in the horse mane, groin, on the shoulders and legs (Figure 4).

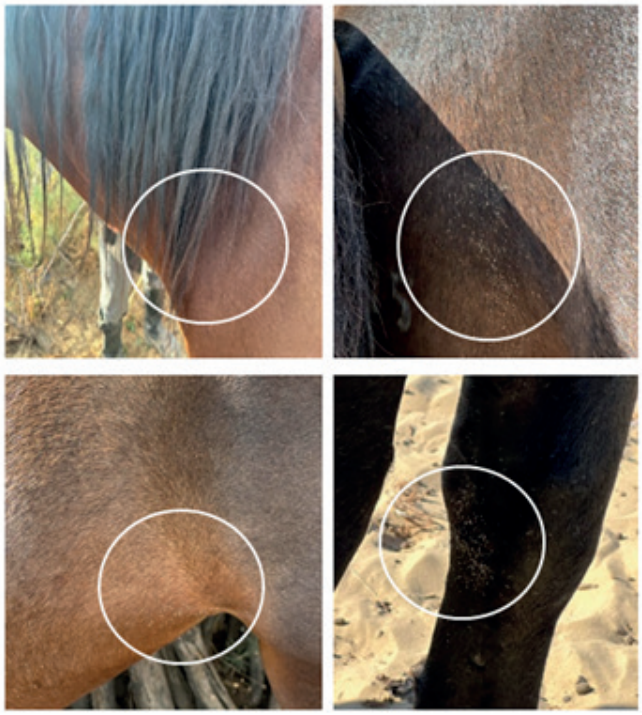
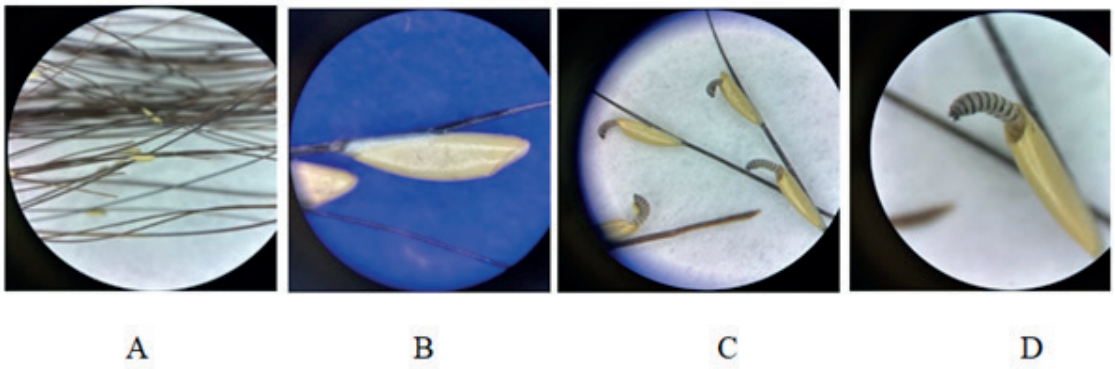


Figure 4 – Sites of *Gasterophilus intestinalis e*gg deposits in horses

The eggs were light and yellowish. They were wedge-shaped, wider at the apex, tapering toward the base, 1.27 mm in length. The attachment region extended only slightly beyond the midpoint of the egg. The operculum was rounded and egg-shaped (Figure 5 A, B). The eggs were kept in the laboratory at room temperature; 20-25 days later, they hatched into first-instar larvae measuring 1.05 to 1.10 mm in length (Figure 5 C, D). In the eggs collected after a drop in air temperature, the larvae were dead.



A, B – eggs; C, D – first-instar larvae

Figure 5 – *Gasterophilus intestinalis* eggs and larvae under microscope

Identification of adult flies and parasitic larvae was conducted in the laboratory using stereoscopic microscopy based on morphological characteristics, with the aid of identification keys. We examined 3,106 larvae and 15 adult specimens.

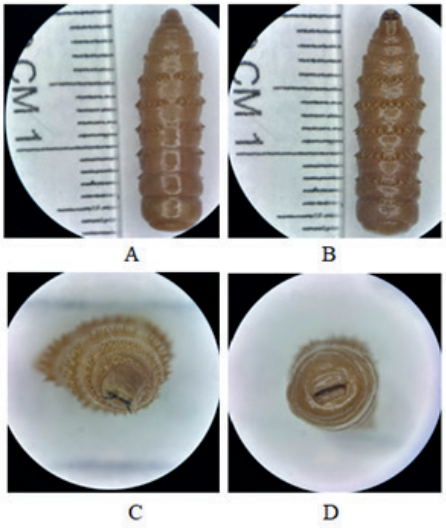
All larvae extracted from the stomachs of horses had a bullet-shaped body, with a pointed anterior end and a broad posterior end, measuring 16-17 mm in length. Unlike larvae of other botflies from the family Hypodermatidae, the larvae we found (2nd and 3rd instars) possessed mouth hooks (Figure 6 B, C). Additionally, in contrast to larvae of the family Oestridae, there was a well-developed median spine between the mouth hooks.

In *Gasterophilus intestinalis* larvae parasitizing horses, two rows of small, forward-pointing hooks are present beneath the sensory organ in the pseudocephalon (Figure 6). Second- and third-instar larvae

typically attach to the non-glandular part of the stomach mucosa, near the junction with the esophagus. These larvae remain immobile for 9-12 months.

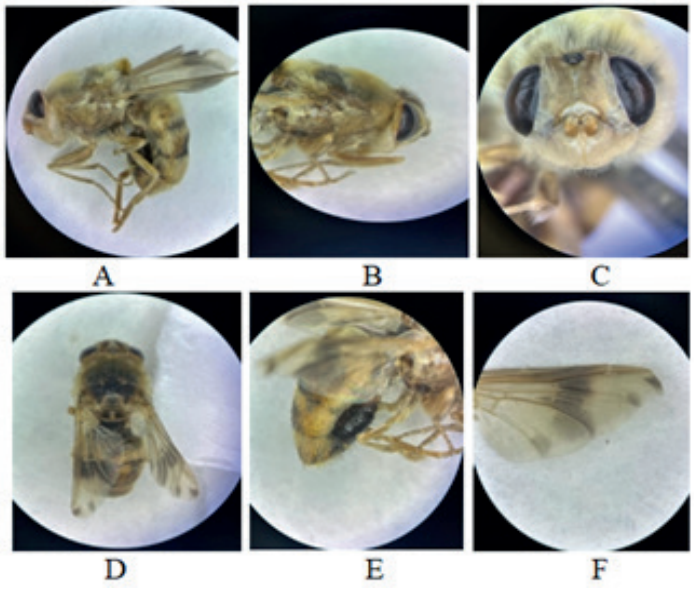
Third-instar larvae are relatively large, ranging from 12.7 to 19.1 mm in length. Both larval stages are adapted to life in the gastrointestinal tract due to their rounded body shape, narrow hook-like mouthparts, and spines – key distinguishing features of *Gasterophilus intestinalis* compared to other botfly larvae of the same genus [9]. Third-instar larvae are characterized by a yellowish coloration.

Based on the combination of these morphological traits, all examined larvae were identified as *Gasterophilus intestinalis* (*De Geer,* 1776) (commonly known as the horse botfly or large stomach botfly).



A – ventral view, B – dorsal view, C – cranial view, D – caudal view
Figure 6 – Second-instar larvae of *G. intestinalis*

All of the 15 collected specimens of botflies belonged to *Gasterophilus intestinalis* (*De Geer*, 1771) (large stomach botfly or horse botfly) (Figure 7). The adult fly typically measures 12 to 15 mm in length, is yellowish-brown, and does not have pronounced sexual dimorphism. It has 9 to 11 mm spotted wings. The botfly has small, non-functional mouthparts. A female's abdomen is elongated with a long ovipositor underneath.



A – habitus, left lateral view, B – head and thorax, C – head, frontal view,
D – habitus, dorsal view, E – abdomen, F – wing
Figure 7 – Female *Gasterophilus intestinalis* under the microscope

Summing up the results, infestation of horses with *Gasterophilus intestinalis* in the Atyrau region occurs not only in autumn but also in spring and summer. In autumn, it begins in the second or third decade of August and the first or second decade of September; it ends by November, or, if the weather is dry and warm, in the first decade of November.

Statistical Analysis of Botfly Larvae Infestation in Camels and Horses Across Atyrau and Kyzylorda Regions. To assess statistically significant differences in botfly larvae infestation rates among camels and horses between Atyrau and Kyzylorda regions, Pearson's chi-square (χ^2) test was employed. The analysis was performed separately for three datasets: clinical examination of camels, clinical examination of horses, and postmortem examination of horses. A two-tailed test was used, with the significance threshold set at $\alpha = 0.05$.

In the first group (camels, clinical examination), 66 animals were examined. Infestation was detected in 9 of 25 animals (36%) in Kyzylorda region and 7 of 41 (17.07%) in Atyrau region. The χ^2 test revealed no statistically significant interregional difference ($\chi^2 = 2.09$; $P = 0.149$), despite the apparent disparity in prevalence.

In the second group (horses, clinical examination), infestation was documented exclusively in Atyrau region (2 cases among 21 animals), with no infected animals detected in Kyzylorda region (0 of 14). The χ^2 value of 0.20 ($P = 0.656$) likewise indicated no significant regional variation.

The third group (horses, postmortem examination) showed infestations in both regions: Kyzylorda (3 of 9 cases) and Atyrau (8 of 16). Here too, the χ^2 test found no statistically significant difference ($\chi^2 = 0.15$; $P = 0.699$).

A supplementary case-control analysis was conducted, treating infestation status as a binary dependent variable and region as an independent factor. For camels, the odds ratio (OR) was 1.53 (95% confidence interval [CI]: 0.61–3.82; $P = 0.364$). While statistically non-significant, this suggests a potential epizootiological trend warranting investigation with larger samples.

The analysis identified no statistically significant interregional differences in infestation rates. However, the observed prevalence values merit attention within epizootiological surveillance frameworks. Future studies will incorporate expanded sample sizes, seasonal stratification, and additional risk factors to refine epidemiological understanding and enhance prevention strategies.

Conclusion

Botfly myiasis of horses and camels has been detected in the Aral and Kazaly districts of the Kyzylorda region and the Isatay, Makhambet, and Kurmangazy districts of the Atyrau region.

In the autumn, the prevalence of cephalopinosis in camels was 36% in the Kyzylorda region and slightly lower (17.1%) in the Atyrau region.

In the Atyrau region, 9.5% of *rhinoestrosis* cases among horses occurred in the autumn.

In the spring, the prevalence of *gastrophilosis* in horses in the Kyzylorda region was 33.3%. In the Atyrau region, it reached 50%.

The high level of botfly infestation in autumn may be associated with free-range intensive grazing, which contributes to the spread of the parasite. These assumptions are based on the high intensity of infestation in individual animals and their overall body condition. It is known that in severe cases of botfly myiasis, animals may stop eating and lose weight. We think that the acquired data does not reflect the full picture of the parasitic burden caused by botflies in camels and horses. Obviously, more animals should be examined, but there are obstacles. First, livestock owners often frown upon the idea of capturing and immobilizing their animals. Second, most of the farm animals graze on free-range pastures, where botfly infestation occurs in the first place. This is indirectly confirmed by our failure to find botfly pupae in the potential pupation substrates on farm premises. In other words, if livestock spent most of their time in enclosures, mature botfly larvae would get into the substrate, where they would be easier to detect than in the open desert or steppe.

Furthermore, the presence of other dipterans, especially flies (*Muscidae*), collected near and from the animals, poses an additional risk of infestation and is an annoyance for the animals. Infestation and fresh lesions induced by the larvae attract other insects, promoting secondary contamination. We think that livestock infestation with botfly larvae can be prevented by 1) treating the skin with chemical or biological insecticides that are non-toxic to animals and humans; 2) applying repellents to coat and skin

to deter adult botflies; 3) conducting regular disinsection procedures in enclosures and farmyards and using pheromone traps for adult botflies.

Summing up, research conducted in the Atyrau and Kyzylorda regions in 2024 revealed the presence of myiasis caused by botfly larvae in camels and horses. Botflies were represented by 3 species: *Cephalopina titillator,* the causative agent of camel cephalopinosis, *Rhinoestrus sp*, the causative agent of equine rhinoestrosis, and *Gastrophilus intestinalis,* the causative agent of equine gastrophilosis. The presence of *Cephalopina titillator* larvae in the nasal cavities of camels was detected in the first and second decades of September. Infestation of horses with *Rhinoestrus sp.* was detected in September in the Atyrau region only, and *Gastrophilus intestinalis* was found in May, June, and September. Flights of adult botflies were observed in September. Based on the findings of larvae at different developmental stages in the nasal cavities of camels (*Cephalopina titillator*), stomachs (*Gastrophilus intestinalis*), and nasal passages (*Rhinoestrus spp*.) of horses, as well as eggs on the horses' hair coat, we can assume that animal infestations in the studied region occur during the active flight period of adult botflies, twice a year - in May and September. Therefore, when preventing botfly larval infestations, it is necessary to consider the flight periods of specific botfly species, larval development, their emergence into the environment, and pupation.

Authors' Contributions

AA: Designed and supervised the study and drafted the manuscript. ZZ, AZh: Statistical analysis and drafted the manuscript. SB, BA, AY: Designed and conducted the study. SK: Conducted the study and drafted the manuscript. EK: Drafted the manuscript and translated. All authors have read, reviewed, and approved the final manuscript.

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Conflicts of Interest

The authors declare that they have no competing interests.

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UDC 619:616.98:579.852.11 **Research article**

Bacteriological monitoring of infectious epididymitis of rams

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Abstract

Background and Aim. Currently, infectious epididymitis of rams (IER) is registered in over 100 countries, including Kazakhstan. The aim of this work is to conduct bacteriological monitoring of infectious epididymitis of rams in the territory of the Republic of Kazakhstan.

Materials and Methods. A total of 1.205 biological samples (907 whole blood samples and 298 tissue specimens) were collected by the Laboratory of Brucellosis from sheep flocks in 17 regions of Kazakhstan. Serological and bacteriological methods were used. Biological properties of the isolated *Brucella* cultures were determined by studying their culture-morphological, tinctorial, biochemical properties, carbon dioxide demand during their growth, ability to excrete hydrogen sulfide, growth on media with dyes - basic fuchsin and thionine, reaction with tripaflavin and R and S sera, thermoagglutination reaction and White-Wilson staining.

Results. As a result of research of 1205 samples of biomaterial 2 cultures of *B.ovis* species were isolated (from one animal of Zhambyl and from the second one of Turkestan regions). Both Brucella strains received strain passports containing descriptions of their phenotypes and genotypes and documentation required for further strain depositing.

Summarizing the results of bacteriological studies with bioassay it can be stated that the study of biological properties of pathogens circulating in the epizootic focus is one of the main links of epizootological control of diseases, allowing to reliably identify sources and reservoirs of infection, to build a scientifically based effective scheme of anti-epizootic measures aimed at preventing infection of humans and animals.

Conclusion. The genus and species affiliation of the isolated brucella cultures to the species *B. ovis* in terms of their biological properties was confirmed by the results of a biological assay on guinea pigs. The results of the conducted bacteriological monitoring indicate the presence of sporadic cases of IER in some economic entities of the Republic of Kazakhstan, which requires increasing the coverage of the studied sheep population during the planned mass diagnostic activities.

Keywords: *B.ovis*; bacteriological study; biosafety; brucella culture; incidence; strain.

Introduction

Despite the conducted anti-epizootic measures, especially dangerous diseases of social and economic importance, such as infectious epididymitis (IEB), *brucellosis,* etc., continue to be registered among animals on the territory of our republic to a greater or lesser extent [1-4].

According to the official data of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan (CVCS of the MoA of the RK), the relative incidence rates of IER in Kazakhstan are quite low, which were equal to 0.008; 0.002 and 0.004% in 2021, 2022 and 2023 respectively.

Thanks to the practical veterinary control of IEB, the main components of which are general organizational and economic, special veterinary and sanitary measures, including diagnostic tests and vaccination of animals, it was possible to achieve a significant reduction in the intensity of the epizootic situation on this disease [5-9].

The above-mentioned indicators of morbidity on IER indicate single cases of infection manifestation on the territories of separate economic entities of the regions of our republic.

However, given the high contagiousness of the causative agent of infectious epididymitis and its pathogenic properties, the nature of the course of the infectious process leading to a decrease in reproductive functions in males, to abortion and stillbirth in ewes, sporadic cases of infection among animals registered annually, it can be stated that the problem of absolute eradication of the circulation of bacteria of the genus *Brucella*, including species ovis on the territory of the Republic of Kazakhstan is still not solved [10-15].

According to the statistical reports on the incidence of OCE in 2021-2023 kindly provided by CVCS, single cases of the disease occurred in the western, northern, eastern and central parts of Kazakhstan, i.e., everywhere across the country except the south.

So far, IER has been reported in over 100 countries, including Kazakhstan.

The fight against IER, both in Kazakhstan and worldwide, is based on the detection of sick animals through diagnostic tests and their timely isolation, followed by a set of veterinary and sanitary measures.

At the same time, prolonged complement fixation reaction (PCFR) is the only serologic test officially regulated by the veterinary legislation of the Republic of Kazakhstan for detection of animals with IER among cattle.

To check the epizootic state of economic entities on this disease in our country before the beginning of the breeding campaign, clinical and serological studies of all rams-producers in breeding farms and companies where artificial insemination of animals is carried out. Breeding males intended for sale are also subjected to mandatory control tests for IER disease. The remaining sheep stock is serologically tested twice in 1 and 2 months after calving, as well as once in 2-4 weeks before mating and artificial insemination.

Those positively reacting according to the results of serological tests are recognized as sick and slaughtered. In case of detection of rams diseased with IER, the economic entity is declared unfavorable and restrictions are imposed. At the same time, it is prohibited to transfer animals from the unfavorable flock to other flocks and farms.

The pathogenicity of a circulating infectious agent affects the dynamics of the infection in the epizootic focus. In light of this, it is important to do a bacterial culture test on all the specimens collected from sick animals in order to isolate a pure culture of the causative agent, make a correct diagnosis, and develop an adequate prevention and control plan.

Materials and Methods

A total of 907 ovine whole blood samples and 298 tissue specimens (fragments of ovine parenchymal organs, lymph nodes, aborted fetuses, testicles and epididymides, etc.) were collected by the Laboratory on livestock farms and at a few slaughterhouses that deal with highly dangerous pathogens. The samples came from 17 regions of Kazakhstan; most of the whole blood samples were from the Zhambyl, Turkestan and Zhetysu regions, where sheep raising is prominent and the small ruminant population is quite significant.

IER surveillance conducted by the Laboratory of Brucellosis of Kazakh Scientific Research Veterinary Institute included serological and bacterial culture testing.

The specimens were collected on the farms that were deemed epizootically safe and on the farms with complement fixation reaction (PCFR)-positive animals.

All laboratory tests were carried out in compliance with the Veterinary Law of the Republic of Kazakhstan (2005) and the Interstate Standard GOST 34105-2023 of Armenia, Kazakhstan, Belarus, Kyrgyzstan, and Russia (2023) [16, 17].

The biological characteristics of the isolated Brucella cultures were determined by studying their cultural, morphological, tinctorial and biochemical properties; their need for CO2 for growth; ability to produce hydrogen sulfide; sensitivity to basic fuchsin and thionine dyes; agglutination with trypaflavine; agglutination with anti-R/anti-S sera; heat agglutination, and White & Wilson staining with crystal violet.

Results and Discussion

In Kazakhstan, bacterial culture tests are used in addition to serological testing to improve the accuracy of the definitive diagnosis and more effectively control the incidence and spread of brucellosis and OCE.

In our study, bacterial culture tests were performed to detect and identify the causative agent. Tissue smears were examined for *B. ovis* under the microscope. The obtained whole blood and tissue samples were plated onto solid and liquid culture media (meat-peptone-liver-glucose-glycerol broth (MPLGGB) and meat-peptone-liver-glucose-glycerol agar (MPLGGA) supplemented with 10% serum), and incubated in a heating block. The cultures were observed for 30 days.

From a total of 1,205 biological samples (907 whole blood samples and 298 tissue specimens from 57 sheep), two *B. ovis* cultures were isolated: one from the Zhambyl region (1 animal) and an-other from the Turkestan region (1 animal).

The epizootic map in Fig.1 shows sample collection sites (black dots) and local farms in the Zhambyl and Turkestan regions where two isolated *B. ovis* cultures came from (red dots). Notably, these regions have been considered epizootically safe for the past few years, according to the statistical reports by CVCS.

A possible explanation is that the Republican Veterinary Laboratory does not have every flock in the country serologically tested for IER every year. Breeding farms where breeding rams are kept undergo serological testing more often than other livestock producers. So, we hypothesize that due to low coverage of the sheep population by IER testing, the source of the infection (a sick animal) remains in the flock, transmitting it to other animals and promoting its spread to other areas. The dangers of IER are underestimated: this pathogen causes abortions and deaths in ewes and necessitates premature culling and slaughtering of breeding rams. IER contributes to infectious pathology, preventing the growth of sheep population and restraining the intensive development of sheep raising, one of the important sectors of Kazakhstan’s economy.

Results of Geographical sites of sample collection for IER testing and *B. ovis* isolation presented in Fig. 1.

Fig. 1 shows that *B. ovis* cultures were isolated from the specimens from the Zhambyl and Turkestan regions in the south of Kazakhstan. One of the cultures was isolated from the whole blood of a ram from Tuimekent, a village in the Bayzak district of the Zhambyl region. The Tuimekent farm was the only one out of 5 farms inspected for IER where PCFR-positive animals were detected and morbidity was quite high (2.5%). No infected animals were detected on other 4 farms in the region. The presence of *B. ovis* infection was further confirmed by bacterial culture tests.

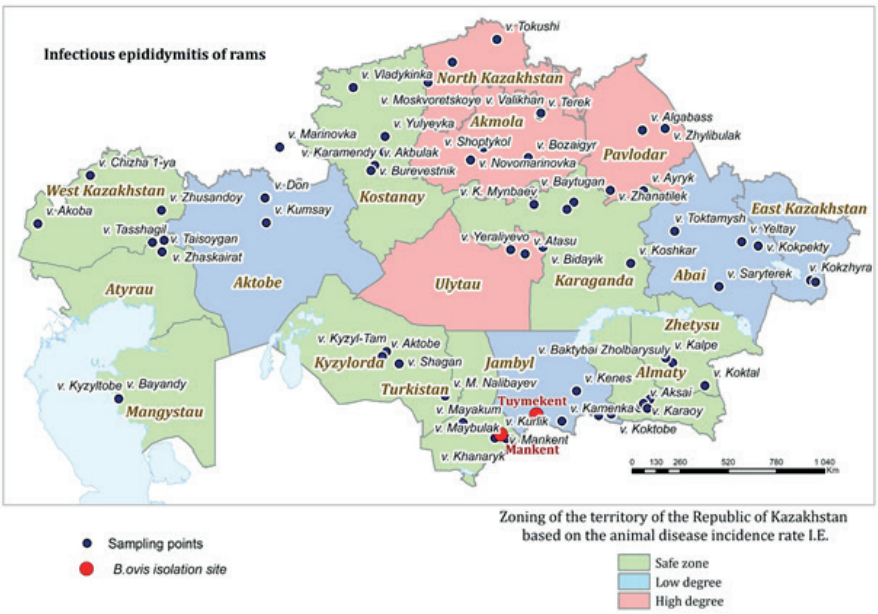

Figure 1 – Geographical sites of sample collection for IER testing and *B. ovis* isolation

Another *B. ovis* culture was isolated from the testicles of a ram slaughtered due to suspected IER at the DS-Brothers slaughterhouse (Mankent, the Sayram district of the Turkestan region). The antemortem PCFR was positive and the antemortem physical examination revealed clear signs of chronic IER: fibrous overgrowth in the testis and the enlarged, lumpy epididymis. The cut surface revealed multiple variously sized inflammation sites filled with greenish, creamy caseous material. Figure 2 - shows the testis of the slaughtered ram with suspected IER.

Figure 2 – Clinical signs of IER in a breeding ram from the Turkestan region

Typical clinical signs of the infection (enlarged scrotum) shown in Fig.2 suggest epididymitis caused by *B. ovis*.

Identification of a breeding ram infected with OCE is important for predicting the spread of the infection in the flock where the animal is used to serve healthy ewes and in other flocks in the neighborhood: in rural areas flocks from different farms often share grazing grounds and water sources and thus can come in contact with each other.

In such cases we recommend conducting repeated large-scale serological testing of all the flocks in the area once every 20-25 days until two subsequent negative results are achieved. This strategy will help to detect both chronically and newly infected animals and improve the epizootic status of the farm.

Phenotypical analysis of the isolated Brucella cultures included their identification to the species level. Their cultural, morphological, tinctorial, biochemical and antigenic properties were studied using conventional methods: description of colony morphology, microscopy of Gram-stained samples, slide serum agglutination tests (with monospecific anti-Brucella abortus and anti-Brucella melitensis sera and anti-R/anti-S sera), trypaflavine agglutination test, heat agglutination test, and White & Wil-son staining.

We found that isolated *B. ovis* cultures grew well in slanted MPLGGA tubes in a heating block at 37-38 ºC in a CO_2-containing atmosphere and in MPLGGB supplemented with 10% of blood serum, pH 7.0-7.2. The colonies were small or medium in size, not very convex, measuring 0.2-3.0 mm in diameter, grayish-white or amber in color, appearing transparent in transmitted light (Figure 3,4).

Under the microscope, the colonies appeared as small, short, non-motile rods or coccobacilli that did not form spores or capsules. The colonies were Gram-negative but stained red with safranin (Kozlovsky staining). The growth of *B. ovis* cultures was observed for the samples collected from two rams (one PCFR-positive animal and another PCFR-positive animal with clinical signs of the infection). The growing coccobacilli did not differ in size or morphology from other Brucella species, had a rough phenotype and tested positively in the heat agglutination and trypaflavine agglutination tests (1:500).

Figure 3 and 4 show growth of *B. ovis* as well separated individual colonies transparent in transmitted light and as dense bands formed by actively growing coalescing colonies.

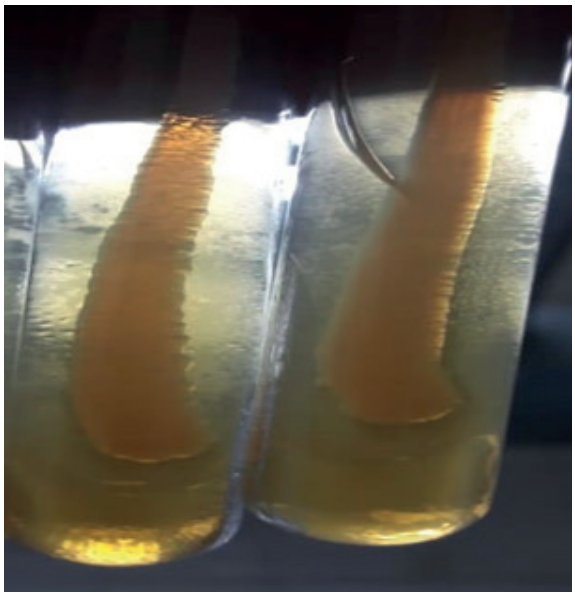

Figure 3 – Active growth of *B. ovis* isolated from the testicles of an infected ram (the Turkestan region) in MPLGGA tubes in the Laboratory of Brucellosis of Kazakh Scientific Research Veterinary Institute

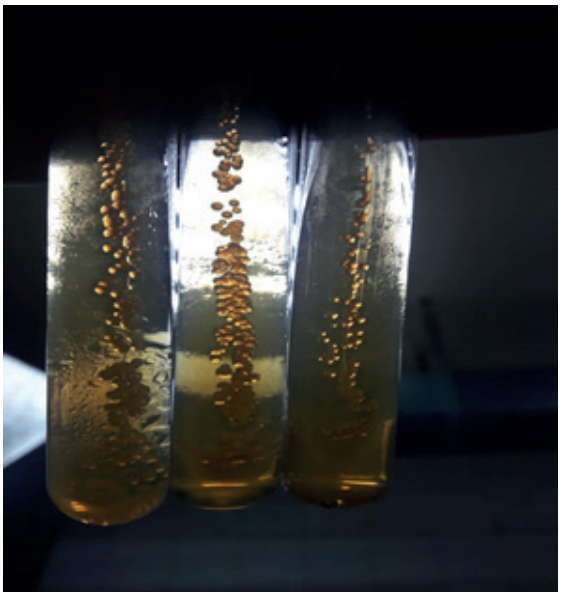

Figure 4 – Growth of *B. ovis* isolated from the whole blood of an infected ram (the Zhambyl region) in the Laboratory of Brucellosis of Kazakh Scientific Research Veterinary Institute

The characteristics of the studied cultures are provided in Table 1.

Table 1 – Phenotypic characteristics of epizootic cultures of B. ovis isolated from biomaterial samples collected from ram

Culture	Slide agglutination test				V-B staining	Heat agglutination	Need for CO_2	Production of H_2S (mm)	Brucella growth in culture media containing:		Agglutination with monospecific sera		Growth in culture media		Catalase	Oxidase
	S =	S #	R+	Trypaflavine					Fuchsine	Thionine						
									1:50000 - 1:100000	1:25000 -50000-1:100000	antiabortus	antimelitensis	MPLGGB	MPLGGA		
B. ovis 1	-	-	+	+	+ R 100%	+	+	-	-	+	-	-	UO	Typ.	+	-
B. ovis 2	-	-	+	+	+ R 100%	+	+	-	-	+	-	-	UO	Typ.	+	-
Control *B.ovis* 63/290	-	-	+	+	+ R 100%	+	+	-	-	+	-	-	UO	Typ.	+	-

Note: *UO – uniformly opaque; Typ. – typical growth; S= – negative serum; S # – positive polyspecific serum; R+ – positive ovis serum*

Table 1 shows that B. ovis cultures did not agglutinate with control S Brucella-positive or negative sera and agglutinated with R Brucella ovis-positive serum. The isolated cultures did not grow on the culture media containing fuchsin at 1:50,000 – 1:100,000 dilutions and grew on the culture media in the presence of thionine at 1: 25,000 – 50,000–100,000 concentrations, did not produce H2S, exhibited catalase activity and were oxidase-negative. Following staining with crystal violet (the *White & Wilson* method), the cultures appeared deep purple-blue, i.e. were rough Brucella variants.

Thus, the morphological, tinctorial, cultural and biochemical characteristics of the cultures isolated from a small ruminant were typical of rough Brucella; therefore, the cultures were identified as *B. ovis.*

To confirm that the isolated cultures and the cultures that exhibited typical biological properties of B. ovis during primary culture on culture media were *B. ovis,* an inoculation test was carried out on 16 Guinea pigs. Materials used for the test included suspensions of the internal organs, lymph nodes, testicles and epididymides of rams from the Turkestan and Zhambyl regions and the aborted fetuses and whole blood samples collected from PCFR -positive animals. The Guinea pigs were challenged with the suspensions in the laboratory setting in compliance with the biosafety guidelines. The Guinea pigs were observed for 30 days; then their blood was collected for serological testing. After that, the animals were euthanized and their internal organs and lymph nodes were harvested to prepare suspensions that were further plated on MPLGGB and MPLGGA in biosafety cabinets. The analysis of the obtained cultures identified two of the resulting cultures as *B.ovis,* and another two, as *B. melitensis.*

Virulence of two *B. ovis* cultures isolated from the infected animals was studied on 4 Guinea pigs using the fast Korotich-Golot method modified by *I.A. Kosilov.*

The Guinea pigs were intracutaneously injected with 0.1 cm^3 of the suspension of 10 billon isolated Brucella cells. On day 4 after the injection, all Guinea pigs developed edema at the injection site that appeared firm and measured up to 2.5 cm in diameter. On day 17, one animal died in each group; the rest of the Guinea pigs died 4 days later, which suggests that the injected cell cultures were virulent. At necropsy, the fallen animals appeared emaciated, with parenchymal hyperplasia and enlarged lymph nodes. Brucellas isolated from the tissue of the fallen Guinea pigs had the same cultural, morphological, tinctorial, biochemical, and antigen properties as stable R forms of B. ovis.

The results of our study are consistent with the reports of other researchers from across the world who seek the pathways to eradicate brucellosis [18, 19].

Summing up the results of the bacterial culture tests, we conclude that studying the biological properties of pathogens circulating in epizootic foci is one of the key elements of epizootic surveillance: it ensures reliable detection of infection sources and reservoirs and helps to elaborate sciencebased, methodologically sound measures for disease prevention and control in animals and humans.

Conclusion

Two *B. ovis* cultures have been isolated from the samples of ovine whole blood and tissues (one sample from the Zhambyl region and another from the Turkestan region). The analysis of their phenotypes showed that their morphological, tinctorial, cultural, and biochemical properties were consistent with those typically observed in rough Brucella forms (*B. ovis*).

The isolated cultures were identified to the genus and species levels as *B. ovis,* which was further confirmed by the inoculation test on Guinea pigs.

Strain passports with phenotype descriptions were prepared for the isolated strains of *B.ovis* (SHAFA-1 and SHAFA-2). They will be stored in the Museum of Microorganisms.

Detection of new epizootic strains of brucellosis on the territory of the Republic of Kazakhstan, including R-forms allows to replenish the collection of brucellosis.

New epizootic Brucella strains, including their R forms, will be an invaluable addition to the unique collection of Brucella strains started in 1937, when the Laboratory of Brucellosis was founded at Kazakh Scientific Research Veterinary Institute. The collection encompasses a wide range of reference, vaccine and epizootic cultures. Following the resolution of the Government, the collection was entrusted to the National Reference Center for Veterinary Medicine in order to create a unified gene pool system for highly dangerous infectious agents and to maintain biodefense in Kazakhstan. Some of the strains from the collection are instrumental in developing biological preparations for diagnosing and preventing brucellosis in animals and humans.

Considering the results of our bacterial culture tests, we recommend that instead of using a random testing strategy, the entire sheep population should be subjected to repeated serological testing to make sure that all sources of the infection have been detected. This will help to eliminate the disease on the affected livestock farms.

Authors' Contributions

AM, ShB and AB: conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. FB, AI, NO, FS, GK, AT, KB and BL: conducted the final revision and proofreading of the manuscript. All authors have read, re-viewed, and approved the final manuscript".

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UDC 575.174.015.3:619:616.98:578.833-036.2:636.22/.28 **Review article**

Bovine Pestiviruses (*Flaviviridae, Pestivirus*) genomic diversity and global distribution

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Abstract

The bovine viral diarrhea virus (BVDV) is a member of the genus *pestivirus* of the family Flaviviridae and is capable of infecting cattle in many countries; it is characterized by genetic diversity and various diverse clinical manifestations. Bovine pestiviruses belong to three species: *Pestivirus bovis* (BVDV-1), *Pestivirus tauri* (BVDV2) and *Pestivirus braziliense* (BVDV-3 or HOBIE-like pestivirus). There are 21 subtypes of BVDV-1, 4 of BVDV-2, and 4 of BVDV-3. The most widespread in the world, BVDV-1 is widespread in cattle and is most often detected in European countries. The largest number of subtypes of this virus have been identified in cattle in Italy and China. The virus is wides pread in the Central region of the Russian Federation (subtypes 1a and 1m). A number of BVDV-1 subtypes have been detected in Turkey, including BVDV-1a, 1b, 1c, 1d, 1f, 1h, 1i, 1l, 1r, and 1v. A total of 11 subtypes are presentin native and imported animals in Siberia: 1a (5%), 1b (35%), 1c (5%), 1d (10%), 1f (20%), 1g (2.5%), 1i (2.5%), 1j (5%), 1k (5%), 1p (5%), and 1r (5%). BVDV-2 is the most virulent and is found less frequently, primarily in the United States, Canada, Brazil, Argentina, Uruguay, in European countries (Germany, Slovakia, Turkey, and Italy), and in Asian countries (South Korea, Japan, and Mongolia). Three subtypes have been identified in Siberia: 2a (25%), 2b (10%) and 2c (5%). BVDV-3 circulates in Europe, Asia, and South America. The main route of virus introduction is via contaminated biological products. In Russia, BVDV-3 of the Italian Brazilian group (3a) was identified in seven lots of fetal serum. The existence of virus polymorphism complicates disease diagnosis and reduces the effectiveness of vaccination and control programs.

Keywords: review; *pestiviruses*; cattle; viral diarrhea; genetic polymorphism; species; subtypes; distribution.

Introduction

Bovine viral diarrhea virus (BVDV) is a worldwide disease in cattle, causing widespread outbreaks and significant economic losses; it can infect a wide range of domestic and wild species, including sheep, goats, deer, camelids, pigs, and wild ruminants [1]. It is a *pestivirus* of the *Flaviviridae family*, which also includes other important animal viruses, such as classical swine fever virus and border disease virus. BVDV is a single-stranded RNA virus that can be taxonomically divided into three species: *Pestivirus bovis* (commonly known as BVDV-1), *Pestivirus tauri* (BVDV-2), and *Pestivirus brazilense* (BVDV-3 or HoBi-like pestivirus), which are further divided into subtypes based on genetic analysis [2-5]. At least 21 subtypes of BVDV-1 (1a-1u), 4 subtypes of BVDV-2 (2a-2d), and 4 subtypes of BVDV-3 (3a-3d) have been described [6]. However, additional subtypes have recently been suggested [7].

BVDV is an important pathogen causing reproductive, respiratory, and gastrointestinal diseases in cattle. In addition, it causes endemic infections and significant economic losses in cattle herds worldwide.

Most often, bovine *pestiviruses* are distributed in those countries where industrial animal breeding is developed with high concentrations in limited areas in the absence or non-observance of preventive diseases. The epizootic state is determined by the pathogenetic mechanisms by which these viruses are preserved in cattle populations. *Pestiviruses* of all types usually cause the same forms of pathologies in animals: acute infections with immunosuppression, enteritis, resorption of embryos, abortions at different stages of pregnancy, congenital malformations of the fetus, and the birth of weakened non-viable calves, infertility, pathology of the respiratory system and diseases of the mucous membranes [8-15]. Many researchers pay attention to the possibility of contamination of biological preparations with pestiviruses, which include embryonic serum, cell culture lines used in the biotechnological industry in the production of vaccines for humans and animals, trypsin, other biotechnological preparations, embryos, stem cells, sperm from breeding bulls, etc. [16-23].

The forms of clinical manifestation and the features of the course of the disease depend on the following factors. The main one is the virulence of the virus strain infecting the animal. The state of the animal's immune system and its milk production also play a role. In this case, the conditions of feeding and keeping animals should be taken into account in each specific case [24, 25]. The characteristics of the epizootic state and the stationarity of foci of infection are always directly dependent on the constant circulation of the virus and its evolution, as well as new foci of infection. During the emergence of new disease outbreaks, a wide range of clinical manifestations of the disease may be observed, as described in the scientific literature [26-28].

The literature describes cases of the pathogen spreading among cattle associated with the use of vaccines that were contaminated with non-cytopathogenic strains of pestivirus during production. It is known that cell cultures and fetal serum are used in the production of viral vaccines, which may contain non-cytopathogenic strains of viruses [18-20].

The purpose of this review was to obtain new scientific information on the distribution of genetic diversity of *pestiviruses* among cattle in different countries of the world.

Pathogen

All *pestiviruses* have a complex genome structure, which is represented by a single-stranded positively charged RNA consisting of 12.3 thousand nucleotides. It has an open reading frame (ORF) about 4000 codons long, encoding four structural proteins (C, Erns, E1, and E2) and eight nonstructural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), flanked at the 5′ and 3′ ends by untranslated regions (5′ UTR and 3′ UTR) [9, 29]. The nonstructural proteins of the virus are involved in viral replication, transcription, and translation, individually or together [9]. Among all regions of the viral genome, the 5′UTR, Npro, and E2 regions are widely used for differentiation (comparative studies) and phylogenetic analysis [29, 30]. The 5′UTR is the most conserved region, contains secondary structures, and acts as an internal ribosomal site of insertion and regulates the conversion of the ORF to the active state upon its insertion into an animal cell; it is most often used for phylogenetic analysis [9].

Numerous studies have proved that changes in the *pestivirus* genome can be caused by three different processes, which are accompanied by the appearance of different mutants (subtypes) of the virus. These include: (1) accumulation of point mutations due to errors in RNA-dependent RNA polymerase; (2) non-homologous RNA recombination; and (3) homologous RNA recombination. It has been established that these mutations in their frequency coincide with the frequency of these processes in other representatives of RNA viruses. As a rule, one point mutation per replication cycle is introduced into the *pestivirus* genome [31, 32]. The average rate of evolution of the virus strains for the 5′UTR region was estimated to be 9.3×10-3 substitutions/site/year, with a confidence interval between 4.8 and 14.7 substitutions per 1000 nucleotides [26]. Taxonomically, BVDV is divided into three species: BVDV-1, BVDV-2, and BVDV-3. The role of each of these three *pestivirus s*pecies in the development of pathology in cattle has not yet been studied in detail. It has been established that all *pestiviruses* are divided into cytopathogenic (cause changes in the monolayer of cell cultures) and non-cytopathogenic (do not cause changes in the structure of the monolayer of cell cultures) [9, 28, 31]. The identified variability of pestiviruses affects the effectiveness of disease diagnostics and control programs. There is no information yet on cross-protection between existing *pestivirus* species [33, 34]. BVDV-1a and BVDV 1b are the most common viruses worldwide, and they, along with BVDV-2a, are included in most commercial vaccines [35].

Epizootology

It is known that viruses can use a strategy based on the following principles to circulate in a cattle population: "infect and disappear" (relay transmission) and "infect and persist". In the first case, this is accompanied by the development of an acute transit form of infection. With this form of infection, susceptible animals transmit the virus to other animals. In the second case, animals develop a persistent form of infection, in which the virus evades the host's immune system. The mechanism of such evasion is characteristic only of *pestiviruses* [36]. Animals with a transit form of infection are short-term and dead-end sources of the virus. Persistently infected animals play a decisive role as a permanent endogenous source of the pathogen in the herd. They maintain a state of stationary distress on the farm [15].

Sources and routes of pathogen transmission

Viruses use a complex strategy for their survival in animal populations, which is based on two principles. They are based on relay transmission of the virus, in which they "infect and disappear" from the body of the infected animal, and also - "infection and preservation" in the body. During relay transmission of the virus, acute clinical forms of infection occur in animals (transit infection). In the second case, the virus is constantly present in the body of the infected animal, causing a persistent form of infection. In this case, the virus evades the effects of the host's immune system using special mechanisms that have no analogues in other viruses [36]. It has been established that animals with a transit form of infection are temporary sources of the virus. Animals with a resistant form of infection serve as a permanent endogenous source of infection in the herd. They play a major role in maintaining the state of stationary distress on farms [12, 15, 25].

The virus is transmitted horizontally. The main modes of transmission are airborne and feco-oral. In this case, the virus was transmitted from an infected animal to a susceptible animal via aerosol inhalation or swallowing of material contaminated with body secretions (saliva, eye and nasal discharge, urine, feces, uterine discharge, and amniotic fluid). There is also a vertical route of transmission from the mother to the offspring [12, 15].

Distribution of virus species

The most widespread species among animals worldwide is BVDV-1, but it is more often registered in European countries. Analysis of nucleotide sequences showed that the majority of virus isolates were BVDV-1 (88.2%). BVDV-2 was detected much less frequently (11.8%). BVDV-1b was the most common, followed by 1a and 1c [37]. The largest number of BVDV-1 subtypes (up to 21) were found in cattle in Italy [38] and China [39].

In Russia, studies on the phylogenetic analysis of virus isolates are limited. A wide distribution of BVDV-1 in cattle in the Central region of the Russian Federation has been established [40]; two antigenically distinct strains of the virus, 1a and 1m, have been identified in the populations of domestic cattle and wood bison [41].

In the Siberian region of Russia, 11 BVDV-1 subtypes were found to circulate among native and imported animals on dairy farms: 1a (5%), 1b (35%), 1c (5%), 1d (10%), 1f (20%), 1g (2.5%), 1i (2.5%), 1j (5%), 1k (5%), 1p (5%), and 1r (5%). The predominant subtype was BVDV-1b, which was detected in both native animals and those imported from other countries with different clinical forms of the disease [36-37]. BVDV-1c and BVDV-1d were detected in serum samples from calves with clinical forms of respiratory tract lesions. These calves were born from heifers imported from Holland and France. BVDV-1f was detected in a calf born from a heifer imported from Holland and in three serum samples from cows and heifers of local breeds from three regions of Siberia. BVDV-1i was detected in the blood serum of a calf of domestic origin. BVDV-1p was detected in a calf born from a heifer from Germany. BVDV-1a was detected in the internal organs of aborted fetuses and in the blood serum of calves of Austrian and Dutch origin. BVDV-1g was detected in a calf of German origin, and BVDV-1k was detected in a calf from a heifer from France. Contamination of fetal serum samples and cell cultures with the BVDV-1j subtype was established [42, 43]. BVDV-1f was also detected in Slovenia [44] and Austria in calves with persistent infection. Cases of detection of this virus subtype have been described in Italy [45] and Turkey, but without description of clinical syndromes of the disease. BVDV-1i was first identified in samples from the United Kingdom (England and Wales) in 1997 [4, 46]. This subtype was first reported in the United Kingdom, where the incidence of cattle affected by this subtype increased from 3% to 6% over a decade [47]. One strain, 436FaUY/052014, was isolated and classified

as BVDV-1i in Uruguay [48]. A case of acute outbreak of severe pneumonia and hemorrhagic enteritis in calves caused by BVDV-1r has been described in the western region of Turkey [49]. Acute clinical forms of fibrinous pneumonia caused by these virus subtypes have been reported in calves in Russia. It was established that these calves were born both to heifers imported from Austria and Germany and to local cows from the Novosibirsk region. Circulation of a large number of BVDV-1 subtypes has been established among domestic cattle breeds and in imported animals in Siberia [42, 43]. Worldwide, BVDV-2 is less common than BVDV-1. It has been isolated from cattle in the United States [50], Canada [51], Brazil [52], Argentina [53], Uruguay [48], European countries (Germany [54], Slovakia [55] and Italy [26]) and Asia (South Korea [56], Japan [57] and Mongolia [58]). It has been established that the main route of introduction of this subtype of the virus into European countries is fetal blood serum and other biological products, in particular vaccines [26, 59]. BVDV-2 is a more virulent species than BVDV-1. It has been established that it has four subtypes (2a-2d) [59]. Most often, UGO is detected in the United States and Canada, where it reaches 50% of all isolated strains [15]. In Russia, in the Siberian region, three subtypes of BVDV-2 have been identified in animals of imported and domestic origin: 2a (25%), 2b (10%), and 2c (5%) [42-43, 60]. It has been determined that the main etiological agents causing damage to the reproductive organs and the development of systemic infection with severe hemorrhagic syndrome in cattle are subtypes 2a and 2b of the second type of virus [15, 51-53]. In Siberia, BVDV-2a was first isolated in 2008 from a locally-originating cow that had aborted; BVDV-2b was detected in heifers imported from the United States during an outbreak of mass abortions and in calves born up to 30 days old with enteritis and pneumonia [42 ,43, 60].

In Brazil, the following BVDV-2 subtypes were found within the species: BVDV-1a (35.9%), BVDV-2b (31.4%), BVDV-1b (10.1%), BVDV-1d (6.7%), BVDV-2c (2.2%), and BVDV-1e (1.1%). BVDV-2c and BVDV-1e were detected for the first time in this study in Brazil [61]. BVDV-2c is a rare subtype. It was detected during massive acute outbreaks of the disease in seronegative animals of different ages in the period from 2013 to 2014 on farms in Germany and the Netherlands. During this period, a sharp decrease in milk production in infected cows, an increase in body temperature, respiratory damage and the development of hemorrhagic enteritis in calves, heifers and cows were observed [62, 63]. Later, in 2016, the virus caused a massive outbreak of the disease among small cattle in southern Italy [64]. We were able to detect the presence of this subtype of the virus in cattle with persistent and transit forms of infection. The animals were imported to Russia from Germany. We should be cautious about the detection of this virus subtype in our country should be treated with caution, since it has not been detected in biological products (vaccines, fetal serum, cell cultures) [42, 43]. The scientific data we have obtained indicate that the maximum spread of the virus in Russia occurred in 2006-2015 and was due to the mass import of highly productive imported animals, which was associated with the intensification of animal husbandry.

At the same time, cases of detection of individual virus subtypes in native animals kept in closed farms, where other animals were not brought from outside, force us to reconsider this conclusion. We assume that these viruses could have been present in the animal population of Siberia for a long time, but the original source cannot be established [43].

The spread of BVDV-3 is limited to several specific regions. It is due to the use of biological products contaminated with the virus. It was established that BVDV-3 was first isolated from a batch of fetal serum in Germany. Serum was collected from animals in Brazil and packaged and repackaged in Europe [65]. Isolate D32/00_HoBi was considered the prototype of the Brazilian group of viruses. BVDV-3 was later identified in cattle from South America [66-68], Asia [69-71], and Europe [13-14]. Other authors have identified genetically distinct subtypes with regional distributions, including the Thai [68], Indian [69], and Italian groups. Thus far, the presence of four genetic groups of this virus (BVDV-3a–d) has been established. There are reports in the international literature on the detection of the BVDV-3 genome in fetal serum. For example, *M. Giammarioli* et al., were able to detect BVDV-3 in 57.7% of the fetal serum lots tested by PCR. They were obtained between 1992 and 2013, filtered and treated with gamma irradiation. Seven lots came from South America, and one from Australia. The origin of the remaining serum lots was unknown. Thanks to the phylogenetic analysis, the authors were able to classify the detected virus as a Brazilian group of viruses and establish that it was imported to Italy with fetal serum [72]. Later, they tested 90 lots of commercial serum, which was manufactured

in the United States and packaged in Europe. BVDV-3 was not detected in these serum lots, but BVDV-1 was present in 19 of them, and BVDV-2 in one [73]. According to other researchers, BVDV-3 was detected in a commercial vaccine against peste des petits ruminants in the Republic of Tajikistan [74]. Data have been published on the presence of the virus genome in 7 of 18 fetal serum samples from two manufacturers used in Russia. Viral RNA was detected in two series of fetal blood serum (manufactured by Biolot LLC) and in five series (manufactured by PAA Laboratories). All virus isolates identified by us were assigned to the Italian subgroup [42, 43]. We also established the etiological role of BVDV-3 in the occurrence of mass respiratory diseases in calves. In addition, the virus was isolated in cell culture [75].

Many researchers believe that BVDV-3 affects the decrease in the fertilization rate, plays a role in the etiology of abortions, the development of systemic infection and enteritis in calves and adult animals.

We have established the etiological role of the virus in the occurrence of mass outbreaks of the disease on three large dairy farms. The source of infection was presumably a live vaccine against lumpy skin disease. Analysis of the results of sequencing the nucleotide sequences of three viral isolates obtained from animals established their identity with the BVDV-3 vaccine strain [76]. There is an opinion of Italian researchers that the low frequency of BVDV-3 detection in Italy and the absence of its circulation in other European countries confirm the hypothesis that it was brought with contaminated biological products, and not with infected animals [26]. These results are consistent with the results of other researchers [76].

Conclusion

The conducted analysis of literary sources indicates that BVDV-1 is most widespread worldwide among the cattle population. BVDV-2 is the most virulent. It is most often detected in North American countries. The main source of pathogens is cattle in international trade and contaminated biological products. The spread of BVDV-3 is currently limited to several regions of South America, Europe and Asia. The primary source is contaminated biological products.

According to the literature in Russia, widespread and fairly high heterogeneity of viral diarrhea viruses circulating among cattle of domestic and foreign origin has been established. Phylogenetic analysis revealed the circulation of 12 subtypes of BVDV-1, 3 of BVDV-2, and 1of BVDV-3. The predominant subtypes are BVDV-1b and BVDV-2a. The main reason for the wide distribution and high level of heterogeneity of BVDV-1 in Russia is the intensification of livestock farming, which is accompanied by an increase in the concentration of animals in limited areas, the movement of animals associated with the livestock trade. In addition, the import of highly productive animals from other countries and the lack of a state control program are of great importance. The circulation of two new subtypes of BVDV-2 (b and c) in Russia has been established. It is necessary to be careful about their detection. It is known that no vaccine has been developed against BVDV-3 in the world. The lack of drugs for the prevention of BVDV-3 creates favorable conditions for the spread of the virus in the cattle population worldwide. In addition, it is important to introduce systematic control of biological products to prevent the spread of the pathogen. Biological products contaminated with non-cytopathogenic strains of the virus should be considered as potential sources of the introduction of emerging species and subgenotypes of bovine *pestiviruses* into new regions and countries of the world. The composition of virus subtypes in the cattle population should be monitored continuously. This approach helps maintain effective diagnostic methods and control measures and serves as an early warning system for the introduction of new *pestivirus* subtypes into naive cattle populations.

Authors' Contributions

TG and AG: conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript".

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Live Turkey Herpesvirus vaccine against Marek's Disease: development, stabilization, and immunobiological evaluation

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Abstract

Background and Aim. Marek's disease (MD) is a highly contagious and economically significant viral infection of poultry, caused by Marek's disease virus (MDV), an alphaherpesvirus that induces lymphomas, paralysis, and immunosuppression in chickens. Kazakhstan currently lacks a domestically produced MD vaccine, resulting in dependence on imports and logistical challenges. This study aimed to develop and evaluate a national live vaccine against MD using a cloned strain of turkey herpesvirus (HVT, strain AV-0007), with optimized production and preservation technologies.

Materials and Methods. The AV-0007 strain was propagated in chicken embryo fibroblast (CEF) cultures using stationary, roller, and suspension cultivation methods. Virus yields were optimized by adjusting MOI, nutrient media, and harvest times. Stabilizing media for cryopreservation and freeze-drying were formulated. Experimental vaccine preparations in both forms were assessed for sterility, safety, biological activity, and immunogenicity in one-day-old chickens. Virus titers were measured using the focus-forming unit (FFU50) method, and immunogenicity was evaluated by virus neutralization tests.

Results. Virus titers exceeded 10^6 FFU50/cm^3 across optimized cultivation methods. Cryopreserved and freeze-dried vaccines preserved high viral activity after stabilization and storage. Both forms met international standards for sterility and were non-pathogenic in chicks even at 10× immunizing doses. Immunized birds developed virus-neutralizing antibodies with titers ranging from 1.67 to 2.33 log FFU50, indicating strong immunogenicity and protective potential.

Conclusion. The AV-0007-based vaccine formulations demonstrated safety, stability, and high immunogenicity. The study confirms the feasibility of producing a domestic Marek's disease vaccine in Kazakhstan and provides a foundation for the local development of poultry vaccines aligned with international quality standards.

Keywords: chicken embryo fibroblasts; immunogenicity; live vaccine; Marek's disease; turkey herpesvirus.

Introduction

Kazakhstan possesses significant potential for the advancement of its poultry industry, a sector anticipated to be pivotal in guaranteeing national food security and enhancing agro-industrial exports [1]. This potential is fundamentally reliant on the capacity to manage viral illnesses that jeopardize poultry health and productivity. Marek's disease (MD) is a notable lymphoproliferative condition induced by the extremely contagious alphaherpesvirus known as Marek's disease virus (MDV), which affects hens.

The ailment is marked by advancing paralysis, immunosuppression, and lethal lymphomas, frequently leading to significant economic detriment in commercial flocks [2, 3, 4, 5].

Currently, MD is solely controlled via immunization. The current vaccinations, however, do not provide sterile immunity [6]. While they reduce symptoms and the risk of death, they do not stop the virus from multiplying or spreading, which allows for the development of stronger MDV types. This constraint has initiated a worldwide quest for more efficacious vaccinations that can provide enhanced, preferably sterilizing, immunity [7, 8, 9].

Live vaccines made from turkey herpesvirus (HVT) [10] are the standard in commercial use because they don't cause disease in poultry and are similar enough to harmful MDV to trigger an immune response. These vaccines are usually made using fibroblast cultures from specially selected chicken embryos that are free from specific diseases, followed by steps to grow the virus, stabilize it, and store it using freezing or drying methods. The process is complicated and requires a lot of resources, involving large-scale cell growth in special containers, and is mainly used by major companies like Merial (France), Intervet (Netherlands), and ARRIAH (Russia) [11].

Despite the introduction of attenuated MDV strains [12, 13] and recombinant vector vaccines [14, 15, 16], they either pose production difficulties or generate diminished immune responses, particularly in flocks with previous MD exposure. Moreover, the specific production technology employed internationally remains undisclosed, creating a technological disparity for domestic manufacturers.

The lack of a domestically developed vaccine for MDV in Kazakhstan is both an epizootic and economic risk. This study fills the gap by creating a national technology for the production of a live vaccination against Marek's disease with a cloned strain of HVT. The objective was to develop a vaccine that complies with worldwide criteria for safety, immunogenicity, and viability while being tailored to local production conditions.

Materials and Methods

Materials: The research used hatching eggs from commercial sources and a specific type of turkey herpesvirus (strain AV-0007) that had a remaining biological activity of $10^{3.75}$ FFU/cm³, stored at -50 °C, along with Eagle's nutrient medium (with and without calcium), fetal bovine serum, and various tools and materials for growing cells. This included incubators, thermostats, roller devices, suspension culture fermenters, and lab glassware like 1.5 dm³ mattresses and 0.5 dm³ roller flasks. This encompassed incubators, thermostats, roller devices, suspension culture fermenters, and laboratory glassware, including 1.5 dm³ mattresses and 0.5 dm³ roller flasks.

Incubation of chicken embryos: Developing chicken embryos (DCE) were acquired by incubating hatching eggs at 37-38 °C with a humidity of at least 50%, continuous air exchange, and periodic axial rotation. The ideal durations for egg storage before incubation, together with factors like temperature, humidity, aeration, and frequency of egg rotation, were established to enhance embryonic development.

Preparation of chicken embryo fibroblasts (CEF): Fibroblast cultures (CEF) were obtained from embryos of differing ages. The improved settings included the optimal age of the embryos for trypsinization, the total number of cells from each embryo, removal of feather debris, trypsin concentration, pH level, how often and how long to use trypsin, and the conditions for different culture methods like stationary, roller, and suspension techniques. Media replacement times for both nutrition and maintenance phases were established.

Virus biomass production: The amount of turkey herpesvirus produced was improved by looking at factors like the age and number of cells in the CEF culture, along with the condition of the cell layer before infection. Multiplicities of infection (MOI) from 0.1 to 0.001 $FFU_{50/cell}$ were evaluated. Viral titers were assessed at multiple times following infection to ascertain the optimal harvest time. The effect of freeze-thaw cycles on viral infectivity was also examined.

Vaccine production and formulations: Experimental vaccine batches were made using the AV-0007 virus strain at a concentration of $10^{6.75}$ FFU_{50/cm^3}, grown in CEF using the suspension method. Two formulations were developed: freeze-dried (lyophilized) and cryopreserved. Three vials of each formulation were utilized for testing. Freeze-dried samples were reconstituted in 0.9% sodium chloride and amalgamated, while cryopreserved samples were thawed in a 40 °C water bath for 1 minute and thereafter mixed.

Sterility testing: Sterility was assessed in accordance with GOST 28085, using standard microbiological culture techniques [17].

Safety assessment: Safety was assessed in 10 one-day-old chicks, each administered an intramuscular injection of 10 immunizing doses ($10^{4.0}$ $FFU_{50/0.2\ cm^3}$) of the vaccine. Birds were monitored for a duration of 10 days. The lack of clinical symptoms and death suggested that the immunization was deemed safe.

Biological activity testing: The amount of virus in both vaccine types was measured by making ten times weaker solutions and testing them on layers of CEF cells. Each dilution was injected with ten flasks. Focus-forming units (FFU) were enumerated, and titers were determined by the Reed and Muench methodology.

Immunogenicity testing: Immunogenicity was assessed in chicks inoculated with $10^{3.0}$ $FFU_{50/0.2cm^3}$. Specific antibodies were found in blood samples taken after vaccination using a test that checks for the ability to neutralize the turkey herpesvirus strain used in the study.

Statistical analysis: All experimental data were analyzed using conventional descriptive and inferential statistical techniques. Quantitative results are expressed as mean ± standard deviation (SD). Group means comparison (e.g., virus titers, antibody levels) was conducted utilizing one-way analysis of variance (ANOVA) accompanied by Tukey's post-hoc test to identify statistically significant differences among groups. A p-value of less than 0.05 was deemed statistically significant.

Results and Discussion

Determination of optimal cell density, cultivation period, and MOI for efficient virus accumulation. Determining the best conditions for infecting the CEF culture is a crucial step in increasing virus yield in vaccine manufacturing. This work included creating a single layer of chicken embryo fibroblasts (CEF) by placing 4×10^5 cells in each cubic centimeter and letting them grow for 4 days. On the third day of cultivation, the cells were infected with turkey herpesvirus at three distinct multiplicities of infection (MOIs): 0.1 FFU/cell, 0.01 FFU/cell, and 0.001 FFU/cell.

Following a 96-hour incubation period at 37.0-37.5 °C, all experimental flasks underwent a single freeze-thaw cycle (-40 °C for 24 hours, then thawed at ambient temperature), after which the virus titer was assessed by titration on a monolayer of fresh CEF cells.

Table 1 illustrates that the viral titer escalated in direct correlation with the MOI. At the minimal MOI of 0.001 FFU/cell, the total viral titer attained $10^{4.75}$ FFU/cm³. Conversely, at MOIs of 0.01 and 0.1 FFU/cell, the titers rose markedly to 106.00 and $10^{6.50}$ FFU/cm³, respectively. This illustrates a distinct positive association between infection dose and the resultant viral concentration in the biomass.

Table 1 – Virus accumulation in the CEF depending on the dose of infection

Seeding concentration of CEF cells, cells/m³	Age of cell culture, days	MOI (FFU/cell)	Virus Titer (FFU/cm³, Mean ± SD)
4×10^5	4	0.1	$10^{6.50\pm0.10}$
		0.01	$10^{6.00\pm0.08}$
		0.001	$10^{4.75\pm0.12}$

The findings indicate that an MOI of no less than 0.01 FFU/cell is necessary to attain optimal viral yields for subsequent downstream processing and vaccine formulation. Although 0.1 FFU/cell produced the best results, using a slightly lower amount of 0.01 FFU/cell might be more cost-effective for large-scale production, while still generating a good amount of virus.

Determination of the optimal virus harvesting period and evaluation of freezing as a virus collection method. To ascertain the ideal timing for harvesting the virus from infected CEF cultures and to assess the efficacy of the freezing-thawing procedure for virus collection, virus-infected cell cultures were incubated under diverse conditions and durations. CEF monolayer cultures (stationary and roller) and suspension cultures were cultured at 37 °C and sampled at various time intervals: 72, 96, and 120 hours post-infection. In each instance, fifty percent of the cultures saw a singular freeze-thaw cycle (frozen at -40 °C for 24 hours, then thawed at ambient temperature), whilst the other samples were processed immediately without freezing.

The virus titer was assessed in all instances using monolayer titration. The results shown in Table 2 indicate that the highest amount of virus was found on the fifth day (96 hours) after infection for both stationary and roller monolayer cultures. The virus titer in stationary monolayers after freeze-thaw attained $10^{6.25}$ FFU/cm³, but in roller monolayers, the titer peaked at $10^{6.75}$ FFU/cm³. The figures exceeded those acquired without the freezing technique, where the titer was around 0.5-1.25 log units lower.

Conversely, in suspension cultures, the peak titers were recorded earlier, at 72 hours post-infection, attaining 105.50 FFU/cm³ without freeze-thaw and $10^{5.25}$ FFU/cm³ with freeze-thaw. Viral titers in suspension cultures decreased markedly at subsequent time points, signifying a reduction in cell viability and viral reproduction.

Table 2 – Virus titers established during production under different conditions

Virus-infected cell culture	Incubation period before collection of viral mass, days					
	3		5		7	
	Freezing. Defrosting.	No freezing	Freezing. Defrosting.	No freezing	Freezing. Defrosting	No freezing
CEF monolayer stationary	$10^{4.50\pm 0.10}$	not tested	$10^{6.25\pm 0.09}$	$10^{5.50\pm 0.11}$	$10^{4.25\pm 0.13}$	not tested
CEF monolayer roller	$10^{5.00\pm 0.10}$	not tested	$10^{6.75\pm 0.07}$	$10^{6.25\pm 0.08}$	$10^{5.50\pm 0.07}$	not tested
CEF suspension	$10^{5.25\pm 0.09}$	$10^{5.50\pm 0.08}$	$10^{2.75\pm 0.14}$	not tested	not tested	not tested

The results indicate that for monolayer cultures, especially those cultivated via the roller method, the virus should be harvested 96 hours post-infection after a freeze-thaw process, which facilitates virus release. In contrast, for suspension cultures, the ideal harvest time is 72 hours post-infection, and prolonged incubation results in diminished viral yields, irrespective of freezing.

Optimization of suspension culture parameters for enhanced virus titer. Due to the destitute virus yield in suspension cultures under conventional conditions, adjustments were implemented to improve virus productivity. The changes included using Eagle's nutritional medium with double the usual amount of amino acids and increasing the fetal bovine serum (FBS) level to 15%. The MOI was raised to 0.05 FFU/cell to evaluate if elevated initial infection doses would enhance viral yield.

Suspension cultures were inoculated with CEF at doses between 2.1×10^6 and 2.4×10^6 cells/cm³ and incubated for 72 and 96 hours. The virus titer was assessed in all instances using monolayer titration. Table 3 shows the best conditions for growing the virus in suspension culture: a cell concentration of 2.4×10^6 cells/cm³, a multiplicity of infection (MOI) of 0.05 FFU/cell, and 15% fetal bovine serum (FBS). Under these circumstances, the viral titer reached a maximum of $10^{6.75}$ FFU/cell at 72 hours. Prolonged incubation to 96 hours led to diminished titers, corroborating prior findings.

Table 3 – Titers of turkey herpes virus in suspension cultivation in CEF culture, $FFU_{50/cell}$

Fibroblast concentration, cells/cm³	Dose of seed virus (MOI), $FFU5_{0/cell}$	Concentration of blood serum in the nutrient medium, %	Timeframe for determining virus titer, h	
			72	96
2.3×10^6	0.01	10	$10^{5.50\pm 0.08}$	$10^{4.25\pm 0.09}$
2.1×10^6	0.01	15	$10^{6.00\pm 0.06}$	$10^{5.50\pm 0.07}$
2.4×10^6	0.05	15	$10^{6.75\pm 0.08}$	not tested

The data show that increasing nutrient and serum levels, as well as a higher multiplicity of infection (MOI), significantly boosts virus production in suspension cultures. The time-dependent aspect of virus growth is a crucial element, as extended incubation negatively impacts titer.

Stability of intra- and extracellular Turkey Herpesvirus and development of preservation strategies. The stability of turkey herpesvirus inside and outside of cells was studied to see if there would be a loss of strength during normal storage and handling. This investigation was a vital measure for formulating efficient preservation strategies for forthcoming vaccine manufacture.

A suspension of live CEF cells containing the virus (intracellular virus) was segregated into two groups for this purpose. One portion was maintained at 4-6 °C, whereas the second underwent freezing at -20 °C for 3 hours, then thawed at ambient temperature, and then stored at 4-6 °C. Viral levels were measured right after treatment and again after 24 hours of storage using standard testing methods on CEF cell layers.

Table 4 illustrates that the internal virus exhibited greater stability than its extracellular counterpart. Following 24 hours of storage at 4-6 °C, the intracellular virus titer diminished marginally from $10^{6.00}$ to $10^{5.75}$ $FFU_{50/cell}$ (-0.25 log). The virus outside the cells, which came from broken cells after freezing, dropped from $10^{5.50}$ to $10^{4.75}$ $FFU_{50/cell}$ in the same period, showing a decrease of 0.75 log while thawing and another 0.5 log in the next 24 hours.

Table 4 – Titers of intra- and extracellular turkey herpesvirus under storage conditions

Material under study	Initial titer of virus in suspension	Terms and Conditions		Status of the virus	Research time, hours	
		processing	storage, °C		0	24
Chicken embryo fibroblast suspension containing turkey herpes virus	$10^{6.00}$	-	4-6	Intracellular	$10^{6.00}$	$10^{5.75}$
		Freezing at minus 20 °C and defrosting	4-6	Extracellular	$10^{5.50}$	$10^{4.75}$

The data demonstrate that the internal virus maintains stability during refrigerated storage, whereas the extracellular virus is considerably more susceptible to freeze-thaw cycles and extended exposure at 4-6 °C. Consequently, it is advisable to store virus-containing solutions intracellularly and safeguard them with stabilizing chemicals during freezing or drying procedures.

Preservation of virus biomass via cryopreservation and freeze-drying. Two preservation procedures, cryopreservation and freeze-drying, were developed to guarantee the long-term storage and biological stability of the vaccine virus. For each approach, virus-laden CEF suspensions were categorized into two groups: those with protective additives and those without.

The frozen samples were kept safe using a mixture made of 70% Eagle's nutritional medium, 20% fetal bovine serum, and 10% dimethyl sulfoxide. Following stabilization, samples were frozen at -70 °C and subsequently transferred to liquid nitrogen (-196 °C) for storage. Samples were made stable using a mix of 4% peptone, 8% sucrose, and 1% gelatin, and then dried out using a careful freezing method.

Biological activity was evaluated prior to stabilization, subsequent to stabilization, and following three months of storage. The findings are encapsulated in Table 5.

Table 5 – Virus titer before and after stabilization and storage (3 months)

Biomass form of turkey herpes virus	Virus titer in biomass before stabilization	Virus titer in biomass after stabilization	Virus titer in biomass after stabilization and storage for 3 months	Change in virus titer after storage, lg FFU_{50}
Cryopreserved with protective additive	$10^{6.75}$	$10^{6.25}$	$10^{6.25}$	0.0
Cryopreserved without protective additives	$10^{6.75}$	$10^{6.25}$	$10^{5.75}$	0.5
Sublimated with protective additive	$10^{6.00}$	$10^{5.75}$	$10^{5.50}$	0.25
Sublimated without protective additives	$10^{6.00}$	$10^{5.75}$	$10^{2.25}$	3.50

The data indicates that samples with protective additives preserved their biological activity nearly intact, exhibiting minimal loss after three months of storage. Conversely, virus biomass devoid of additives exhibited a notable decline in infectivity, especially in the freeze-dried state, when the titer fell by 3.5 log. This finding underscores the essential importance of adequate stability in maintaining vaccination viability.

Establishment of methods for standardization of freeze-dried and cryopreserved Turkey Herpesvirus. The evaluation of vaccine quality is grounded in factors that positively influence the vaccinated organism while ensuring no harm to the animals or the environment. Therefore, the methods used to ensure both types of vaccines are safe include checking for cleanliness from unwanted germs, measuring the strength of the vaccine virus, and testing how well it triggers an immune response, as well as assessing the physical and technical quality of the vaccine. A biological model vulnerable to Marek's disease was selected from this list of approaches as the target for evaluating the preparation's safety. The biological model consisted of one-day-old chicks. The procedures outlined in GOST 28085 "Biological preparations. Methods for determining sterility" were selected to evaluate sterility. The biological activity was evaluated using a titration approach to quantify the turkey reproductive herpes virus through plaque- or focus-forming units in a monolayer culture of chicken embryo fibroblasts contained in penicillin vials. A biological model susceptible to the Marek's disease virus, specifically one-day-old hens, was selected to evaluate the vaccine's immunogenicity and safety. Standard methods were used to check the physical and technical features of the vaccine, such as how it looks, how well it dissolves (only for the dry form), the condition of the packaging, how well it is sealed, the internal vacuum (only for the dry form), nitrogen levels in the Dewar flask (only for the liquid form), and the correctness of the labels. Table 6 presents the compilation of selected and established methodologies for the standardization of the cryopreserved and freeze-dried turkey herpes virus vaccine.

Table 6 – List of methods for standardization of turkey herpes virus vaccine in cryopreserved and dry forms

Vaccine parameter being assessed	Method of evaluation by vaccine forms		Indicators for forms	
	Cryopreserved	Dry	Cryopreserved	Dry
1	2	3	4	5
Appearance, color, purity from impurities	Frozen liquid of yellow color, without impurities	Dry tablet-shaped porous mass without impurities of cream color	Corresponds	Corresponds
Presence of vacuum in vaccine vials	-	Vacuum according to GOST 28083	-	Vacuum according to GOST 28083
The presence of liquid nitrogen in the Dewar vessel in which the vaccine is stored	Liquid nitrogen with signs of boiling and evaporation	-	Corresponds	-
Solubility	-	For up to 2 minutes in physiological sodium chloride solution	-	Corresponds
Sterility	GOST 28085		Corresponds	Corresponds
Harmlessness	Inoculation with the vaccine intramuscularly at a dose of at least 10^4 $PFU_{50/0.2}$ cm^3 in one-day-old chickens in an amount of at least 10 heads		Harmless	Harmless

Continuation of table 6

Biological activity by virus titer, FFU50/cm3	Titration on a monolayer culture of chicken embryo fibroblasts by infecting the cell culture with tenfold dilutions of the vaccine in at least 4 flasks	Not less than $10^{6.0}$	Not less than $10^{6.0}$
Immunogenicity by antibody titer in vaccinated chickens, lg	Immunization of at least 20 one-day-old chickens by intramuscular administration of the vaccine at a dose of 10^3 $FFU_{50/0.2}$ cm^3.	Not less than 1.5	Not less than 1.5

The information in Table 6 shows that the main qualities of the vaccine, like being free from germs, being safe, working effectively through virus titer, and triggering an immune response, are all standardized using the same methods. The dry vaccine formulation has supplementary methods for assessing the look and color of the preparation, as well as verifying the presence of a vacuum within the vials containing the vaccine. The evaluation of the cryopreserved preparation includes not just the main procedures but also checking the vaccine's appearance and color, as well as confirming the presence of liquid nitrogen in the Dewar vessel used for its transport.

Preparation of experimental samples of freeze-dried and cryopreserved forms of the vaccine against Marek's disease, and their standardization according to immunobiological properties. Samples of freeze-dried and cryopreserved Marek's disease vaccine were made from turkey herpes virus with a strength of $10^{6.75}$ FFU_{50/cm^3}, using special chemicals meant for freeze-drying and cryopreservation. One hundred vials of freeze-dried and cryopreserved vaccine formulations were manufactured.

The vaccine's qualities were standardized by checking for cleanliness, safety, how well it works based on virus levels, and its ability to trigger an immune response.

Three vials of the vaccine were utilized for assessing each parameter, both freeze-dried (dry) and cryopreserved (liquid). The freeze-dried vaccine from the three vials was mixed with a saltwater solution, combined into one vial, and the average of that sample was used for testing. The cryopreserved vaccine was thawed in a water bath at 40 °C for 1 minute, pooled, and an average sample was collected for testing.

Determination of vaccine sterility. The results of the sterility tests, done according to GOST 28085 standards, showed that both vaccine types are free from unwanted germs. In the vaccine samples grown on specific nutrient media, no germs were found during the observation period. In the vaccine samples grown on special nutrient media MPA, MPB, MPPB, and Sabouraud, no germs were found during the observation time.

Determining the safety of a vaccine. To assess safety, ten one-day-old chickens were vaccinated intramuscularly with an average sample of each preparation form containing 10^4 $FFU_{50/0.2cm^3}$ of turkey herpes virus, administered at a dose of 0.2 cm^3. The chickens underwent clinical observation for a duration of 10 days. The safety was evaluated according to the clinical status of the birds. The outcomes of evaluating this parameter from the experimental vaccine series are presented in Table 7.

Table 7 – Data on the safety assessment of the experimental series of the vaccine against Marek's disease

Test drug	Number of vaccinated chickens, heads	Vaccine dose, $FFU_{50/head}$	Results of observation over 10 days	Vaccine evaluation
Marek's disease vaccine dry	10	10^4	0/10	Harmless
Marek's disease vaccine dry	10	10^4	0/10	Harmless
Note: The denominator is the number of vaccinated chickens, the numerator is the number of chickens with general and local pathologies detected				

The information in Table 7 shows that all 10 chickens given the freeze-dried Marek's disease vaccine and all 10 chickens given the cryopreserved Marek's disease vaccine, at a dose ten times higher than the usual amount, stayed healthy and alive for 10 days after vaccination, proving that the vaccine is safe for chickens.

Determination of biological activity of vaccine preparations. The biological efficacy of the freeze-dried and cryopreserved experimental vaccine formulations was assessed by measuring the viral titer in monolayer cultures of CEF cells utilizing the focus-forming unit (FFU) method. To achieve this, 10-fold serial dilutions were created using pooled average samples of each vaccine formulation (reconstituted or thawed), and 10 flasks of monolayer CEF cultures were inoculated with each dilution.

Focus-forming units were detected and enumerated 96 hours post-infection. The virus titers were subsequently determined by the Reed and Muench method. Table 8 reveals that the freeze-dried vaccine exhibited a titer of $10^{6.00}$ $FFU5_{0/cm^3}$, whereas the cryopreserved variant demonstrated a marginally elevated titer of $10^{6.50}$ FFU_{50/cm^3}.

Table 8 – Results of titration of experimental series of freeze-dried and cryopreserved Marek's disease vaccine

Material under study	Virus dilutions						Virus titer
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Marek's disease vaccine dry	10/10	10/10	9/10	6/10	0/10	0/10	$10^{6.0}$
Marek's disease vaccine liquid	10/10	10/10	10/10	10/10	0/10	0/10	$10^{6.5}$
Note: in the denominator is the number of flasks with infected cell culture, in the numerator is the cell culture in flasks with focus-forming units							

These findings show that both vaccination formulations maintain robust biological efficacy post-processing and stabilization. The cryopreserved version showed slightly better ability to infect, possibly because the viral material was handled more gently compared to the lyophilization process. Both formulations, however, achieved the requisite potency level of $\geq 10^6$ FFU_{50/cm^3}, thereby qualifying for immunogenicity assessment.

Evaluation of vaccine immunogenicity. To evaluate the immunogenic potential of the vaccine, one-day-old hens were inoculated with $10^{3.00}$ $FFU_{50/0.2\ cm^3}$ of either the freeze-dried or cryopreserved vaccine formulation. Blood samples were taken from the vaccinated hens (15 in each group) 21 days after vaccination and tested with a virus neutralization test using a turkey herpesvirus strain at a strength of $10^{6.50}$ FFU_{50/cm^3}.

The difference in virus neutralization levels before and after vaccination was used to evaluate the production of specific antibodies. Table 9 shows that the average level of neutralizing antibodies was 1.67 log FFU_{50} in hens given the freeze-dried vaccine and 2.33 log FFU_{50} in those given the cryopreserved version. This result signifies that both vaccinations elicited the generation of virus-specific antibodies at levels adequate to provide protective protection.

Table 9 – Titer of virus-neutralizing antibodies in blood serum samples of chickens vaccinated with freeze-dried and cryopreserved Marek's disease vaccine

The vaccine used for the vaccination	Number of serum samples in the pool	The titer of the virus in a mixture with blood serum collected		Difference in virus titers
		before vaccination	21 days after vaccination with the vaccine	
Marek's disease vaccine dry	15	$10^{6.00}$	$10^{4.33}$	$10^{1.67}$
Marek's disease vaccine liquid	15	$10^{6.50}$	$10^{4.17}$	$10^{2.33}$

The information in Table 9 shows that the amount of turkey herpes virus mixed with blood serum samples from chickens before they were vaccinated with freeze-dried Marek's disease vaccine was about 106.00 FFU50, while it was about $10^{6.50}$ FFU_{50} when mixed with serum samples from chickens before they received the cryopreserved Marek's disease vaccine. Conversely, the titer of the same virus in a mixture with serum samples from the same chickens 21 days post-vaccination was $10^{4.33}$ FFU_{50} and $10^{4.17}$ FFU_{50}, respectively. The disparity in viral titers was 101.67 and $10^{2.33}$ FFU_{50}, respectively, within the mixture of blood serum samples obtained before and after inoculation with freeze-dried and cryopreserved vaccines.

The observed disparity in viral titers is large and ensures the presence of robust protection in the bodies of vaccinated animals.

The standardization results show that the experimental vaccine series, whether cryopreserved or freeze-dried, do not contain any harmful microbes and are safe for one-day-old chickens when given a much higher dose ($10^{4.0}$ FFU_{50} per chicken) through an injection into the muscle. The Marek's disease vaccine, made from the "AV-0007" strain of the turkey herpesvirus, shows it can effectively trigger an immune response in chickens when given as an injection at a dose of $10^{3.0}$ FFU_{50} per bird. In immunized hens, antibodies are produced at a titer of 1.67-2.33 lg FFU_{50} after 21 days.

To mitigate the significant danger of Marek's disease in industrial poultry, farmers implement preventive measures by vaccinating all chicks on their first day of life. The lack of such procedures results in the affliction of juvenile birds and their widespread mortality [18]. In our nation's chicken business, a vaccine manufactured abroad is utilized specifically for the prevention of Marek's illness. The vaccines coming from faraway countries, even with regular air shipping, often don't meet the needs of chicken farms because of problems like late deliveries, breaking storage and transport rules, and not having enough for all the baby birds since they weren't ordered early enough. The utilization of imported vaccinations creates a degree of dependency on pharmaceutical partners and suppliers. The aforementioned reasons underscore the importance of developing a homegrown vaccination for Marek's disease as well as other perilous infectious diseases. Investigations involving the turkey herpes virus, which underpins the vaccination for Marek's disease, are among the most intricate [19, 20, 21, 22].

This paper discusses the successful creation, standardization, and first tests in live hens of a vaccine made from the AV-0007 strain of turkey herpesvirus (HVT), showing strong safety, effectiveness, and ability to provoke an immune response in one-day-old hens.

This work's principal achievement was the finding of optimal viral generation parameters in CEF culture. Our results indicate that the virus production is directly affected by the multiplicity of infection (MOI), with titers rising proportionately from 0.001 to 0.1 FFU/cell. An MOI of 0.01 FFU/cell was found to be both affordable and effective, generating viral levels over 10^6 FFU/cm³, similar to those seen in standard vaccine production using traditional HVT strains.

Our study of growing methods showed that using the roller monolayer method, particularly with a 96-hour incubation followed by freeze-thaw treatment after infection, resulted in the highest virus levels (up to $10^{6.75}$ FFU/cm³). This finding supports previous research that shows gentle rolling and better air flow during incubation improve the health of fibroblasts and how well the virus replicates. Even though suspension cultures initially had lower yields, changes to the nutrient mix and the number of infections significantly improved results, proving that suspension systems can work well for large-scale production.

An essential component of this study was the examination of viral stability under diverse storage and processing conditions. We noted that intracellular virus (inside living CEF) exhibited substantially greater stability than extracellular virus in solution, which experienced substantial destruction during freeze-thaw cycles. This conclusion corresponds with previous research indicating that cell-associated HVT had more infectivity than free virus, owing to its protection against physical and enzymatic destruction.

So, all later versions, like frozen and dried vaccine preparations, used the virus inside fibroblast suspensions. This method was crucial for maintaining the virus's biological activity during extended storage periods.

The research further illustrated the significance of integrating stabilizing chemicals in vaccination preservation. Cryopreserved and freeze-dried virus samples kept higher levels of the virus when they

were mixed with the right protectants (FBS and DMSO for cryopreservation; peptone, sucrose, and gelatin for freeze-drying). In contrast, the virus levels dropped by up to 3.5 log in freeze-dried samples that didn't have stabilizers. Conversely, viral titers decreased by as much as 3.5 log in freeze-dried samples without stabilizers. This finding highlights how important the formulation is for keeping vaccines effective and matches earlier studies on freeze-dried viral vaccines.

The tested vaccination series, checked according to veterinary standards and GOST rules, showed strong sterility, safety, and ability to trigger an immune response. Both freeze-dried and frozen versions produced strong neutralizing antibody responses (1.67–2.33 log FFU50), similar to or better than the levels usually associated with protection against Marek's disease. Both the freeze-dried and frozen versions of the vaccine produced strong antibody responses (1.67–2.33 log FFU50), which are similar to or better than the levels usually associated with protection against Marek's disease. No adverse reactions or fatalities were documented during safety assessments, further corroborating the appropriateness of these formulations for neonatal chicks.

This research establishes a basis for the domestic manufacturing of a Marek's disease vaccine in Kazakhstan, diminishing dependence on imported vaccines and enhancing food security and poultry health infrastructure. Using the AV-0007 strain as a base for the vaccine, along with improved growing and stabilizing methods, offers a practical and expandable way to produce vaccines locally.

While the AV-0007-based vaccine demonstrated strong immunogenicity and biological activity under laboratory conditions, it is important to contextualize these results against existing commercial Marek's disease vaccines. Widely used HVT-based vaccines, such as those produced by Merial (France), Intervet (Netherlands), and FGBI ARRIAH (Russia), typically achieve virus titers of 10^6–10^7 $FFU_{50/cm3}$ in production, with established immunogenicity inducing antibody responses in the range of 1.5–2.5 log FFU_{50} in SPF chickens.

In our study, both the cryopreserved and freeze-dried formulations reached titers $\geq 10^{6.0}$ $FFU_{50/cm3}$ after optimization, which is consistent with the international standard for live poultry vaccines. Furthermore, the virus-neutralizing antibody response induced in one-day-old chicks (1.67–2.33 log FFU50) aligns with or slightly exceeds those reported for commercial HVT vaccines under controlled conditions. These results indicate that the vaccine candidate is competitive in both potency and immunogenicity.

Notably, commercial production protocols often involve industrial-scale roller bottle systems or bioreactors, which are not fully accessible in Kazakhstan. However, the methods proposed in this study – including suspension culture in optimized media – offer a scalable and cost-effective alternative adapted to local infrastructure. This supports the feasibility of establishing national production capabilities without requiring immediate large-scale investment in industrial platforms.

Additionally, the inclusion of both cryopreserved and lyophilized forms provides flexibility in storage and distribution, comparable to international vaccine options, many of which offer only one preservation form. The stability results obtained here, especially for formulations with protective media, are on par with imported analogues, which typically guarantee viability for 3-6 months under cold chain conditions.

This study focused on small-scale production and initial testing in living animals; future research should focus on larger pilot batches, longer tests for immune response (more than 21 days), and trials in real chicken farming settings. Furthermore, investigating the genetic stability of the AV-0007 strain and its effectiveness in conjunction with other vaccines (e.g., bivalent or trivalent formulations) could improve its practical use in strategies for managing poultry diseases comprehensively.

Conclusion

A production matrix lot of the cloned turkey herpesvirus strain (AV-0007) with a baseline titer of $10^{5.5}$ FFU_{50/cm^3} was refreshed and used as a foundation for generating high-yield viral biomass. Standardized protocols for obtaining developing chicken embryos (RCE) and preparing chicken embryo fibroblast (CEF) cultures were established using both stationary and roller monolayer methods, as well as for maintaining fibroblast viability in suspension. Technological parameters for virus propagation in CEF cultures were developed and optimized across all three cultivation systems-stationary monolayer, roller monolayer, and suspension-resulting in consistent production of viral suspensions with titers of at least $10^{6.0}$ FFU_{50/cm^3}.

A scalable technological scheme for the production of HVT biomass in CEF culture was successfully developed. Formulations of stabilizing media were also optimized to preserve viral infectivity during cryopreservation and lyophilization. Using these systems, experimental and pilot series of the Marek's disease vaccine were prepared and evaluated. In accordance with international standards for veterinary viral vaccines, comprehensive methods for vaccine quality assessment were selected and implemented, including sterility testing, safety evaluation in one-day-old chickens, determination of biological activity by viral titer, and immunogenicity testing via virus-neutralizing antibody titers.

These results validate the feasibility of developing a domestically produced vaccine against Marek's disease in Kazakhstan, which meets international quality benchmarks for biological safety and efficacy.

Authors Contributions

LK and BM: Conceptualization, formal analysis, designed the study, writing - original draft. BM, LK, AT: Conducted an extensive literature review and analyzed the data. GZh, TT, AT, K.B: ШInvestigation. BM, LK, AT: Bata Curation, Writing - Review & Editing. BM: supervision. All authors have read, reviewed, and approved the final manuscript.

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Prevalence of zoonotic intestinal protozoa infections of cats in Central Asia and border regions

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Abstract

Totally 97 sources from digitalized databases were analyzed with aim to assess the epidemiological significance and the introducing risk of feline intestinal zoonotic protozoa infections into Kazakhstan from neighboring countries. It was concluded that pet and stray cats in China, Russia, Central Asian countries and Kazakhstan serve as reservoirs for *Toxoplasma gondii, Cryptosporidium spp*., and *Giardia duodenalis.* They pose a significant threat to both humans and animals but are often overlooked due to a lack of awareness at the regional level. The diseases caused by them can be classified as neglected feline and human parasitic infections in Kazakhstan. To implement the One Health Concept, it is necessary to consolidate research and coordinate the work of public health, veterinary, and ecological services at an intergovernmental level for the study the molecular epidemiology and systematic monitoring the cat-borne zoonotic protozoa among definitive and intermediate hosts' populations and environment.

Keywords: *Felis catus;* cryptosporidiosis; giardiasis; toxoplasmosis; prevalence.

Introduction

The domestic cat (*Felis catus*) is one of the most popular synanthropic animals. According to 2024 statistical data, the cat population in Kazakhstan was approximately 101.2 thousand, with its population density increasing nearly 1.2 times over the last decade. Russia leads Europe in the number of cats (37.5 million) and ranks fifth in the world after the United States, Brazil, China, and Japan [1]. Sociological surveys indicated that 57% of Russians keep cats at home [2], with 18% of them owning two or more pets [3]. In Moscow and St. Petersburg alone, the cat population reaches 2.953 million, having grown by 20% over the past three years [4]. Furthermore, official sources, mass media, and social networks highlight a global and persistent trend of increasing stray cat populations in urban areas and food service locations, where access to food waste contributes to their survival and proliferation [5-8].

Given that cats can be carriers of numerous zoonotic diseases and have a high level of contact with humans, *Felis catus* holds significant medical and veterinary importance [6-14]. It is well established that 61% of the 1.415 known human infectious pathogens are zoonotic. According to WHO, WOAH, and FAO standards, zoonoses pose a serious threat to human health, particularly in low-income countries, where the burden of these diseases is often underestimated due to limited surveillance and funding [15-20]. For example, in Kyrgyzstan, where small-scale nomadic livestock farming plays an important economic role, factors such as poverty, limited research, poor healthcare and veterinary infrastructure, inadequate hygiene, and close interaction between humans, livestock, and other animals place up to 70%

of the population at constant risk of zoonotic infections [21-27]. Therefore, a quantitative assessment of the impact of zoonotic diseases in Central Asian countries is considered essential for determining healthcare priorities [15-20].

Among household cats parasitic diseases are fifth in frequency of diagnosis [28]. It has been shown that stray cats serve as sources of environmental contamination in urban areas by zoonotic parasitic pathogens, which can accumulate and remain viable in the environment for extended periods [29-33].

Given the increasing populations of both pet and stray cats and dogs, a systematic assessment of the current epidemiological status of parasitic diseases among carnivores in urban ecosystems is highly relevant for organizing an effective antiparasitic control system.

Preliminary results of the research project No. AR19679420 “Study of the genetic diversity of zoonotic parasites of cats circulating in Kazakhstan” funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan, revealed the dominance of protozoan pathogens, particularly *Giardia*, in the intestinal microbiome of *F.catus* subpopulations in the country's major cities [34].

Intestinal parasitic protozoa cause serious illnesses in their hosts. Intracellular coccidian, for instance, contribute to extensive cellular destruction, dysfunction of the digestive and other organ systems, and overall intoxication of the body [34]. *Giardia* infections cause micro traumas to intestinal epithelial cells, disrupt parietal digestion, enhance fermentation processes, and accelerate the evacuation of digested material. Intestinal protozoan infections are often associated with various allergic reactions [35]. As a result, pathogens such as *Cryptosporidium* and *Giardia* lead to severe diarrheal diseases, especially in children in developing countries [36-44]. Infection with *Toxoplasma gondii* can result in various pregnancy outcomes. The detection of IgM antibodies to *T. gondii* is associated with screening for congenital malformations in newborns [45, 46].

This review aims to assess the epidemiological significance and the risk of introducing feline intestinal zoonotic protozoa infections into Kazakhstan from neighboring countries.

The search for literary sources was conducted in databases such as Web of Science, PubMed, Scopus, Elsevier, Springer, Google Scholar, and eLibrary. Latin names of pathogens and author names were used as keywords. A total of 97 sources were analyzed, including recent publications on the epidemiology of intestinal zoonotic protozoa infections transmitted through cats in neighboring countries. Among them, 34 studies were from China, 32 from Russia, and 21 from Central Asia.

Prevalence of cat-associated protozoa infections among the human population in Central Asia and other bordering countries. Certain regions of Kazakhstan and neighboring countries, a notably high epidemiological burden of protozoan zoonoses, potentially transmitted by cats, has been reported. This is attributed to several risk factors, including poor hygiene, unsafe drinking water, poverty, overcrowding, and frequent contact with animals in household settings [47, 48].

Toxoplasmosis. Currently, up to 50% of the world’s human population is infected with toxoplasmosis, which in most cases remains latent [49, 50]. In East Kazakhstan, ELISA testing revealed that 16% of 504 residents were seropositive, with prevalence increasing with age [49]. Isolated clinical cases have been documented among immunocompromised individuals [51-53]. In Kyrgyzstan, over a 10-year period, 5.1% (11 of 216) of congenital toxoplasmosis cases were fatal, while population seropositivity reached 6.69% [20].

In Nanjing, China, a study of 6,849 pregnant women found that 6.4% tested positive for T. gondii antibodies, with 19.9% being IgM-positive and 80.1% IgG-positive. In a study of 1,032 newborns who were divided into normal and malformed groups based on their health status, IgM-positive rates were 0.6% in the normal group and 28.13% in the malformed group. The difference was statistically significant ($p < 0.01$). The primary risk factors for T. gondii infection were contact with animals and poor dietary habits [45].

Cryptosporidiosis. In the last decade of the 20th century, there were reported 383 cases of human cryptosporidiosis (15.8% in target patient groups), 75.7% of which were in children, predominantly under two years of age in Turkmenistan. Clinically, the infection was characterized by pronounced symptoms of severe diarrhea with hemorrhagic colitis. The main sources of infection were birds and animals, including home pets [56, 62].

In China, a retrospective epidemiological analysis of human *Cryptosporidium* infections (1987–2018) involving at least 200,054 people from 27 provinces revealed an average prevalence of 2.97%. Zoonotic species identified in humans in this region include *C. felis*, as well as six *C. hominis* subtypes [56-62].

Giardiasis. In Kazakhstan, 1,397 people were diagnosed with giardiasis in 2024, with an incidence rate of 6.97 per 100,000 population [53].

In Uzbekistan, giardiasis prevalence among the population ranged from 1.9% to 2.6% between 2018 and 2020. The share of *giardiasis* among identified intestinal protozooses reached 98% [63].

In China, the average infection rate with *G. duodenalis* is 0.85% (n=23,098), with the highest rate (9.46%) reported in Shanghai [64-66].

Travel-associated protozooses. GeoSentinel network data from january 2007 to december 2019 reported Western European tourists returning from international trips with infections caused by *G. duodenalis, Cryptosporidium spp.,* and other intestinal protozoa. A total of 2,517 protozoa cases were recorded, including 82.3% *giardiasis* and 11.4% *cryptosporidiosis*. Most travelers (64.4%) undertook long journeys (18–30 days). Giardiasis was most frequently contracted in Southern and Central Asia (45.8%) and sub-Saharan Africa (22.6%), while *cryptosporidiosis* was more common in sub-Saharan Africa (24.7%) and Southern and Central Asia (19.5%) [67].

Thus, *giardiasis, cryptosporidiosis*, and *toxoplasmosis* – zoonotic protozooses with *F. catus* as a potential source – are cosmopolitan diseases prevalent among the human population of Kazakhstan and all bordering countries. These infections carry a high potential for cross-border spread due to global mobility of people and pets. The scarcity of official and scientific data on infection rates with specific protozoan pathogens in some Central Asian countries is explained by weak organization of specialized parasitological studies.

Infection rates of intestinal zoonotic protozooses in cats in Kazakhstan and bordering countries. Although cats are recognized as a source of several dangerous intestinal protozoa zoonoses, studies on this group of parasitosis among *F. catus* in Central Asian countries are extremely limited and are confined to a few isolated publications.

Toxoplasmosis. In Kazakhstan, serological testing revealed positive results in 25% of adult cats and 3.5% of kittens under one year old. Using coprological methods, *T. gondii* oocysts were identified in 1.6–5.6% of *F. catus* [69].

Toxoplasmosis is considered an endemic infection in Kostroma Oblast [70], Krasnodar and Perm Regions [71], Saint Petersburg [72], and other regions of Russia [73]. In Voronezh Oblast, cats and dogs play a key role in maintaining epidemiological tension, with infection rates of 52% and 36%, respectively [74]. In Voronezh, *T. gondii* oocysts were found in the feces of 20.59% of cats [75]. Among 84 domestic cats with outdoor access, 50% were seropositive; 71.5% of these were adults and 28.5% kittens. Among 126 stray cats, 60% were seropositive from which 84.6% were adults and 15.4% kittens [74]. A serological screening of pet carnivores in Tatarstan revealed 15.8% positivity. In Kazan, 34.9% of tested cats were seropositive [76]. In Perm, 35.1%, in Vologda, 32%, and in Moscow, 33.8% tested cats were positive. Coprological methods revealed *T. gondii* oocysts in 7.3% of cats [78].

In China, *T. gondii* was first isolated from cats in Fujian Province in the mid-20th century, and since then, extensive studies have been conducted to understand the parasite's epidemiology and biology [79, 80]. For instance, 100% infection was identified via immunological testing of 43 stray cats in Shanghai [79]. Overall, the average seropositivity in home cats in China ranges between 15-25% [82-84].

Thus, as the definitive host, the cat plays a crucial role in transmission of *T. gondii* and is the main source of infection for humans. Serological studies show that infection rates vary significantly depending on the region and type of cats, with stray cats generally being more frequently infected than pet ones [84].

Other protozoa infections in cats. Coprological studies the cat population in Moscow revealed a relatively diverse species composition of intestinal protozoa. Cysts of *Giardia spp.* were detected in 4.07%, and the subfamily *Toxoplasmatidae* oocysts were found in 0.48% of *F. catus* [85, 86]. A higher protozoan infection rate was recorded among stray animals, with Giardia spp. cysts found in 5.8% of samples, averaging 84.5 ± 9.1 cysts per a microscope field [87]. In the Volga region, the most frequently reported protozoa in *F. catus* were *Giardia, Isospora,* and *Sarcocystis* genera [88].

During coprological examination of feces from 164 cats in Almaty Metropolis, Kazakhstan, the prevalence of *Giardia* was significantly higher ($p<0.05$) in shelter cats (26.1%) compared to pet cats (4.2%). The infection rate was also higher in young cats (18.8%) than in adults (8.3%). The antigen presence was nearly twice higher in diarrheic cats than in those with firm stool. *G. duodenalis* prevalence was slightly lower in males than in females [34].

The bibliography on the molecular epidemiology of *Cryptosporidium spp.* and *G. duodenalis* in cats remains quite limited, although such studies are critical for assessing infection levels, genetic identity, and the public health potential of these parasites. For example, in a molecular study of fecal samples from 346 domestic cats in eastern China, *Cryptosporidium spp.* was detected in 2.3%, and G. *duodenalis* in 1.4% of animals. Three cats had mixed infections of *Cryptosporidium spp*. and *Tritrichomonas foetus,* while other mixed infections were not observed [89].

In Guangdong Province, China, PCR and genomic sequencing were used to identify and genotype *Cryptosporidium spp.* and *G. duodenalis* in fecal samples from 418 cats. The overall infection rates were 6.2% for *Cryptosporidium spp.* and 3.6% for *G. duodenalis.* Purebred cats were more susceptible to C*ryptosporidium spp.* and *G. duodenalis* (12.4% and 10.8%, respectively). Cats under 6 months of age had a *Cryptosporidium spp*. infection rate of 13.6%, significantly higher than older animals [90-97].

Thus, the level of cat infection with zoonotic protozoa generally correlates with human infection rates in the reviewed regions and contributes to an unfavorable environmental safety status regarding these parasitic diseases.

Conclusion

The data presented, along with preliminary pioneering research in Kazakhstan, suggest that cats in Central Asia and neighboring countries serve as reservoirs for several zoonotic protozoan pathogens, including *Toxoplasma gondii, Cryptosporidium spp*., and *Giardia duodenalis*. These parasites pose a significant threat to both humans and animals but are often overlooked due to a lack of awareness at the regional level. These pathogens and the diseases they cause can be classified as neglected feline and human parasitic infections in Central Asia.

Given the high epidemiological burden and real risk of cross-border transmission, comprehensive studies on the molecular epidemiology of cat-borne zoonotic protozoa and systematic monitoring among human populations, definitive and intermediate hosts, and in the environment are essential. To implement the One Health concept, it is necessary to consolidate scientific support and coordinate the work of public health, veterinary, and ecological services at an intergovernmental level.

Authors' Contributions

LL, AE, VK and AB: Conceptualization, methodology. DM, LS: Investigation and formal analysis. DM: Writing-original draft. DM, AE, LS: Writing-review and editing. LA: Project administration. All authors read, reviewed, and approved the final version of the manuscript.

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Epizootic situation and comprehensive diagnosis of equine salmonellosis-associated abortion in the Republic of Kazakhstan

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Abstract

Background and Aim. Abortion associated with equine salmonellosis is an infectious disease that significantly affects the productivity of herd-based horse breeding in the Republic of Kazakhstan. The disease is characterized by late-term abortions and is associated with considerable economic losses in the agricultural sector. The aim of this study was to detect *Salmonella* DNA using molecular genetic methods.

Materials and Methods. The study was based on pathological material collected from horses in nine regions of Kazakhstan during the period from 2023 to 2025.

Results. PCR analysis revealed that the proportion of positive samples ranged from 25% to 33.3%, depending on the year. Enzyme-linked immunosorbent assay (ELISA) of 309 equine serum samples detected antibodies to *Salmonella abortus equi* in 29 cases (10.6%). Microbiological investigations using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) confirmed the presence of *Salmonella spp.*, as well as opportunistic microorganisms such as *Enterobacter hormaechei, Pantoea agglomerans*, and *Escherichia hermannii.*

Conclusion. The obtained results confirm the circulation of the pathogen among horses and emphasize the need for ongoing epizootiological surveillance. Furthermore, they highlight the importance of continued molecular and genetic studies, including whole genome sequencing, to enhance understanding of the disease's epidemiology and to optimize preventive measures.

Keywords: abortion; diagnostics; epizootiology; horses; MALDI-TOF; salmonellosis.

Introduction

Herd-based horse breeding holds a significant position in the agricultural sector of the Republic of Kazakhstan. As of January 1, 2025, the equine population exceeded 4.2 million, placing the country among the leading nations within the Commonwealth of Independent States in terms of horse numbers. Horses serve not only as draft and transport animals in remote areas, but also as important sources of

meat and milk. The production and processing of mare's milk into koumiss holds cultural and economic importance. However, the characteristic practices of herd management –particularly year-round grazing – create favorable conditions for the spread of infectious diseases, among which salmonellosis-associated abortion poses a particular threat.

Equine abortion remains one of the most significant problems in horse breeding, leading to substantial economic losses and reduced genetic potential in herd populations. It is estimated that infectious causes account for approximately 30–50% of all equine abortions globally, with bacterial pathogens being responsible in over 60% of confirmed infectious cases .Among these, *Salmonella enterica* subsp. *enterica* serovar abortus equi (*S. abortus equi*) holds particular importance as an abortifacient pathogen causing systemic infection and placentitis in pregnant mares [1].

Severe outbreaks of *S. abortus equi* have been reported in foals in Italy, with high morbidity and mortality rates, particularly in mixed infections [2]. Similar cases have been reported in Argentina [3] and central Italy [4], where *S. abortus equi* was identified as a primary abortifacient agent. In Xinjiang, China, a seroprevalence of 20.9% was detected among 971 horses tested using ELISA [5]. Australian studies on stud farms also confirm the circulation of multiple *Salmonella* serotypes [6], while pooled environmental testing in equine hospitals in the U.S. supports the pathogen's persistence in clinical settings [7].

Further research from Italy has shown public health implications associated with *S. abortus equi* in horses slaughtered for meat [8]. Russian researchers have described thrombohemorrhagic syndromes in young livestock associated with salmonellosis [9]. In Argentina, *S. abortus equi* was recognized as an emergent cause of abortion in equines [3].

In Kazakhstan, foundational research was provided by *Sultanov* et al. [1], while recent molecular characterization of local isolates was carried out by *Mussayeva* et al. [10]. According to *Issabekov* et al. [11], bacterial abortion remains a persistent problem in the northern regions of Kazakhstan. These findings are supported by official data from the Committee for Veterinary Control and Supervision of the Ministry of Agriculture, which reported 217 cases of equine abortion between 2021 and 2024, with bacterial causes identified in 132 cases (60.8%) and *S. abortus* equi confirmed in 14.3% of these [12].

New serological approaches using recombinant outer membrane proteins have been proposed for diagnostic improvements [13], while recent experimental vaccine development in Kazakhstan demonstrates active efforts toward prevention [14].

The aim of this study is to assess the current epizootic status of salmonellosis-associated abortion in horses in Kazakhstan. This is achieved by detecting *S. abortus equi* DNA using PCR, evaluating antibody prevalence via ELISA, performing microbiological identification using MALDI-TOF MS, and analyzing the regional and temporal dynamics of positive cases.

Materials and Methods

Ethical approvals

This study was approved by the Local Ethics Committee of the Faculty of Veterinary Medicine and Animal Husbandry Technology at the S. Seifullin Kazakh Agrotechnical Research University, meeting held on November 8, 2023 (Protocol No. 3).

This study was conducted within the framework of the project IRN AR22783162 "Epidemiology and Molecular Genetic Analysis of the Causative Agent of Equine Salmonellosis-Associated Abortion" and covers the period from 2023 to 2025. Research materials including serum samples and pathological specimens were collected from horse farms located in the Karaganda, North Kazakhstan, Abai, Mangystau, Pavlodar, Atyrau, Ulytau, West Kazakhstan, and Akmola regions. A combination of molecular biological, serological, and microbiological diagnostic methods was employed to detect *Salmonella* infection.

PCR was used to detect *Salmonella* DNA in pathological material collected as part of epizootiological surveillance. According to the summary analysis of the epizootic situation, a total of 34 samples were examined. These samples were collected from 15 aborted equine fetuses originating from farms located in the Karaganda, North Kazakhstan, and Akmola regions. From each fetus, pathological material was sampled from internal organs – primarily liver, lungs, spleen, and intestines – depending on the condition and integrity of the carcass. Sampling was performed by veterinary personnel during the spring and autumn periods of 2023-2025, which coincide with seasonal abortion peaks in herd-based

horse breeding. The samples were placed into sterile containers, cooled to 4 °C immediately upon collection, and transported to the laboratory within 24 hours for further molecular analysis.

Diagnosis was performed using a certified real-time PCR test kit from VetFactor. DNA extraction was carried out using the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Germany). Amplification was performed using a CFX96 thermal cycler (Bio-Rad, USA). Reliability of the results was ensured through the inclusion of positive and negative control samples.

An ELISA was conducted in 2025 on 309 serum samples collected from horses across nine regions. The ELISA plates were sensitized with a thermostable extract of *S. abortus equi* (10 μg/mL). An anti-horse conjugate (Cusabio, China) diluted 1:2000 was used, along with TMB substrate (Immunobiotech, Russia) and a stop solution of 0.02 M H2SO4 Serum samples were diluted from 1:100 to 1:800, and in some series up to 1:6400. Optical density was measured at 450 nm. A result was considered positive if the optical density was twice (or more) of the negative control.

Microbiological analysis was conducted in 2025 on samples from aborted horse fetuses obtained from farms in the Karaganda and Kostanay regions. The analysis included tissue samples from the liver, lungs, kidneys, spleen, and intestines.

One gram of biological material was collected from each organ and transferred under sterile conditions for further processing. The samples were thoroughly homogenized in 9 mL sterile physiological saline, maintaining a 1:10 ratio to ensure uniform bacterial distribution. The homogenized material was allowed to stand briefly to allow larger tissue fragments to settle, after which the suspension was centrifuged to remove residual tissue debris. The resulting supernatants were incubated in a thermostat under optimal temperature conditions, creating an environment conducive to the activation and initial proliferation of any Salmonella present.

The prepared suspensions were streaked onto Endo agar (for *Enterobacteriaceae detection*) and Salmonella-Shigella (SS) agar using a sterile Drigalski spatula. The plates were incubated aerobically at 37 °C for 18-24 hours. Colonies with characteristic morphology were subcultured for purification.

Real-time PCR was conducted using a certified VetFactor diagnostic kit (VetExpert, Kazakhstan), optimized for *Salmonella spp*. detection. DNA extraction from pathological samples was performed with the QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Each PCR reaction (25 μL) included 5 μL of DNA template, primers/probes from the kit, and Taq polymerase mix. Amplification was carried out on a CFX96 thermal cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. Fluorescence was detected in the FAM channel. Each run included positive and negative controls to validate results.

Indirect ELISA was developed using a thermostable extract of *Salmonella abortus equi* (10 μg/mL) coated on 96-well microplates in carbonate buffer (pH 9.6), incubated overnight at 4 °C. After blocking and washing, horse serum samples (diluted 1:100 to 1:6400) were added and incubated at 37 °C for 1 hour. Detection was carried out using horseradish peroxidase–conjugated anti-horse IgG (Cusabio, China; 1:2000), followed by TMB substrate and a 0.02 M sulfuric acid stop solution. Absorbance was measured at 450 nm. A result was considered positive if the OD value was at least twice that of the negative control.

Identification of the isolated microorganisms was conducted using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The analysis was performed at the National Center for Biotechnology of the Republic of Kazakhstan (Astana, Kazakhstan). Colonies grown on Endo and Columbia agar were transferred to a polished steel target plate using a sterile loop. After air drying, 1 μL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution was applied to each spot. Spectra acquisition was carried out using a Bruker Microflex LT mass spectrometer (Bruker Daltonics, Germany). Identification was performed using the Bruker Biotyper software and database. Score values ≥2.0 were considered reliable for species-level identification.

Results and Discussion

PCR

According to epizootiological surveillance data collected between 2023 and 2025, a total of 34 pathological samples from horses across nine regions of Kazakhstan were tested. Positive results were observed in 5 out of 16 samples (31%) in 2023; 2 out of 6 samples (33.3%) in 2024; and 3 out of 12

samples (25%) in 2025. This confirms the continued circulation of *Salmonella spp*. among herd-based horse populations, particularly in the Karaganda, North Kazakhstan, and Akmola regions.

ELISA

In total, 309 serum samples from mares across nine regions were analyzed. Antibodies to *S. abortus equi* were detected in 29 cases, accounting for 10.6% of all samples tested (Table 1).

Table 1 – Number of samples tested for *Salmonella abortus equi*

№	Region	Number of Samples Tested	Number of Positive Samples	Prevalence, %
1	Karaganda	39	7	17.94
2	Pavlodar	40	3	7.5
3	Akmola	31	5	16.12
4	North Kazakhstan	34	7	20.58
5	Mangystau	30	0	0
6	Abai	38	4	10.52
7	Atyrau	33	2	6.06
8	Ulytau	37	1	2.7
9	West Kazakhstan	27	0	0
	Total	309	29	

To visualize the seroprevalence of Salmonella abortus equi in different regions, a bar chart was constructed (Figure 1). The highest prevalence rates were observed in North Kazakhstan (20.6%) and Karaganda (17.9%) regions, while no antibodies were detected in samples from Mangystau and West Kazakhstan regions.

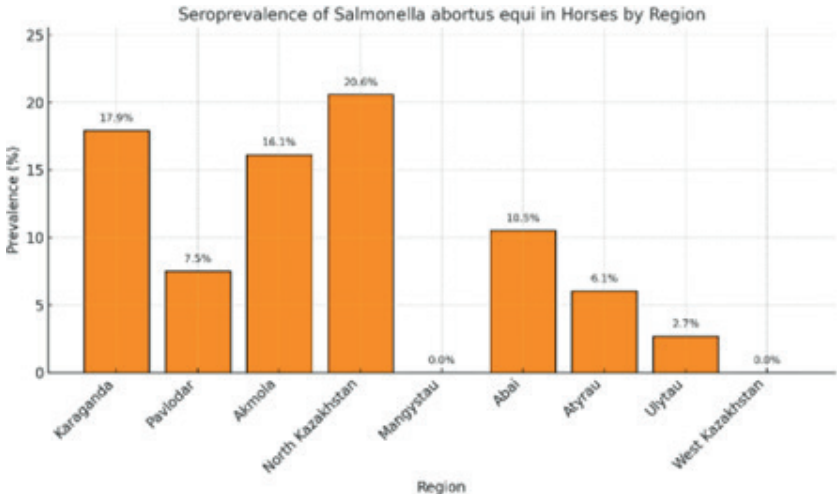

Figure 1 – Regional distribution of seroprevalence rates (%) of *Salmonella abortus equi* in horse serum samples from nine regions of Kazakhstan

In certain cases, high antibody titers of up to 1:1600 were observed, which may indicate persistent carriage or prior infection.

Microbiological Investigations

A bacteriological analysis was conducted on aborted equine fetal tissues received from farms located in the Karaganda and Kostanay regions. MALDI-TOF mass spectrometry performed on isolated colonies revealed the presence of *Enterobacter hormaechei* in lung-derived samples. This microorganism belongs to the *Enterobacteriaceae* family and is considered an opportunistic pathogen, capable of causing various infections in both animals and humans, particularly in immunocompromised individuals.

Further MALDI-TOF analysis identified colonies isolated from a liver sample as *Lelliottia amnigena*. Also, a member of the *Enterobacteriaceae* family, this is an environmental and commensal organism occasionally associated with opportunistic infections (Figure 2).

Rank (Quality)	Matched Pattern	Score Value
1 (-)	Salmonella sp (enterica st Stanley) 15 LAL	1.662
2 (-)	Salmonella sp (enterica st Dublin) Sa05_188 VAB	1.596
3 (-)	Salmonella sp (enteritidis) 25089078 (PX) MLD	1.569
4 (-)	Salmonella sp (enterica st Hadar) Sa05_506 VAB	1.563
5 (-)	Salmonella sp (choleraesuis) 08 LAL	1.506

Figure 2 – Results of mixed-sample analysis: proteins characteristic of *Salmonella spp.* identified

Colonies isolated from liver and lung samples were cultured on Endo agar and Columbia agar for further investigation. Colonies obtained from the spleen were distributed as follows: three Petri dishes with Endo agar, and one with Columbia agar. Subsequent analysis will help clarify the species identity of the isolates and confirm the presence of *Salmonella spp.* (Figure 3).

Result Overview

Organism names which are in blue and underlined are linked to the matching hint table below.

Analyte Name	Analyte ID	Organism (best match)	Score Value	Organism (second best match)	Score Value
A7 (+++) (A)	A7	Salmonella sp	2.437	Salmonella sp	2.4
B7 (++) (A)	B7	Salmonella sp	2.220	Salmonella sp	2.01
C7 (+++) (A)	C7	Salmonella sp	2.334	Salmonella sp	2.23
D7 (+++) (A)	D7	Salmonella sp	2.437	Salmonella sp	2.311
E7 (+++) (A)	E7	Salmonella sp	2.345	Salmonella sp	2.297
F7 (+++) (A)	F7	Enterobacter hormaechei	2.482	Enterobacter hormaechei	2.462
G7 (+++) (A)	G7	Enterobacter hormaechei	2.4	Enterobacter hormaechei	2.379
H7 (+++) (A)	H7	Enterobacter hormaechei	2.488	Enterobacter hormaechei	2.468
A8 (++) (A)	A8	Escherichia hermannii	2.237	Escherichia hermannii	2.191
B8 (+++) (A)	B8	Escherichia hermannii	2.394	Escherichia hermannii	2.346
C8 (+++) (B)	C8	Lelliottia amnigena	2.403	Lelliottia amnigena	2.381
D8 (+) (B)	D8	Lelliottia amnigena	1.95	Lelliottia amnigena	1.917

Figure 3 – MALDI-TOF mass spectrometry result of a colony isolated from the spleen

The following pathogens were identified in tissue samples from the liver, lungs, intestines, and spleen of aborted equine fetuses from farms in the Karaganda region: *Salmonella spp., E. hormaechei, Escherichia hermannii, and L. amnigena.* In contrast, samples from farms in the Kostanay region (Vladimirovka village) revealed the presence of *Escherichia fergusonii* (kidneys), *Pantoea agglomeran*s (lungs, liver, spleen, and heart), and *Mixta calida* (intestines). The use of MALDI-TOF mass spectrometry confirmed the polyetiological nature of microbial associations. In several cases, the isolation of *Salmonella spp.* coincided with the presence of opportunistic bacteria, which may enhance the pathogenicity of the infectious process.

The data obtained confirm the ongoing epizootic concerns regarding salmonellosis-associated abortion in horses within several regions of the Republic of Kazakhstan. Although no official outbreak foci have been reported, the detection of relevant species via PCR and ELISA indicates ongoing pathogen circulation and the potential threat posed by latent or chronic carriers. The proportion of PCR-positive results from samples collected between 2023 and 2025 ranged from 25% to 33%. This may be

influenced by regional disease prevalence, as well as the quality of pathological material collected. The highest proportions of positive cases were recorded in the Karaganda, Akmola, and North Kazakhstan regions, aligning with serological and microbiological findings from 2025.

ELISA testing confirmed the presence of antibodies to *S. abortus equi* in 10.6% of serum samples. High antibody titers (up to 1:1600) observed in some animals suggest prior infection or an ongoing chronic condition. The positive results observed in samples obtained from multiple regions – from Abai to Atyrau – point to a broad geographic distribution of the pathogen, with no clear regional confinement.

Noteworthy are the microbiological findings from aborted fetuses, where MALDI-TOF mass spectrometry not only identified *Salmonella spp.*, but also co-isolated several opportunistic bacteria, including *E. hormaechei, E. hermannii, P. agglomerans, E. fergusonii, L. amnigena*, and *Mixtacalida.* These findings highlight the polyetiological nature of the disease, wherein multiple microorganisms may concurrently contribute to pathogenesis. Mixed infections are likely to exacerbate clinical severity and complicate diagnostic efforts.

The results underscore the importance of incorporating comprehensive diagnostic methods including PCR, ELISA, and MALDI-TOF in infectious disease surveillance systems for herd-based horse breeding. This is particularly relevant for remote areas of Kazakhstan where veterinary infrastructure is limited and horses remain integral to traditional lifestyles and local economies.

This study not only confirmed the presence of *Salmonella* infection in horses but revealed important aspects of its polyetiology and regional epizootic patterns, providing a foundation for the development of targeted preventive and veterinary measures.

Conclusion

These findings confirm the widespread prevalence of salmonellosis-associated abortion in horses in Kazakhstan, and demonstrate the continued circulation of *S. abortus equi* among the equine population. The use of diagnostic methods such as PCR, ELISA, and microbiological testing facilitates accurate pathogen identification and improves diagnostic outcomes. Moreover, this study highlights the polyetiological nature of the disease, evidencing the necessity for further investigation into the interactions of multiple microorganisms in equine salmonellosis pathogenesis. The continued use of molecular and serological techniques is essential for effective surveillance and prevention strategies.

Authors' Contributions

TB, ZhB and GE: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript., DSH, AK, AS, IA and SA: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript".

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Conflicts of Interest

The authors declare that they have no competing interests.

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UDC 619:616.9-036.22(574) (045) **Research article**

Impact of anthropogenic factors on the epidemiology of anthrax in Kazakhstan

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Abstract

Background and Aim. Despite significant success in the fight against anthrax, cases of animal and human disease are still registered in Kazakhstan. The presence of many permanently unfavorable points with numerous anthrax burial sites contributes to the maintenance of epidemiological unfavorability in the country. Moreover, active human activity in potentially dangerous zones often contributes to the activation of the epidemic process and the emergence of new infection outbreaks. The purpose of this research was to study the degree of influence of various anthropogenic factors on the epidemiology of anthrax in Kazakhstan.

Materials and Methods. The analysis object was information about anthrax outbreaks in Ka-zakhstan from 1933-2024. The study materials were data from the cadastre of stationary points unfavorable for anthrax, materials for statistical veterinary reporting, and our own records obtained during expedition trips. The data were analyzed using system modeling (Monte Carlo method) and spatial-geographical analysis via the Moran autocorrelation method.

Results. In total, 4,089 outbreaks of anthrax in Kazakhstan occurred during the study period. In chronological terms, 5 historical periods were distinguished, and the analysis of the available epidemiological data for 1933–2024 indicates that the main periods of increase and decrease in the annually registered anthrax outbreaks correspond to certain periods of economic and socioeconomic change in the country and the introduction of antiepizootic measures against this infection.

Conclusion. The analysis shows a pronounced uneven temporal and spatial distribution of anthrax foci and significant differences in the dynamics of the epizootic process in Kazakhstan. At the moment, new information is being formed on the factors influencing the ecology and epidemiology of the disease, which are associated with various forms of organization and management of the economy, urbanization of the population, changes in their social conditions and the influence of other anthropogenic factors.

Keywords: anthrax; anthrax burial sites; epizootic process; epizootic situation; Kazakhstan.

Introduction

Modern problems associated with the epizootic process of diseases in farm animals depend on the influence of several anthropogenic factors that aggravate the epizootic situation and contribute to the manifestation of an infectious disease in certain areas. Many authors (*S. Chowdhury* et al., 2021; *C. An* et al., 2023; *C.D. Reddell* et al., 2023; *I. Abirova* et al., 2023) point to the role of human activity in triggering the natural geographical and economic factors affecting the epizootic process of a disease [1, 2, 3, 4].

Moreover, outbreaks of such infections cause enormous economic losses for the country's agricultural sector and, more importantly, pose a serious threat to human health and life [4, 5, 6]. In this respect, studying potential sources and/or factors of pathogen transmission in a certain territory is critical. Such sources are often epidemiologically significant veterinary animals on which special sanitary and epidemiological requirements are imposed [7]. In this context, the role of veterinary epidemiology is also important, as this scientific field studies the epizootic process of especially dangerous infections and the potential impact of objects of epidemiological importance on the infection process [8].

Despite many years of success in combating anthrax, cases of animal and human disease are still registered in the Republic of Kazakhstan [9, 10]. Anthrax infection is a typical anthropurgic infection that is territorially confined and actively manifests itself, mainly in the warm season [11].

The existence of many permanent anthrax-unfavorable points (PAPs) with numerous burial sites for the corpses of animals that died from anthrax contributes to the maintenance of epizootologically and epidemiologically unfavorable conditions in the country. Research has shown that almost every fourth settlement in the Republic of Kazakhstan is permanently unfavorable for anthrax [12].

Currently, in almost all regions of the Republic of Kazakhstan, outbreaks of anthrax occur against the background of sporadic morbidity [9, 13]. The development of such a situation is facilitated by the changing socioeconomic living conditions of the population and the influence of numerous natural and anthropogenic factors that require further study [14, 15, 16, 17].

In this context, it is critical to establish the general territorial distribution patterns of anthrax in the Republic of Kazakhstan and the factors that preserve the activity of stationary unfavorable points for anthrax (SNP). Therefore, the present study aimed to determine the degree of influence of various anthropogenic factors on the epidemiology of anthrax in Kazakhstan.

Materials and Methods

The objects of the study were data on the registration of cases of Anthrax throughout the Republic of Kazakhstan in the period from 1933 to 2024. Statistical data from the veterinary accounting and reporting of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan, data from the Cadastre of inpatient Anthrax-affected areas of the Republic of Kazakhstan (2002) [18], and our own records obtained during expedition-ary trips to livestock farms were also used as primary materials. In total, 4,089 anthrax outbreaks were registered in the territory of the Republic of Kazakhstan during the analyzed period.

For statistical processing of the data obtained, the basic principles of statistical analysis, with system modeling (Monte Carlo method) and spatial and geographical analysis, using the Moran automatic correlation technique [19], were used.

The Monte Carlo method is a group of numerical techniques that utilize random sampling to simulate various processes and solve mathematical problems. The essence of this method lies in the repeated execution of random experiments, the results of which are then analyzed to obtain a statistical estimate of the parameter of interest [20].

The spatial autocorrelation method (Global Moran’s I index) enables the analysis of spatial autocorrelation based on both the locations of objects (such as anthrax foci) and their attribute values. Using the provided set of objects and their associated attributes, the tool assesses whether the observed spatial pattern is clustered, evenly distributed, or random [21].

Results and Discussion

The long-term concept of combating anthrax in Kazakhstan (formerly the territory of the USSR) was based on the large number of registered and unregistered anthrax foci (burial sites) in the country, which

pose a constant potential threat of new outbreaks of the disease. The mainte-nance of epizootologically and epidemiologically unfavorable conditions in the country is facilitated by the presence of many SNPs with numerous burial sites for animals that died from anthrax. From 1933-2024, 1,767 SNPs were registered in Kazakhstan, in which >1,760 people and >25,000 animals became infected.

The long-term dynamics of the number of registered outbreaks of anthrax in Kazakhstan reveal a complex and ambiguous epidemiological situation (Figure 1).

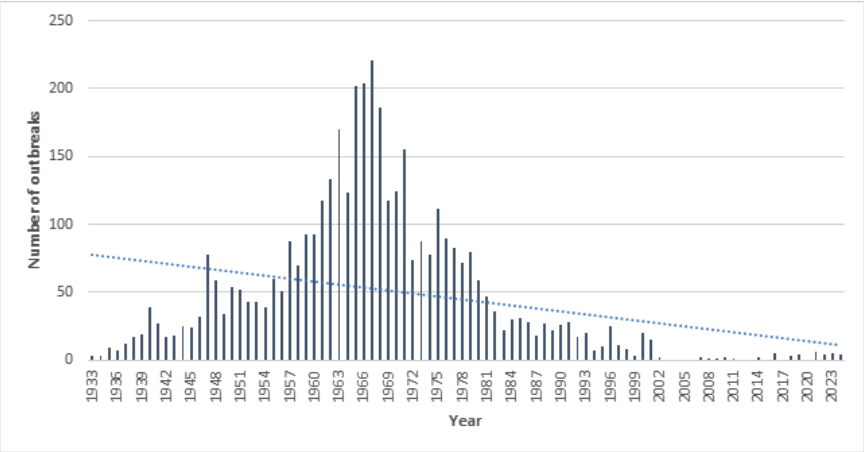

Figure 1 – Time dynamics of the registration of anthrax outbreak
s in Kazakhstan from 1933-2024

The figure demonstrates that, overall, there is a downward trend in the annual number of outbreaks across the country over the entire study period. However, it should be noted that the number of outbreaks varied significantly during different time intervals, ranging from isolated cas-es to several hundred outbreaks per year. Furthermore, no anthrax outbreaks among animals were offi-cially registered from 2003-2006.

Naturally, the dynamics of the epizootic process of anthrax over such a significant period are influenced by many factors, some of which are anthropogenic. Thus, the results of human economic activity, such as increasing the number of susceptible animals, expanding farms, organizing anthrax burial sites, conducting construction and excavation works, and implementing mass preventive measures, undoubtedly impacted the dynamics of the anthrax epizootic process. In this context, the analysis of the available epizootological data from 1933-2024 suggests that the main periods of increase and decrease in the annually registered anthrax outbreaks correspond to certain periods of socioeconomic and agricultural change in the country and the stages of the introduction of antiepi-zootic measures against this infection (Table 1).

Table 1 – Summary of anthrax outbreaks in Kazakhstan across various historical periods from 1933-2024

Period, years	Total number of outbreaks during the period	Number of outbreaks by animal species				Minimum number of outbreaks per year	Maximum number of outbreaks per year
		Cattle	Horses	Pigs	Small ruminants		
1933-1953	615	415	34	22	144	3	78
1954-1968	1850	1051	90	95	614	39	221
1969-1983	1236	747	73	30	386	22	155
1984-2001	346	218	34	5	89	3	31
2002-2024	42	30	7	2	3	0	6

In total, 4,092 anthrax outbreaks occurred in Kazakhstan during the study period. In chronological terms, 5 historical periods (1933-1953, 1954-1968, 1969-1983, 1984-2001, and 2002-2024) corresponded to the stages of increase and decrease in annual morbidity and were associated with the peculiarities of various political decisions, economic activities, and veterinary supervision processes in the republic.

During the first period (1933-1953), there was a significant increase in annual morbidity (615 outbreaks officially registered), most likely due to the development of the national economy and the increase in the number of farm animals. Moreover, the organization of measures for disinfecting and disposing of dead animals was weak. For example, according to data from the National Statistics Committee, there were 1.8 mln heads of cattle and 2.2 mln heads of small cattle in Kazakhstan in 1933; by 1953, their numbers had reached 4.1 and 18.2 mln heads, respectively [22].

In 1951, with the adoption of new veterinary and sanitary rules regulating the mandatory burning of anthrax corpses without prior dismemberment to stop further contamination of the soil with anthrax bacilli, a slight decrease in the incidence rate was noticeable from 1951-1954 (Figure 1).

However, starting in 1955, there was a significant increase in the registration of disease outbreaks. The second period (1954-1968) demonstrated the highest incidence of anthrax in animals in Kazakhstan. In total, 1.850 outbreaks were registered during this historical period; the average number of outbreaks per year was 123, and the maximum number (221) for the entire observation period was registered in 1967. One of the most likely reasons for this high outbreak prevalence is the state campaign to develop virgin lands that began in 1954 and significantly affected Kazakhstan. A massive influx of people (>2 mln) began, with individuals moving to Kazakhstan from all over the USSR to develop virgin lands. Accordingly, the growth of the rural population contributed to the growth of the livestock population (the number of small cattle and pigs increased 4-fold from 1941–1961). In addition, the mass plowing of virgin lands led to the removal of anthrax spores from the soil surface in the territories of old and/or unaccounted anthrax burial sites. Subsequently, the erosion of soils by wind contributed to the spread of anthrax spores across significant distances. All these factors significantly increased the likelihood of animal contact with anthrax spores, which was confirmed by studies by Soviet scientists [23].

The republic has immunized susceptible animals against anthrax since the beginning of the 1950s; however, mass vaccination of the entire susceptible population of farm animals has been applied only since 1961. Thus, although a downward trend in the annual incidence of anthrax has been observed since 1969, the situation remained tense until the early 1980s. In total, 1.236 outbreaks of anthrax were registered in Kazakhstan from 1969-1983, with an average of >80 outbreaks annually.

Since 1983, the incidence rate has been stabilized at an average of 20 outbreaks per year (a total of 346 outbreaks were registered between 1984 and 2001), facilitated by the establishment of control over compliance with veterinary legislation, alignment and compliance with veterinary and sanitary rules at facilities of epidemiological significance, and wider susceptible livestock vac-cination coverage [24].

The collapse of the USSR led to a profound crisis in the agro-industrial complex of all post-Soviet republics, including the Republic of Kazakhstan. The established economic system was disrupted, and former state and collective farms began to disband due to bankruptcy, which was accompanied by a significant migration of the rural population to cities, leading to a sharp reduction in the number of all farm animal types. For example, from 1991-1998, the number of cattle in the Re-public of Kazakhstan decreased from 9.592 thousand heads to 3.958 thousand heads, that is, an almost 2.3-fold decrease; the same trend was observed for other farm animal types [25, 26]. That is, the decrease in the number of animals susceptible to anthrax in the country has to some ex-tent influenced the dynamics of the epizootic process and contributed to reducing the tension of the epidemic situation.

The gradual improvement of the economic situation in Kazakhstan since the late 1990s, the attraction of large investments in livestock farming, and the improvement of veterinary services have also contributed to the improvement of the epizootic situation, reflected in the registration of anthrax cases since 2002. A total of 42 outbreaks of anthrax were registered from 2002-2024.

Due to the systematic implementation of complex preventive and antiepidemic measures, the areas of anthrax registrations in Kazakhstan have gradually changed. Unlike the situation in the 1950s to the 1970s, outbreaks of the disease have virtually ceased in vast territories, including the Atyrau, Kostanay, and Mangistau regions, where no cases of the disease have been observed in humans or animals for

the past 20-30 years (Mangistau – 50 years). Nevertheless, outbreaks have been registered in areas that previously experienced intense morbidity, potentially indicating a continuing threat of the anthrax pathogen being carried out from old and/or unaccounted burial sites due to agricultural activities, construction, and other human activities. Various natural factors, such as floods and earthquakes, represent an additional source of infection.

Epidemiologically significant veterinary objects significantly influence the epizootic process of a particular nosological unit in many socially significant zoonoses. The territory of Kazakhstan has historically been considered unfavorable for many diseases of contagious etiology common to humans and animals. Some nosological forms have a natural focal character; other diseases are anthropurgic, meaning that the development of the epizootic process of such diseases depends directly on human activity [6, 14, 15].

For example, the development of virgin fallow lands in Kazakhstan aimed to strengthen national food security and improve the economic indicators of virgin regions. However, this campaign had many negative effects, including negatively impacting the dynamics of the epizootic process of socially significant infections, such as anthrax.

During the intensification of the agro-industrial complex, livestock complexes were organized on state farms, the number of livestock increased, enterprises for the production and processing of meat and dairy products were built, and wool, leather, and fur procurement points were created. There are several challenges affecting animal health and safety. Poor infrastructure, lack of reliable medicines for prevention, insufficient trained veterinary staff, and ineffective disease control make it difficult to protect people from infections spread by animals [22, 25].

In this context, without proper epidemiological control over objects of potential epidemiological significance, the development of virgin lands has worsened the epidemiological situation. As a result, due to an increase in the number of susceptible animals, an increase in the number of objects of epidemiological significance (e.g., livestock complexes, cattle burial grounds, anthrax burial sites, and slaughterhouses), the development of transport logistics, both between farms and among the abovementioned objects, has worsened the epizootic situation regarding anthrax in Kazakhstan [23, 27].

Undoubtedly, the vaccination of susceptible farm animals has significantly affected the dynamics of the anthrax epizootic process in Kazakhstan. In Kazakhstan, specific animal immunization against anthrax began in the 1950s, and since 1961, mass animal vaccinations have been organized in many regions. Even so, in those years, the level of vaccination could not ensure coverage of susceptible livestock, which is explained by the intensive increase in the number of animals, the insufficient vaccine supply at the local level, a lack of personnel, and poor accounting and planning of veterinary measures. Thus, although there has been a tendency toward a decline in the annual incidence of anthrax since 1969, the situation remained tense, and >50 outbreaks were registered in the republic per year until the 1980s [24].

Overall, from 1961-2010, the use of vaccine prophylaxis as part of antiepidemic measures reduced the number of infection foci by 107 times and reduced the incidence of anthrax in animals to isolated cases [27].

Full vaccination coverage of all susceptible livestock, strict control over the implementation of the entire range of preventive measures, and constant epizootological monitoring of the epizootic situation with anthrax in each region of the republic currently allow the country to maintain a stable situation.

Conclusion

Thus, the analysis shows a pronounced uneven temporal and spatial distribution of anthrax foci and significant differences in the dynamics of the development of the epizootic process in the territory of Kazakhstan. At the moment, new information is being formed on the factors influencing the ecology and epidemiology of the disease, which are associated with various forms of organization and management, urbanization of the population, changes in their social conditions and the influence of other anthropogenic factors.

Authors' Contributions

YM and SA: Developed the concept and design of the study. BK and TK conducted a comprehensive literature search, analyzed the collected data, and drafted the manuscript. AM and MB: performed final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript.

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UDC [636.09:004.41]:303.722.4(045) **Research article**

Spatial analysis of rabies using ArcGIS Pro tools

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Abstract

Background and Aim. In line with the One Health concept, which recognizes the interconnectedness of human, animal, and ecosystem health, the global burden of rabies remains relevant given the current increase in zoonotic and vector-borne diseases. For successful rabies control, monitoring the changing patterns of infection spread is vital. This paper is devoted to the spatial analysis of the spread of rabies among animals in Kazakhstan.

Materials and Methods. The Spatial Autocorrelation (Moran's I) and Anselin Local Moran's I statistics of the Geoprocessing tool in ArcGIS Pro were used.

Results. Several types of spatial distribution were noted: clusters in the northeast, south, and west of the country; sparse type in the border areas of the north and northwest; and random distribution in the central and southwestern regions. High-Low Outliers indicating sporadic outbreaks of rabies caused by the migration of infected animals, as well as Low-High Outliers indicating the containment of the epizootic due to preventive measures or natural barriers were also revealed.

Conclusion. The study highlights the need to strengthen control over the spread of rabies, implement measures to prevent the migration of infected animals, and optimize vaccination and monitoring programs. The use of spatial analysis methods allows us to identify epidemiological patterns and develop effective strategies to combat the disease in regions with different risk levels.

Keywords: animal rabies; spatial analysis; clusters; geographic information system technology.

Introduction

Rabies is a major public health problem [1, 2, 3]. Despite significant progress in epidemiological surveillance and prevention, more than 55,000 people die from rabies every year worldwide, making it the deadliest zoonosis [4, 5]. In Kazakhstan, it remains a pressing veterinary and epidemiological problem, especially in rural and border regions. The main sources of infection are wild and stray animals, and the spread of the virus largely depends on natural [6, 7], climatic, and socioeconomic factors.

Modern methods of epidemiological monitoring, including spatial analysis using GIS technologies, make it possible to identify foci of infection, analyze patterns of spread, and develop effective preventive measures [6, 7].

Spatial analysis plays a key role in studying the spread of infectious diseases such as rabies, enabling the identification of patterns and clusters of disease incidence. As noted in the IMAJINE report: "Regional economic growth varies significantly across EU territories depending on local capacities, policies, and historical trajectories" (*Dax* et al., 2020, 15), ArcGIS Pro tools, including Spatial Autocorrelation (Global Moran's I) and Cluster and Outlier Analysis (Anselin Local Moran's I), are widely used to assess spatial autocorrelation and identify local clusters, respectively [8, 9].

Global Moran's I is used to measure overall spatial autocorrelation, determining whether the data are randomly distributed, clustered [10, 11, 12, 13], or dispersed. This tool calculates Moran's I, z-score, and p-value to assess the significance of spatial distribution [10, 11, 12, 13].

Anselin Local Moran's I identifies statistically significant hot spots, cold spots, and spatial outliers, providing detailed information at the individual site level [12, 13]. This method is useful for identifying areas with abnormally high or low disease rates.

The application of these methods in ArcGIS Pro allows researchers to effectively analyze spatial data on rabies, identify clusters of cases, and assess the influence of various factors on the spread of infection. The use of such tools contributes to a deeper understanding of epidemiological processes and the development of effective strategies for disease control and prevention [12, 13].

Materials and Methods

Spatial Autocorrelation (Moran's I). To perform Moran's, I index analysis in ArcGIS Pro, data from the rabies database for 2013-2023 converted into point data were used as the input, and the number of rabies cases was used as the identification attribute (Figure 1). The analysis generated a document with full identification characteristics and a corresponding diagram.

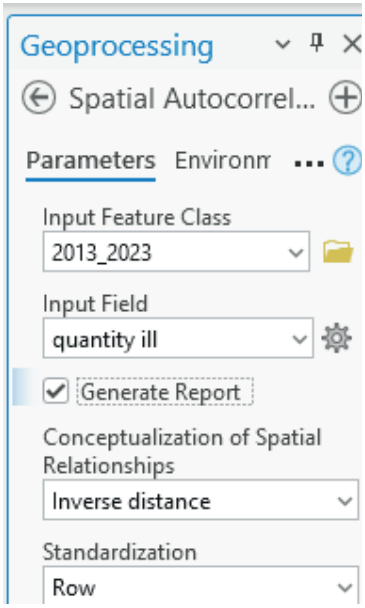

Figure 1 – Setting up the tool parameters: Spatial Autocorrelation (Moran's I)

Cluster and Outlier Analysis (Anselin Local Moran's I). This tool is used in geostatistics to identify [12, 13] spatial clusters and outliers. Anselin Local Moran's I can determine the presence of significant spatial patterns in the data, such as clusters of high or low values, and identify outliers that may indicate anomalies or interesting features [12, 13]. This method can help understand the spatial distribution of the data and identify areas that require further analysis or intervention.

In the context of spatial analysis with Anselin Local Moran's I [12, 13, 14, 15, 16], a High-High Cluster refers to an area where high values of a variable are surrounded by other high values [13, 14, 15]. This indicates positive spatial autocorrelation, where high values tend to cluster together. Similarly, a Low-Low Cluster refers to an area where low values of a variable are surrounded by other low values. This also indicates positive spatial autocorrelation, where low values tend to cluster together [13, 14, 15]. Such clusters may indicate areas of high and low activity or concentration of a particular phenomenon, respectively, that require further investigation.

In the same context, a High-Low Outlier refers to an area where a high value of a variable is surrounded by low values [13, 14, 15]. This indicates negative spatial autocorrelation, where the high value is an anomaly among the low values. Similarly, a Low-High Outlier refers to an area where a low value of a variable is surrounded by high values, indicating negative spatial autocorrelation wherein the low value is an anomaly [13, 14, 15]. Such outliers may indicate unusual or interesting features in the data that require further analysis.

Results and Discussion

Spatial Autocorrelation (Moran's I). The analysis of spatial autocorrelation using z-score (standardized critical value) and p-value (significance level) revealed the following spatial features:

High z-scores (>2.58, red zones) indicated the clustering of cases, probably [14, 15] related to sporadic outbreaks established among agricultural (western and northeastern regions) and domestic animals (southern regions), in which infection often does not go unnoticed [14, 15].

Low z-values (<2.58, blue zones) indicated a sparse distribution, reflecting the absence of coherent foci, possibly due to isolated cases of infection from migrating animals. This may correspond to rabies outbreaks in previously safe areas (northern regions).

Random distribution (yellow area) indicated the absence of clear patterns of viral transmission, reflected as sporadic outbreaks without obvious territorial references (central and northern regions). These data are shown in Figure 2.

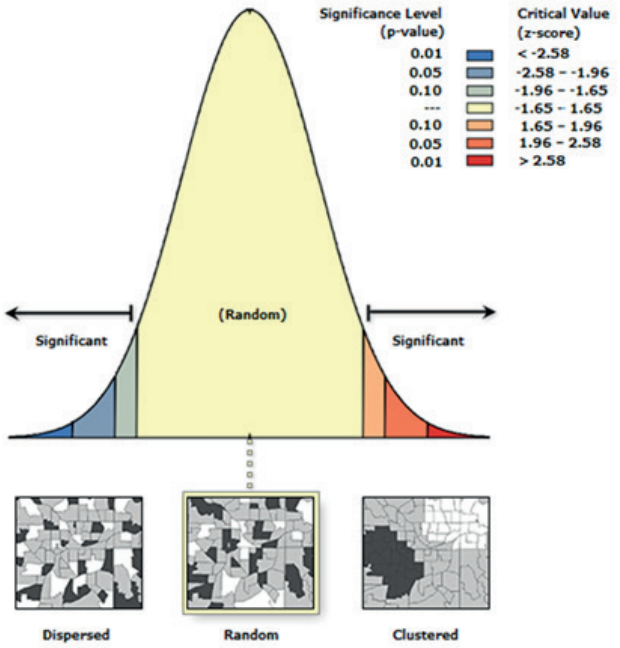

Figure 2 – Spatial correlation results (Moran's I index)

Cluster and Outlier Analysis (Anselin Local Moran's I). This analysis produced a map showing identified clusters and outliers of animal rabies cases (Figure 3).

High-High Clusters (pink dots) [13, 14, 15]. These clusters were identified in the western and northeastern regions, characterized by a high, established incidence of rabies "surrounded" by equally high values, indicating the presence of active natural foci of infection, wherein the virus is actively spread by wild animals among farm and domestic animals [13, 14, 15].

High-Low Outliers (bright red dots): These indicated areas where rabies was detected, surrounded by low values; that is [13, 14, 15], sporadic outbreaks of rabies were registered, brought into an area with overall low incidence.

Low-High Outliers (dark blue dots): These marked territories with a low number of infected animals, surrounded by areas with high infection, which may be due to the effectiveness of vaccination or the presence of natural barriers, such as mountains and rivers. These patterns were established in the territories of northeastern Kazakhstan.

Low-Low Clusters (blue dots): These territories were characterized by low incidence, surrounded by the same low values, which may indicate a controlled epizootic situation, a low population of infected animals, or the effectiveness of veterinary measures. These clusters occurred in southern Kazakhstan, where urban rabies is widespread, stemming from domestic animals. Regarding vaccination effectiveness, it is worth noting that here, domestic animals (cats and dogs) had the highest rates of rabies vaccination, which indicates a significant containment of the spread of rabies [14, 15]; nevertheless, gaps remain.

Not Significant (grey dots): In these territories, statistically significant spatial dependence was not established. These values indicated the predominant territory of the country.

No Neighbors (black dots): For these areas, the available information was insufficient to determine the corresponding characteristics.

Moran's scatterplot (analysis of spatial dependence) plots the relationship between the number of diseased animals and their spatial distribution, with more points located at the extremes of the plot indicating higher levels of clustering or outliers. $R^2=0$ indicates that the data may have low global spatial dependence, but local clusters are present.

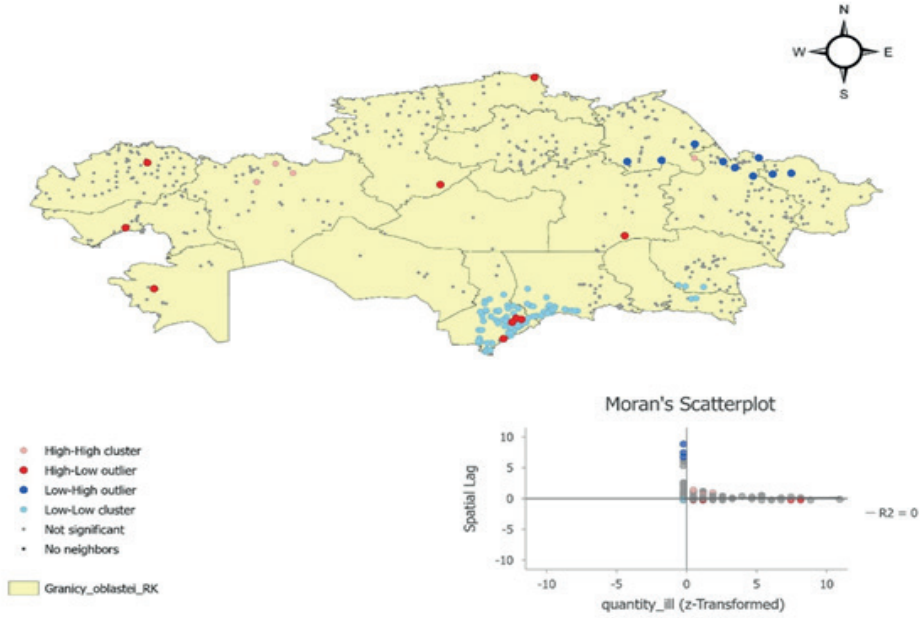

Figure 3 – Cluster and outlier analysis of animal rabies

Spatial autocorrelation (Moran's index 1) showed the following distributions of rabies cases in Kazakhstan:

- Cluster distribution: Foci in the northeast, west (among farm animals), and south of the country (among domestic animals), where infection spread probably occurred through companion animals (south) and wild animals (south, west, and northeast);
- Sparse distribution: Isolated cases of rabies in the border areas of the north and northwestern parts of the country;
- Random distribution: This included cases where the source of infection was migratory animals (central and southwestern regions), while a random, mutually unrelated distribution may have been due to the inaccuracy of epizootological characteristics given scant information about rabies among wild animals; this represents a significant barrier to the creation of an effective system of anti-epizootic measures.

For cluster-based cases, increased control, vaccination, and monitoring measures are needed in high-risk areas. In case of sparse distribution, it is important to monitor animal migration and prevent the introduction of infection into new regions. In cases of random distribution, studies of additional factors (e.g., climate, ecology, and contact with people) are needed.

The spatial analysis method (Anselin Local Moran's I) [12] along with rabies clusters revealed High-Low Outliers in the territories of the Mangystau, Atyrau, West Kazakhstan, Kostanay, North Kazakhstan, Karaganda, and Turkestan oblasts. These can be perceived as sporadic outbreaks of rabies in areas with low or no incidence as a result of accidental contact with infected migrating animals. One such example is the registered sporadic outbreak in the Mangystau oblast in the form of 18 foci with 19 rabies-infected animals (16 agricultural [camels], 1 domestic, and 2 wild animals) in 11 settlements

(villages) from February 14 to March 20, 2018, where the infection source comprised wild fauna (wolves). Subsequently, the infection spread to the territory of the Atyrau region, with 7 outbreaks from March to September, also through a wild animal.

In areas with high disease incidence, Low-High Outliers were established [13], perceived as contained epizootics due to effective preventive measures or the presence of relief features like high mountains or mountain slopes, which impede animal movement.

Conclusion

Modern global trends in the study of problems related to animal and human morbidity are increasingly represented by a system of scientific analysis involving popular approaches such as the use of Big Data, ICT methods, AI, GIS, and other digital technologies; the growing popularity of online databases and the addition of research results to accessible online resources and libraries; the development of modern methods and platforms for widespread use; and the continuity of relevant research worldwide. This is evidenced by a significant increase in scientific publications and conferences in the field of veterinary medicine, frequent studies using molecular genetic methods to determine the genetic characteristics of pathogens, the active use of geospatial analysis methods with subsequent mathematical modeling, and forecasting disease outbreaks and foci. Along with increasing disease spread, the growth of these applications has been facilitated via active promotion by global organizations (e.g., FAO, WHO, OIE) of the One Health concept, which is based on the principle of close interconnection between the health of people, animals, and ecosystems.

The fundamental basis was a verified, reliable rabies database with complete information for the last 10-year period (2013-2023), with 942 rabies outbreaks and 1,243 infected heads among more than 10 animal species ranging from rats to deer. Outbreaks in 584 settlements reflected territorial characteristics, with the most unfavorable zones at various levels as follows: rural: Kokzhyra (Abay region), urban: Semey, district: Urzhar (East Kazakhstan oblast), and oblast: East Kazakhstan (181 outbreaks). The abovementioned unfavorable situation in the eastern part of the country, as well as in the southern, western, and northeastern parts, was expressed by clusters derived from spatial analysis using ArcGIS Pro, indicating a tense epizootic situation via comprehensive analysis along with other conditions.

Spatial autocorrelation analysis using Moran's I index and the Anselin Local Moran's I method allowed the identification of features of rabies distribution among animals in Kazakhstan. The epizootic process was found to have a clustering nature in several regions (northeast, west, and south), requiring the strengthening of preventive measures, including vaccination and monitoring. The sparse distribution in the border areas of the northern and northwestern parts of the country indicates the need to control animal migration to prevent the introduction of infection.

The random spread of rabies cases in the central and southwestern regions may be due to both the migration of wild animals and insufficient epizootological information, which emphasizes the importance of additional research and improved monitoring systems.

The identified High-Low Outliers in certain areas indicate sporadic outbreaks of rabies caused by accidental contact with infected migrating animals. Low-High Outliers in areas with high incidence may indicate the effectiveness of preventive measures or natural barriers limiting the spread of infection.

These results highlight the need for a comprehensive approach to combat rabies in Kazakhstan, including veterinary surveillance strengthening, animal migration control, regular vaccination, and further application of spatial analysis methods to optimize anti-epizootic measures.

Authors' Contributions

AK, BT: Conducted laboratory research and wrote the first draft of the manuscript. SA: Developed the aims, objectives, and methodology of the work; GM and ZB: prepared the article per the publication requirements. EG: Performed statistical analysis and reviewed the manuscript. All authors read, reviewed, and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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The effect of an extruded feed with a symbiotic formulation on the production of clary catfish

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Abstract

Background and Aim. This article presents the results of a study on the effectiveness of extruded feed containing a formulation of symbiotic bacteria on the performance of catfish production and its meat quality as assessed by veterinary and sanitary indicators. The study aimed to develop extruded feed based on a symbiotic formulation and to determine the effectiveness of the feed on the production of clary catfish meat.

Materials and Methods. Experimental work was carried out on the platform of the International Scientific Research center “Fisheries” and S.Seifullin Kazakh Agrotechnical Research University (S. Seifullin KATRU). In total, 135 kg of feed was used to feed young African clary catfish at a rate of 30 g per fish per day over 40 days. The fish were divided into two pools, with 100 individuals in each group. To assess the effectiveness of feed, a weekly total catch of the two pools was carried out to determine the mass of fish. To determine the Fulton fatness coefficient (kc), 20 fish were measured from each group. Veterinary and sanitary assessments of fish meat were carried out using generally accepted organoleptic and physico-chemical research methods.

Results. On the 40th day, a significant difference was observed in all weight gain indicators on day 40: the mean fish weights were 570 ± 17.8 and 565 ± 18.2 g in the Control and Experimental groups, respectively ($P < 0.01$). Indeed, the chemical composition of meat improved in terms of the mass fractions of protein and fat: the mass fraction of protein was 16.22 and 17.38% for the Control and Experimental groups, respectively, and 3.90 and 4.90% for the mass fraction of fat.

Conclusion. The novelty of this research is to develop extruded feed based on a symbiotic formulation and to determine the effectiveness of the feed on the production of clary catfish meat. One formulation of extruded feed with a symbiotic was obtained. The effect of symbiotic feed on the productive performance of the African catfish has been studied. A veterinary and sanitary assessment was carried out to determine the nutritional value of fish meat. All indicators of the amino acid, vitamin, and mineral composition of fish meat in both groups were normal.

Keywords: Clariid catfish; extruded feed; fish meat; symbiotic; veterinary and sanitary assessment.

Introduction

With the development of fish farming intensification, there is an urgent need to develop effective technologies for growing various species of fish, including new aquaculture facilities. Tilapia and catfish are two of the most promising objects of industrial fish farming in Kazakhstan [1]. The African clary catfish (also known as the marbled clary catfish and the Nile clarias) is a favorite among fans of non-pond fish farming due to its adaptability and rapid growth in conditions of closed water supply [2]. Adequate nutrition is an important factor for the proper growth of fish, and this is especially critical when using closed-circuit water supply installations [3-5]. The African catfish is an opportunistic predator that requires animal-based feed. According to domestic and foreign literature sources, it has been established that extruded feed with a symbiotic supplement can serve as a good food source, increasing the performance of fish production and having a direct positive effect on the chemical composition and nutritional value of fish meat [6-10].

In aquaculture, feeding rate and nutritional value are important factors influencing fish growth. Therefore, determining the optimal feeding rate is important for fish production [11]. The growth rate is also affected by the nutrient content in the feed [12]. Thus, the development of a feed extrusion formulation is an important process of mixing various feed components and processing the components by extrusion to meet nutritional needs [13]. In his article, Shaw reported that, in a closed water supply, the replacement of animal protein by 25% led to a low feed conversion rate and protein efficiency in the African catfish, and replacement at 75% reduced the ash content of the body [14].

In this study, we aimed to develop the first dry, water-resistant, full-fledged extruded feed based on a symbiotic formulation and to determine the effectiveness of this feed on the performance of fish meat production.

Materials and Methods

Experiments were conducted at the International Scientific Research Center “Fisheries” S. Seifullin KATRU. Extruded waterproof dry food was produced by extrusion and granulation in the production and testing workshop of NFT-KATRU LLP at the Faculty of Veterinary Medicine and Animal Husbandry Technology of S. Seifullin KATRU. The extrusion process was carried out as follows: the crushed grain was moistened in a screw mixer (moisture was introduced in the amount of 275 - 400 liters per 1 ton of product), after which it entered the receiving chamber of the extruder. In the extruder, the grain was subjected to compaction, compression, and high temperatures, reaching 25-50 atmospheres and 110 °C in the extrusion zone. The processing time of the product in the extruder was 8-10 s. All raw materials used for feed after extrusion and granulation were coated with fish oil and gelatin in order to ensure that the granules had a stable shape.

The obtained strains of lactobacilli were used to produce a symbiotic formulation in an amount of 1.0×10^7 CFU. For this purpose, lactobacilli were diluted in 25 ml of distilled water together with feed yeast. The symbiotic formulation was then added to the general diet after the feed was extruded by spraying it onto the surface of the granules. *Leuconostoc mesenteroides* and *Lactococcus lactis* were added to the main experimental diet in the amount of 10^7 cells/g for eight weeks.

In total, 135 kg of feed was used to feed young African clary catfish at a rate of 30 g per fish per day over 40 days. The fish were divided into two pools to form an experimental and a control group, with 100 individuals in each group. Generally accepted methods in fish farming were used to study the breeding and biological parameters of the fish. To assess the effectiveness of feed, a weekly total catch of the two pools was carried out to determine the mass of fish. To determine the Fulton fatness coefficient (kc), 20 fish were measured from each group, recording the fish mass (M), total length (L), and the length of the fish from the beginning of the head to the end of the scaly coat (l).

Veterinary and sanitary assessments of fish meat were carried out using generally accepted organoleptic and physico-chemical research methods. When conducting physico-chemical studies, a sample was cooked, and the concentration of hydrogen ions (pH) was potentiometrically determined; hydrogen sulfide was determined by heating minced meat and measuring a reaction to peroxidase according to A.M. Poluektov, a reaction to ammonia gas using the Eber method, and ammonia was determined with Nessler reagent. The nutritional value of fish meat was studied using gas chromatography in the Food Safety Laboratory of the Almaty Technological University.

Results and Discussion

We developed a formulation of dry extruded feed for juvenile catfish weighing 508 ± 0.1g with the addition of a probiotic (Table 1). An analysis of the domestic feed raw materials market confirmed the availability of all the components necessary to create a catfish feed formulation. The recipe used mainly vegetable raw materials (soybean meal, crushed wheat, and extruded peas), while fishmeal was used as an animal raw material. The formulation of the extruded water-resistant feed uses mainly raw materials of domestic production, which will reduce the cost of feed compared to imported feed by 30% (at current prices), while the chemical composition of the feed is not inferior to foreign analogs. The scientific justification for each component of the formulation is given. Taking into account the above and having analyzed the fish feed market in Kazakhstan (demonstrating a lack of specialized catfish feeds), we decided to include about 70% fish meal and soy meal in the recipe (the latter is more, since catfish prefer vegetable protein provided by the FAO).

Table 1 – Recipe of extruded feed with symbiotic supplement for catfish

Number	Components	Grams (per 5 kg)
1	Fish flour	1642.5
2	Blood meal	2100
3	Shredded wheat	350
4	Extruded peas	400
5	Potato starch	300
6	Fish oil	50
7	Tricalcium phosphate	50
8	Premix	100
9	Symbiotic supplement (mixture of *Lactobacillus* strains)	7.5

The composition of the extruded feed included crushed wheat and extruded peas in approximately equal proportions. In addition, our formulation included starch and gelatin for water resistance, with amino acids and mineral premix for fish. According to the FAO, the main nutrients for catfish are dry matter (DM), crude protein (CP), crude fat (CF), crude fiber (CF), and ash. All standards were taken from the FAO website. A chemical analysis of commercial and developed feeds was carried out, which was then used in the experiment to evaluate the effectiveness of the feed.

Table 2 – Chemical composition of extruded feed for catfish

Compound feed	Indicators (mean ± M±m, %)					
	Humidity	Protein	Fat	Cellulose	Ash	Starch
Catfish feed of KATRU (Experimental group)	9.5 ± 0.11	43.1±0.12	16.89±0.06	4.64 ±0.05	6.57 ±0.02	18.57 ±0.16
Commercial fish food (Control group)	9.3 ± 0.12	45.2 ±0.21	20.02 ± 0.08	4.43 ±0.04	6.46 ±0.09	14.22 ± 0.16

Chemical analysis showed that the extruded feed we developed for catfish, which included a symbiotic supplement, contained a protein level of 43.1 ± 0.12%, compared with 45.2 ± 0.21% in the commercial feed. The fat content also differed, being 16.89 ± 0.06% in our feed compared with 20.02 ± 0.08 in the commercial feed.

Preliminary experiments were conducted to evaluate the effectiveness of the developed feed on the production of African catfish in a closed-circuit water supply system with a water temperature in the range of 26-27 °C. To control the hydrochemical regime, water was analyzed daily according to the two most important parameters (O_2 and pH), and the temperature regime was determined once every 7 days. In addition to these indicators, a comprehensive analysis of water for the content of NO_2 and NO_3 was performed. The average O_2 content during the observation period was 6.3 mg/l, with fluctuations of

between 5.2 and 7.2 mg/l. The average pH value was 7.3, with fluctuations of 6.8-7.7. The average NO_2 value was 0.3 mg/l, with fluctuations of 0.3-1.5 mg/l, while the same values for NO3 were 30 mg/l and fluctuations of 10–50 mg/l. These results indicate a good hydrochemical regime in a closed-circuit water supply installation during experiments.

The initial weight of catfish was 486 ± 12.3 g in the Control group and a slightly lower value of 480 ± 9.6 g in the Experimental group ($P < 0.05$) (Figure 1). In the following two weeks, we did not observe a significant difference between the groups. In the first week, the control fish gained weight, but the absolute increase was 2.2 ± 0.26 g higher in the Experimental group; the experimental fish were 3.6 ± 0.33 g heavier in the second week. On day 40, we observed a significant difference in all indicators of weight gain ($P < 0.05$): the weight of the Control group fish was 570 ± 17.8 g, compared with 565 ± 18.2 g in the Experimental group ($P < 0.01$). The two length measurements (L and l) were not significantly different between the groups, given that only by the third week of the experiment did the fish differ in weight. However, in terms of fatness (kc), the two groups were significantly different at weeks 3 and 4 ($P < 0.05$). Thus, the assessment of feed efficiency showed a positive result since the relative increase in the Experimental group was 12.4 ± 1.7%, while the practical gain in the Control group was two times less, at 7.6 ± 0.83%. The survival rate of the fish in the two groups was 100%.

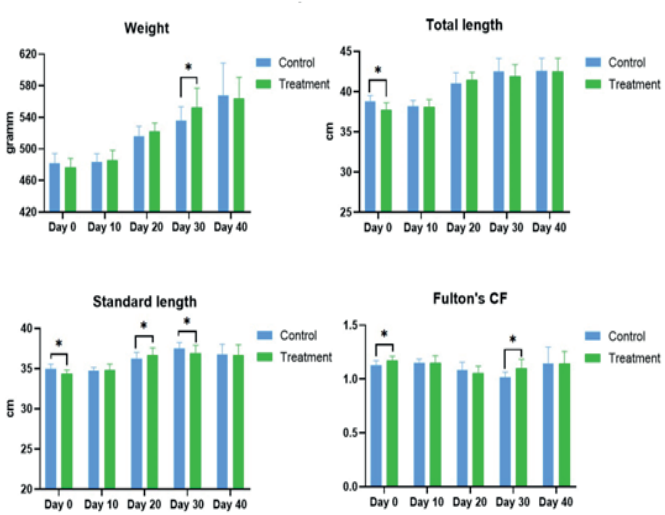

Figure 1 – Fish Breeding and Biological Indicators of African Catfish Fed with Extruded Feed with a Symbiotic Supplement

The Fulton fatness coefficient (kc) at the last measurement was 1.1 and 1.2 in the Control and Experimental groups, respectively. The veterinary and sanitary assessment of catfish meat in the Experimental group - according to organoleptic parameters - was fresh and benign, and the surface of the fish was glossy in appearance. The structure of the meat was dense and elastic. The color of catfish meat is dark gray, and it has a specific smell and taste. When tested by cooking, the color of the broth was transparent, without changes and flakes, which indicated the freshness of the meat. The pH of the meat was 6.6, corresponding to the norm for fresh fish meat. When determining hydrogen sulfide by heating the minced meat, the drop did not stain, which also served as an indicator of its good quality. When setting up the Eber reaction, a cloud of ammonia did not appear, which is considered a negative reaction to ammonia. When reacting with Nessler's reagent, the fish meat extract acquired a greenish-yellow color, which meant that the fish meat was fresh.

In terms of the chemical composition of catfish meat, the mass fraction of protein in the Experimental group was 17.38%, compared with 16.22% in the Control group; for fat, the mass fractions were 4.9 and 3.90%, respectively. The mass fraction of carbohydrates was not detected in either group. In the meat of the experimental fish, a decrease in the concentration of most amino acids was observed: arginine (3.959% compared with 4.969% in the Control group), lysine (1.527 vs. 1.104%), tyrosine (0.735 vs. 0.848%), phenylalanine (1.188 vs. 1.432%), histidine (0.933 vs. 0.906%), leucine + isoleucine (1.612

vs. 1.958%), methionine (0.820 vs. 0.789%), threonine (1.131 vs. 1.228%), and serine (0.905 vs. 1.140%). Nevertheless, all indicators of amino acid composition in both groups were normal. In terms of vitamin composition, approximately the same values of vitamin indicators were observed in both the experimental and control groups. The content of the mineral composition also corresponded to the norm, and their values were the same in both groups.

The demand for extruded mixed feed for fish was estimated at 29.3 million tons in 2008 and is expected to grow in step with increases in global aquaculture production. Since 1995, the production of mixed fish feeds has grown by an average of 10.9% per year [15–16]. Fish meal [17], soy meal [18], wheat [19], and blood meal [20] are mainly used in compound feeds for fish. In particular, African catfish need a large amount of animal protein as they are predators [21]; thus, fish and blood meal are used to increase dietary protein content [22]. Similarly, all of the above feed components were used in our formulation, the bulk of which was fish and blood meal. Wheat and extruded peas were also used as fillers, while gelatin, starch, and fish oil were used to shape the granules and bind the feed components.

The use of extrusion technology for the production of aquaculture feeds makes it possible to obtain pellets with high physical quality for a number of components. When the feed components are extruded, the microbial contamination of the feed is also reduced or eliminated, which makes it possible to obtain food that is safe for consumption. Some studies have also examined the effect of extruded feed with a symbiotic supplement on fish bodies and growth rates, as well as on the chemical composition of fish meat [22, 23]. Feeds prepared with a high content of vegetable protein (such as soy meal, wheat, and other economical sources of vegetable protein) may lack methionine [13]. Vegetable protein sources are poorly absorbed due to anti-nutritional factors and an unbalanced amino acid composition, which can lead to loss of nutrients in meat [3, 13]. Therefore, we included additional methionine in our formulation of the extruded feed. No significant differences in growth, body composition, and nutrient retention were found in fish fed with either extruded or granular feed with the same feeding rate ($P > 0.05$). The channel catfish fed with extruded feed had the same growth rates as those fed with granular feed with the same formulation [24]. The study found that growth - as indicated by the growth parameters and weekly growth - was influenced by the type of feed, with extruded feed having a greater impact than granular [25].

Conclusion

We developed a formulation of dry extruded feed for juvenile catfish weighing 508 ± 0.1g with the addition of symbiotic (mixture of probiotics). The recipe used mainly vegetable raw materials: soybean meal, crushed wheat, and extruded peas, fishmeal.

The chemical analysis revealed that the extruded catfish feed we created, including a symbiotic additive, had a protein concentration of 43.1% ± 0.12%, while the commercially available feed boasted a protein level of 45.2% ± 0.21%. Additionally, our feed exhibited a fat content of 16.89% ± 0.06%, contrasting with 20.02% ± 0.08% found in the commercial feed.

At the start of the study, catfish in the Control group weighed an average of 486 grams, with a standard deviation of 12.3 grams, while the Experimental group had a slightly lower average weight of 480 grams, with a standard deviation of 9.6 grams ($P < 0.05$). By day 40, a notable disparity emerged across all weight gain metrics ($P < 0.05$), with the Control group averaging 570 ± 17.8 g, while the Experimental group averaged 565 ± 18.2 g ($P < 0.01$). Although initial length measurements (L and l) showed no significant difference between the groups, this distinction became apparent in weight by the third week. Notably, the groups exhibited a statistically significant divergence in fatness (kc) during both the third and fourth weeks ($P < 0.05$).

A comparative analysis of catfish meat revealed significant differences in chemical composition between an experimental group and a control group. The experimental group exhibited a slightly higher protein content (17.38%) compared to the control group (16.22%). Fat content also showed a noticeable difference: 4.9% in the experimental group and 3.9% in the control. A detailed amino acid profile revealed a generally lower concentration of most essential and non-essential amino acids in the experimental group's meat. Specifically, arginine (3.959% vs 4.969%), lysine (1.527% vs 1.104%), tyrosine (0.735% vs 0.848%), phenylalanine (1.188% vs 1.432%), histidine (0.933% vs 0.906%), leucine + isoleucine (1.612% vs 1.958%), methionine (0.820% vs 0.789%), threonine (1.131% vs 1.228%), and serine (0.905% vs 1.140%) all showed a reduction in the experimental group.

Authors' Contributions

Supervision, conceptualization, writing - original draft preparation, writing - review and editing AP: methodology, validation, formal analysis, NS, DZh, ZhA, IA, GA, AK: all authors have read and agreed to the published version of the manuscript.

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UDC 619 **Review article**

A comprehensive review of lameness in broilers: infectious and non-infectious factors in the context of Kazakhstan's poultry industry

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Abstract

In recent years, Kazakhstan has experienced steady growth in poultry production and increased poultry meat production, but the prevalence of lameness in broilers remains high. Lameness in broilers is one of the most urgent problems in the modern poultry industry, having a significant impact on the health and welfare of birds, as well as on the economic performance of production. The aim of this review article is to systematize data on causes, diagnosis, prevention and strategies to reduce lameness in broilers. Special attention is given to bacterial chondronecrosis with osteomyelitis (BCO), one of the major infectious causes of lameness caused by pathogens such as *Staphylococcus aureus* and *Escherichia coli*. Both infectious and non-infectious factors are discussed: genetic predisposition, rapid growth, vitamin and mineral deficiencies, poor housing conditions, poor litter quality and high planting density. Modern diagnostic techniques, including bacteriological tests, histology, polymerase chain reaction (PCR), and imaging technologies such as infrared thermography and computer vision are covered. Preventive measures including probiotics, vitamin and mineral supplements, vaccinations, genetic selection, and improved housing and biosecurity have also been analyzed. The prospect for future research aimed at the use of digital technologies, genomic analysis of pathogens and the development of disease-resistant broiler lines are considered. The review provides an up-to-date scientific and practical basis for specialists working in the field of veterinary medicine, zootechnics and industrial poultry production.

Keywords: bacterial chondronecrosis; diagnosis; genetics; lameness broilers; osteomyelitis; prevention.

Introduction

Poultry farming in Kazakhstan in recent years demonstrates stable growth, which is accompanied by an increase in the number of poultry and poultry meat production. Compared to last year (2024), the number of birds as of February 1 (2025) increased by 1.8% and amounted to 45.990.901 heads. Broiler production plays a key role in ensuring food security of Kazakhstan, for this purpose it is necessary to provide the population with quality products. The increase in domestic production of poultry meat, helps to reduce dependence on imports, as well as the creation of new jobs and the development of agriculture. Over the past year, the output of meat in the country shows positive dynamics. Thus, poultry meat production in 2024 amounted to 445.4 thousand tons, which is 7.7% higher than in 2023 (413.6 thousand tons) [1]. In 2023, Kazakhstan imported 118 thousand tons of poultry meat, which is a significant part of the country's total meat imports. However, in the first 11 months of 2024, the volume of poultry meat imports decreased by 10.7% year-on-year to 121 thousand tons. Thus, despite the significant volume of poultry meat imports at present, Kazakhstan is taking active measures to increase its own production in order to achieve food independence in this sector.

Based on observations by *Dr. Adnan Alrubaye* from the University of Arkansas (USA) during visits to broiler farms in Kazakhstan, lameness has been identified as a significant contributor to economic losses in broiler production [2]. Lameness in broiler chickens is a serious problem causing significant economic losses and deterioration of animal welfare. Lameness in broiler chickens has a serious impact on welfare, meat quality, production, food safety and economic losses [3]. Lameness in broilers results in reduced weight gain, increased feed costs, increased culling and additional veterinary costs, causing significant financial losses to producers [4]. Lameness causes significant economic losses in the poultry industry due to decreased meat quality and increased treatment and prevention costs [5]. Foot disease in birds has a negative impact on both their welfare and productivity. Poultry farms should strive to improve housing conditions, optimize diets and regularly monitor poultry health to minimize the risk of lameness. Ultimately, taking care of broiler health not only promotes broiler welfare, but also provides a higher economic return for poultry farms.

The aim of this review article is to conduct a multifactorial analysis of the causes of lameness in broilers, taking into account genetic, infectious, feed and management factors, which will allow the development of effective measures for prevention and improvement of poultry productivity. Bacterial chondronecrosis with osteomyelitis (BCO) is a major cause of lameness, causing significant welfare and health problems in birds. Bacterial pathogens such as Escherichia coli and Staphylococcus aureus can play a significant role in the development of lameness in broilers. These bacteria are easily transmitted from one bird to another, which can make transmission control difficult [3, 6]. The main causes of lameness in broilers include both bacterial infections and a mismatch between muscle growth and skeletal development. The rapid rate of weight gain places excessive mechanical stress on immature bone and cartilage, resulting in the formation of osteochondral fissures, which are then colonized by bacteria, causing abscesses and necrosis. Bacterial chondronecrosis with osteomyelitis is also associated with immunodeficiency caused by stress or inflammation, which allows bacteria to enter the bloodstream and colonize bone growth zones [3, 7]. The next major factor contributing to a significantly increased risk of lameness is housing conditions, such as floor type (wire or litter). Wire floors can create instability and physiological stress in birds. Poor ventilation, litter quality and boarding density affect the incidence of lameness, as poor housing conditions can contribute to deteriorating feet and other skin conditions associated with lameness [8].

Prevention of lameness in broilers, is an important objective to improve animal welfare and economic efficiency in poultry production. Various strategies are used to reduce the incidence of lameness, which include the use of probiotics and vitamins in the diet as well as the use of antibiotics. This promotes bone health and reduces the risk of lameness in broilers [5, 7]. Lameness in broilers is and remains a multifactorial problem associated with bacterial, genetic and environmental factors Management of these factors, including improved housing conditions and the use of various supplements, can significantly reduce the incidence of lameness and improve the overall health of the birds as well as the economic viability of production. Research highlights the importance of early detection and management of this problem through genomic analysis, experimental lameness models and behavioral monitoring.

In the study of lameness in broilers we analyzed scientific publications in peer-reviewed foreign and domestic journals, data from the Bureau of National Statistics of the Republic of Kazakhstan, studies considering infectious and non-infectious causes of pathology, as well as the works of leading researchers in the field of veterinary medicine and poultry farming. Special attention is paid to bacterial chondronecrosis with osteomyelitis (BCHO), works devoted to the influence of housing conditions, feeding, genetics on the development of lameness. Modern diagnostic methods, including histologic, microbiologic, and molecular analysis, are studied.

1. Etiology and classification of lameness in broiler chickens

1.2 Infectious and non-infectious causes

Lameness in broiler chickens is a serious problem in the poultry industry that affects the welfare of the birds and economic performance. Studies show that lameness in broilers can be caused by infectious factors such as bacterial chondronecrosis with osteomyelitis, and non-infectious factors such as genetic parameters and housing and nutritional conditions of birds [9]. As described by *B. Kierończyk* lameness of birds can arise from both infectious factors and non-infectious factors that affect broiler leg health.

Infectious factors play a significant role in the development of lameness in broilers [7]. The main bone and joint infection leading to lameness is bacterial chondronecrosis with osteomyelitis, which is caused by various pathogenic bacteria (Table 1).

Table 1 – Bacterial, viral and fungal pathogens associated with lameness in broilers

Pathogen	Disease / Condition	Clinical manifestations	Diagnostic methods	Sources
Staphylococcus aureus	Infectious arthritis	Swollen joints, lameness, pain.	Bacterial culture, PCR	*Anthney* et al, 2024; *Assumpcao* et al, 2024; *Wideman*, 2016; *Alrubaye* et al, 2015; *Alrubaye* et al, 2020; *Choppa & Kim*, 2023.
Escherichia coli	Colibacillosis	Systemic infection, joint involvement.	Bacterial culture, serology	*Ekesi* et al, 2021; *McNamee & Smyth*, 2000;
Enterococcus spp.	Infectious tendovaginitis	Joint swelling, limited mobility.	PCR, histology	*Do* et al, 2024.
Reovirus	Viral arthritis	Joint deformities, inflammation of tendons	Serology, PCR	*Kierończyk* et al, 2017

The most important pathogens causing BCHO are *Staphylococcus spp.* and *Escherichia coli*, penetrating through the bloodstream, cause necrosis and inflammation leading to bone lesions especially femur and tarsal [3, 10]. In addition to *Staphylococcus spp*. and *Escherichia coli*, other types of pathogenic bacteria are often isolated as well as those associated with bone and joint disease leading to lameness in broiler chickens. Studies indicate that BHO can be caused by different strains of bacteria, making it difficult to diagnose, treat and prevent the disease [11]. Research by *R. Wideman* indicates that BCD is the primary infectious cause of lameness in broilers, often resulting from bacterial infections such as *Staphylococcus aureus* and *Escherichia coli*, these bacteria can spread hematogenously, leading to bone lesions. The pathogenesis of BCO begins with microfractures in the rapidly growing bones due to mechanical overload, which create a favorable microenvironment for bacterial colonization. Once pathogens such as *Staphylococcus aureus* or *Escherichia coli* enter the bloodstream – either through damaged mucosa or skin – they localize in these weakened areas. There, they trigger an inflammatory response, osteolysis, and necrosis. The virulence factors of these bacteria, including adhesins and toxins, exacerbate bone degradation and impair the host's immune response [6, 10, 14]. This complex pathophysiological mechanism makes both diagnosis and prevention challenging.

Non-infectious factors are also an acute problem in the development of lameness in broilers (Table 2).

Table 2 – Risk factors for lameness in broiler chickens

Factor Category	Specific reason	Effect on lameness	Sources
Genetics	Rapid growth	Skeletal deformities, bone fragility	*Wideman*, 2016; *Guo* et al., 2019.
Management	High planting density	Increased stress, trauma to the extremities	*Gocsik* et al., 2017; *Granquist* et al., 2019.
Nutrition	Calcium and phosphorus deficiency	Rakhitis, bone weakness	*Waldenstedt*, 2006; *Alharbi et al*., 2025.
Mechanical factor	Poor quality of bedding	Dermatitis of the paw pads, infections	*De Jong* et al., 2014; *Kierończyk* et al., 2017; *Alrubaye* et al., 2020.

Rapid growth and increased body weight in modern broilers place mechanical stress on immature bones, which can lead to microfractures and lameness [7, 9]. Genetic predisposition also plays a role in the development of BHO. Nutrient deficiencies, especially calcium and phosphorus in the diet, affect bone development and increase the risk of developing lameness. Proper nutrition is critical to keeping birds' feet healthy [9]. Likewise, poor housing conditions, including litter quality and ventilation, can exacerbate skeletal diseases leading to lameness [3, 12]. Non-communicable factors also have their own specific mechanisms of action. Calcium and phosphorus deficiency in the diet disrupts bone mineralization, leading to osteopenia and deformities. Vitamin D3 deficiency reduces calcium absorption and disrupts bone remodeling, making them more brittle. Environmental stresses, such as high planting density and wet litter, cause chronic stress and immunosuppression, which indirectly increases susceptibility to infections. In addition, wet bedding contributes to the appearance of dermatitis and skin damage, through which bacteria can enter the bloodstream [9, 12, 21]. To reduce these risks, management strategies that include improving housing and nutritional conditions are recommended.

Lameness in broilers is a complex problem caused by many factors. Infectious and non-infectious factors play a key role in the development of lameness in broilers. A comprehensive approach to the treatment of lameness, including measures to improve housing and nutritional conditions, can reduce the risk of disease prevalence and economic losses.

1.2 Main types of limb pathologies in broilers

Limb abnormalities in broilers represent a major problem in the poultry industry, affecting the health and welfare of the birds as well as the economic performance of production. They can be related to genetic factors, rapid growth, nutrition and housing conditions.

The main types of limb pathology in broilers include tibial dyschondroplasia (TD), femoral head necrosis (FHN) and valgus-varus deformity (VVD). Dyschondroplasia of the tibia is characterized by abnormal cartilage growth and is common in fast growing broiler breeds. Necrosis of the femoral head This condition is associated with destruction of bone tissue in the femoral head region and often results in lameness. Valgus-varus deformity is a condition in which the limbs are deviated inward or outward, resulting in impaired gait and reduced growth [13].

Bacterial chondrosclerosis with osteomyelitis (BCO) is one of the leading causes of lameness and health problems in modern broilers. The disease results from bacterial bone infections, which leads to bone destruction and is one of the leading causes of lameness [14]. Lameness results in limited mobility and reduced activity of the birds, leading to poor quality of life. It is manifested by an increase in the time spent lying down and a decrease in the time birds spend walking. This condition is also associated with the pain and discomfort that lameness causes, which impairs the overall well-being of the birds [15]. Lameness leads to difficulty in accessing food and water, which can cause dehydration and death. It is also associated with increased culling of birds for slaughter due to pathological changes in carcasses [3]. Lameness causes significant economic losses in the poultry industry due to decreased meat quality and increased treatment and prevention costs [5]. Limb pathologies in broilers include a variety of conditions that have a significant impact on the health and welfare of the birds as well as the economic performance of production.

2 Pathogens causing lameness

Lameness of broiler chickens, is a serious health and welfare problem in the poultry industry, often associated with bacterial infections. Various bacterial species have been identified as causative agents, including known pathogens including *Staphylococcus aureus, Escherichia coli, Enterococcus spp.* The diversity of genome, virulence factors, and transmission routes of these pathogens is critical for the development of effective prevention and treatment strategies. *Staphylococcus spp., Enterococcus spp.* and *Escherichia coli* are the predominant bacterial pathogens isolated from lameness-affected broiler chickens [5].

Outbreaks of severe infectious diseases in birds caused by *Staphylococcus aureus* and *Escherichia coli* occur suddenly and cause serious health problems. These bacteria enter the body from the environment when the body's natural defenses are compromised, often through skin wounds or inflamed mucous membranes. In broilers with developing lameness, the bacteria enter the bloodstream through the skin, respiratory system, or gastrointestinal tract. The bacteria colonize the proximal growth zone of

the rapidly growing femoral and tibial bones, causing necrosis leading to lameness. *S. aureus* shows that the genome continues to evolve, while *E. coli* shows high genomic diversity, indicating frequent host changes and adaptation to different environments [10].

Studies suggest that the use of probiotics such as *Enterococcus faecium* on young chickens helps reduce the incidence of lameness and mitigates the negative effects [16]. The results of this research are valuable to the poultry industry, demonstrating significant benefits in maintaining normal avian health, and help elucidate the effects of preventive probiotic administration on lameness in broilers.

Bone disorders in broiler chickens are caused not only by pathogenic bacteria but also by viruses. Reovirus infection in broiler chickens can impair nutrient absorption, resulting in osteoporosis, bone fragility, femoral head necrosis and tendovaginitis, especially when combined with enteritis and digestive tract lesions [9]. Also in infectious bursitis, pathologic lesions include pitting type hemorrhages in the leg muscles.

Mycotoxins such as deoxynivalenol (DON) and fumonisin (FUM) are secondary metabolites of microfungi that are commonly found in corn and soybean cake-based feeds. These toxins reduce intestinal barrier function and cause immunosuppressive effects that predispose broilers to bacterial chondronecrosis with osteomyelitis. Studies have shown that the presence of DON and FUM in feed increases the incidence of BCHO, especially when kept on wire floors [17]. However, multiple studies indicate the need for further research on these problems.

3 Risk factors for lameness

There are many factors in growing broiler chickens that influence the development and function of the bones and skeletal system. Rapid growth rates in broilers are associated with an increased risk of lameness due to genetic factors and orthopedic disorders. Studies show fast-growing broiler breeds have a higher risk of lameness compared to slow-growing breeds due to their higher body weight and lower activity [18]. Rapid growth puts mechanical stress on immature cartilage, leading to microfractures in bone and subsequent bacterial infection, osteochondrosis and bacterial chondronecrosis with osteomyelitis [7, 9]. Lameness is closely associated with impaired avian health and welfare, including dermatitis of the pads of the feet and burning of the hock joints, which indicate ulcerative and necrotic lesions of the limbs in broilers [12]. Foot problems in broilers result in significant losses. High growth rates in broilers due to genetics significantly increase the risk of lameness and orthopedic disorders. These problems require attention to improve bird welfare and production development.

Housing and management conditions also influence lameness in broilers and is a significant indicator of broiler welfare. The main risk factors include housing density, litter quality and ventilation. High planting density compromises litter and air quality, leading to increased moisture and ammonia, which contributes to dermatitis and lameness. It also limits ventilation, which exacerbates heat stress and worsens the overall condition of the birds [9, 19]. Poor litter quality, especially wet litter, promotes microbial activity, worsening housing conditions. Most of the problems result in leg diseases that lead to reduced ability to walk, causing gait changes [12, 20]. Improving these aspects can greatly reduce the risk of lameness and improve the overall welfare of the birds.

Another major risk factor for lameness is related to nutrition and mineral metabolism, which Another major risk factor for lameness is related to nutrition and mineral metabolism, which include deficiencies in calcium (Ca), phosphorus (P), vitamin D3, and a lack of essential amino acids. These elements play a key role in maintaining bone health and preventing diseases such as chondronecrosis with osteomyelitis. Vitamin D3 deficiency is also critical, as it is necessary for the proper absorption of calcium and phosphorus. Without adequate vitamin D3, even with calcium and phosphorus, bone problems can occur. Ca and P deficiency significantly impairs bone development in broilers, reducing bone mineral density and bone strength, which increases the risk of lameness [21]. The addition of 25-OH vitamin D3 to broiler diets showed a reduction in the incidence of lameness caused by bacterial chondronecrosis with osteomyelitis, emphasizing its preventive efficacy [22, 23]. The use of organic micronutrients such as Availa-ZMC has shown a 20-25% reduction in lameness by improving the integrity of the intestinal barrier and stimulating the immune response [24]. These micronutrients and vitamins are critical for bone health and must be balanced in the diet of birds. Non-essential amino acids also play an important role in overall health, although their direct link to lameness requires further study.

Factors such as injuries during transportation and handling of poultry can arise from a variety of conditions and procedures. The condition of broilers prior to loading and unloading, including their health and fitness, significantly affects the risk of injury and mortality during transportation. External conditions such as temperature and humidity play an important role, they can lead to hyperthermia, while cold and wet conditions can cause hypothermia, which increases the risk of injury. To reduce these risks it is necessary to improve transport conditions, including temperature and humidity control, and to optimize handling procedures, this will help to reduce injuries [9, 25].

Limping in broilers is a multifactorial problem related to genetics, housing conditions, nutrition and mechanical damage. Rapid growth rates and deficiencies in important micronutrients can lead to poor leg health, and high planting density and poor litter quality exacerbate the situation. To reduce the risk of lameness, it is necessary to improve management, optimize nutrition and minimize stressors.

4 Diagnosis of lameness

Diagnosis of lameness in broilers is an important task and includes clinical evaluation of the severity of the abnormality, laboratory tests (bacteriological analysis, PCR, histology) and imaging techniques such as X-rays and ultrasound to detect bone and joint damage. The main cause of lameness in broilers is bacterial chondronecrosis with osteomyelitis (BCO), which causes significant problems in poultry [7].

Clinical studies have shown that the severity of lameness in broilers can range from mild to severe. Characteristic signs include changes in gait, such as decreased speed, step frequency and stride length, as well as lateral body sway. Lameness is also often associated with other health problems such as race joint burns and foot pad dermatitis [7, 12].

Laboratory diagnostic methods, including bacteriologic analysis, often reveal the presence of bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, in the affected tissues. Histologic studies help to identify morphologic changes such as necrosis and osteomyelitis. Genomic analysis of bacterial pathogens allows a better understanding of disease mechanisms and identification of pathogenic elements [6, 10].

Modern imaging techniques such as infrared thermography (IRT) are used to non-invasively assess leg surface temperature, which can help in detecting subclinical signs of BHO. Computer vision and automated activity monitoring systems can be used for early detection of lameness by analyzing changes in bird activity and gait [26, 27]. A comprehensive approach to diagnosing lameness in broilers that includes clinical observations, laboratory tests and advanced imaging techniques can contribute to the timely detection of lameness and improve the leg health of broiler chickens.

5 Strategies for prevention of lameness

There are several strategies for preventing leg health in birds to reduce the incidence of lameness in broilers. These measures include biosecurity and sanitary measures, genetic selection and growth management, sound nutrition and optimization of broiler housing conditions (Table 3).

Table 3 – Strategy for prevention of lameness in broilers

Strategy	Target problem	Realization	Expected results	Sources
Biosafety	Infectious diseases	Strict sanitary measures, control of pathogen introduction	Reducing the incidence of infections	*Assumpcao* et al., 2024; *Alrubaye* et al., 2020; *Ekesi* et al., 2021.
Power management	Mineral deficiency	Balancing the diet for Ca, P, vitamin D, etc.	Bone strengthening	*Waldensted*, 2006; *Alharbi* et al., 2025; *Wideman* et al., 2015.
Control of planting density	Overpopulation	Optimal space per bird	Stress and injury reduction	*Gocsik* et al., 2017; *De Jong* et al, 2014; *Granquist* et al., 2019.

Continuation of table 3

Genetic selection	Skeletal disorders	Breeding for strong bones, stunted growth.	Less lameness	*Wideman*, 2016; *Guo* et al., 2019; *Kierończyk* et al., 2017.
Use of probiotics	Bacterial infections	Adding Enterococcus faecium and others to feed	Reduced incidence of osteomyelitis and lameness	*Alrubaye* et al., 2020; *Do* et al., 2024; *Alharbi* et al., 2024.
Vaccination	Staphylococcus aureus	Electronically processed vaccine	Reduction of arthritis caused by staphylococci	*Assumpcao* et al., 2024; *Choppa, Kim,* 2023.
Mycotoxin control	Weakening of the immune system and bones	Feed sorbent additives, feed quality control	Prevention of predisposition to lameness	*Alharbi* et al., 2024.
Maintaining bedding quality	Mechanical injuries and infections	Timely replacement and moisture control	Reduction of pododermatitis and secondary infections	*De Jong* et al., 2014; *Kierończyk* et al., 2017.

Studies show that the use of a vaccine based on electron beam-killed *Staphylococcus spp.* showed a 50% reduction in the incidence of lameness due to a more effective immune response [5]. The use of probiotics such as *Enterococcus faecium* and PoultryStar® Bro significantly reduces the incidence of lameness by improving gut integrity and barrier function [16, 19]. The use of organic micronutrients such as Availa-ZMC significantly reduces lameness by improving intestinal barrier integrity [8]. Addition of 25-hydroxyvitamin D3 to drinking water reduces the incidence of lameness by improving calcium metabolism and strengthening bone structure [23]. Using wire-floor models, high levels of lameness can be reproduced to evaluate the efficacy of various preventive measures [7]. These interventions improve immune response, gut integrity and bone health, which together reduce the risk of lameness.

6 Prospects and directions for future research

Current research is focused on developing new diagnostic and monitoring methods that can aid in the early detection and management of lameness in broilers. Systems have been developed that utilize computer vision for early detection of lameness in broilers. For example, automated systems such as eYeNamic™ can assess bird activity and predict lameness levels based on image analysis [26]. These systems may become part of animal welfare assessment schemes in the future.

Using machine learning techniques such as decision trees, broilers can be classified based on their walking ability [28]. These models can automatically estimate lameness based on the birds' walking speed. Accelerometers can be used to monitor activity and detect behavioral changes associated with lameness, which requires further research to improve continuous monitoring [18]. Future research should focus on integrating different technologies and methods to create comprehensive monitoring systems that will allow more accurate and timely detection of lameness in broilers.

Genome studies of bacterial isolates from lameness foci show that pathogens can vary significantly between farms, requiring further study to understand disease mechanisms [10]. Genetic studies show that there is an innate difference in the susceptibility of different broiler lines to BHO, opening up opportunities for breeding more disease-resistant lines [7]. One of the major contributors to lameness is bacterial chondronecrosis with osteomyelitis, which is caused by pathogenic bacteria such as *Staphylococcus* and *E. Coli* [10, 14].

Understanding the microbiological aspects of bacterial chondronecrosis with osteomyelitis, including the role of the gut microbiota and bacterial translocation pathways, is an important area of research. Improving microbial balance with probiotics and other supplements may reduce the risk of lameness in

broilers [14]. Probiotics and other supplements may support bone health and overall broiler resistance, which helps reduce the incidence of bacterial chondronecrosis with osteomyelitis.

Research shows that the physical environment, including litter and air quality, can significantly affect foot health in broilers. Lameness is often associated with race joint burns and paw pad dermatitis, indicating the need for improved housing conditions [12]. Future research should focus on developing new diagnostic methods, exploring new probiotic and phytobiotic supplements, integrating genetic, environmental approaches to develop comprehensive strategies for prevention and treatment of lameness in broilers.

Conclusion

This review work revealed that lameness in broilers is a complex multifactorial problem associated with infectious diseases, poor housing conditions, nutritional deficiencies, genetic predisposition and intensive growth of poultry. One of the most commonly diagnosed pathologic conditions associated with lameness is bacterial chondronecrosis with osteomyelitis (BCO). *Staphylococcus aureus* and *Escherichia coli* have been found to be the main bacterial agents in BCHO, causing inflammation and necrosis of bone tissue, especially in active growth areas in fast-growing broilers.

Infectious agents interact with predisposing non-infectious factors such as poor ventilation, poor litter quality, high planting density and micronutrient imbalances in the diet to exacerbate the risk of lameness. The rapid growth rate of modern broilers places mechanical overload on bones and joints, increasing the likelihood of microdamage and bacterial invasion.

Preventive strategies considered, including the use of probiotics (e.g. *Enterococcus faecium*), organic micronutrients, vitamin D3, and vaccination against *S. aureus*, have been shown to reduce the incidence of lameness in experimental conditions. Special attention is paid to the role of modern methods of visual and molecular diagnostics, such as infrared thermography, automated gait analysis and PCR, which allow to detect subclinical cases of lameness and monitor the health of the herd at an early stage.

Thus, effective control of lameness requires an integrated approach that includes both biological and management measures and the use of modern monitoring technologies. The integration of preventive strategies and early diagnosis will significantly improve broiler welfare and reduce economic losses in the poultry industry.

Authors' Contributions

GA: conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. BB: conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript".

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