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UDC 619:616.98.578.636 **Research article**

Immunogenic activity of a prototype activated moraxella vaccine under experimental conditions

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Introduction

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

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

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

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

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

Introduction

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

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

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

Materials and Methods

Ethical approval

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

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

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

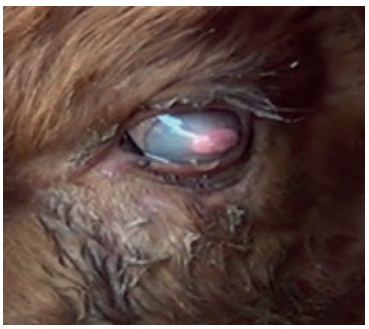

Figure 1 – Abundant ingrowth of superficial blood vessels into the cornea

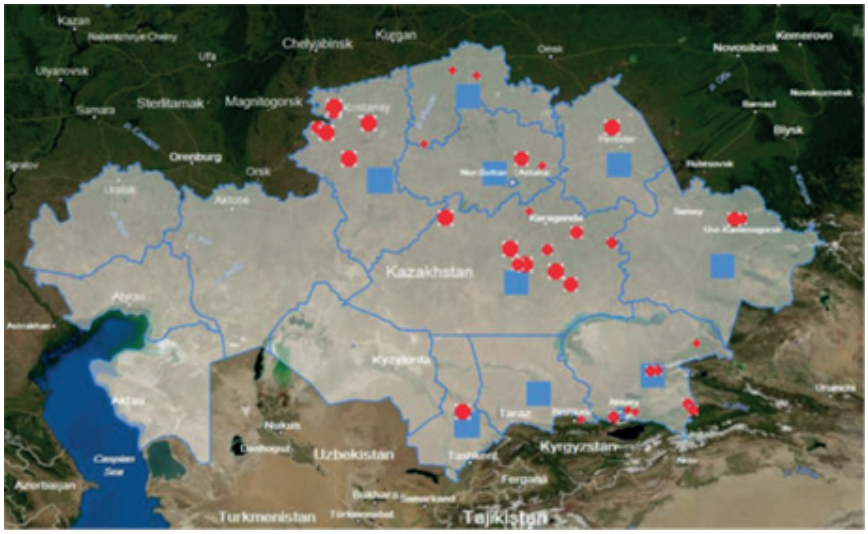

Figure 2 – Epizootic map visualizing the quantitative and qualitative indicators of the moraxellosis epizootic process across various regions of the Republic of Kazakhstan for 2019

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

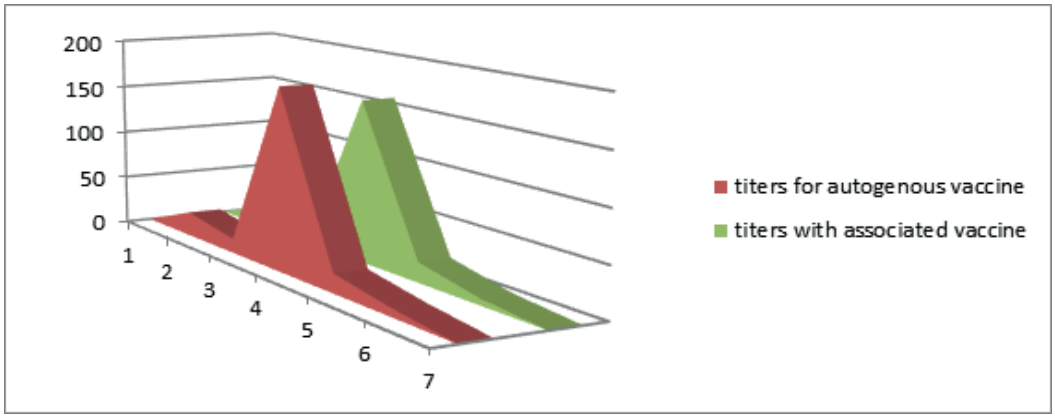

Figure 3 – Titers of specific antibodies in blood serumcattle after immunization with various vaccines

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

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

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

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

Discussion and Conclusion

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

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

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

Authors' Contributions

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

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Competing Interests

The authors declare that they have no competing interests.

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