HERALD OF SCIENCE

OF S. SEIFULLIN KAZAKH AGROTECHNICAL
RESEARCH UNIVERSITY:

VETERINARY SCIENCES

№ 1 (005)

Astana 2024

COMPOSITION OF THE EDITORIAL BOARD

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

Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2024. – N1(005). – P. 4-14. - ISSN 2958-5430, ISSN 2958-5449

VETERINARY SCIENCES

doi.org/ 10.51452/kazatuvc.2024.1(005).1642
UDC 619.616.98.579.841.935.07

USING INDIRECT HEMAGGLUTINATION ASSAY FOR THE DIAGNOSIS OF CATTLE BRUCELLOSIS

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Abstract

The possibility of using indirect hemagglutination assay (IHA) with milk (IHA/m) to differentiate post-infectious antibodies from post-vaccination ones in cows immunized with the *Brucella abortus* 82 was studied. Anti-*Brucella* antibodies by IHA/m were detected in the milk of all lactating animals (n=20) culled on the results of tube agglutination test (AT) and/or complement fixation test (CFT), while the milk ring test (MRT) were negative in 20% of cases. IHA/m, as well as IHA with blood serum (IHA/s), confirmed brucellosis in cows with AT- and/or CFT- negative or questionable results. The correlation coefficient between the results of the two IHA variants was very high (r = 793), and the hemagglutinin titers in the blood serum were significantly higher - 1:760 (+13.3%; -11.7%) than in AT-1:260 (+8.7%; -8.0%) (P≤0.01). Vaccination of cows caused increased production of complement-fixing, agglutinating and precipitating antibodies, which tended to weaken during observation of the animals: 30, 60- and 90-days post-vaccination (p.v.). By the end of the experiment, all vaccinated animals showed questionable AT; positive RID/O-PS and IHA/s were noted in 10% and 20% of cases, respectively. However, complement-fixing antibodies remained at diagnostic values in all animals until the end of the experiment. Despite the intense antibody immune response to the vaccine injection, there were no *Brucella*-specific agglutinins detected by IHA/m and MRT in the milk of cows even on the 30th day p.v. The results show the need for further study on a large population to determine the diagnostic value of IHA/m for differentiating infected from vaccinated animals.

Key words: brucellosis; diagnostics; milk; traditional serological test; indirect hemagglutination assay; post-vaccination antibody.

Introduction

Brucellosis is one of the most common zoonoses in the world, causing significant economic losses and public health problems in more than 170 countries. High levels of humans brucellosis are observed in the Middle East, Mediterranean, sub-Saharan Africa, China, India, Peru, Mexico and other countries [1]. The tense brucellosis epizootic and epidemic situation remains in the countries of Eastern Europe and Central Asia, including the Russian Federation (RF) [2,3] and the Republic of Kazakhstan (RK) [4,

5]. As for RF, in the period from 2011 to 2020, 4 490 and 376 brucellosis-affected farms and flocks were registered with seropositive 95668 cattle and 14533 sheep, respectively. A difficult epidemiological situation has developed in the North Caucasus, Transcaucasia, as well as in the Siberia and the Volga regions. Thus, in the Republic of Dagestan (RD) in 2021, 176 cases of human brucellosis were identified (5.64 per 100 000 population), among which there was a high proportion of minors (1.93 per 100 thousand population), which is associated with traditions individual livestock farming in the republic, when children from an early age actively participate in feeding, maintaining and slaughtering livestock [6]. Kazakhstan, as well as other Central Asian countries, are among the 25 countries with the highest brucellosis incidence [7]. In the RK, 63.4% of rural districts are affected by cattle brucellosis with an average incidence rate of 0.45%. The dynamics of sheep brucellosis incidence has tended to decrease over the years and currently amounts to 0.1%, and the incidence rate of people per 100 000 population ranges from 1.9 to 4.91 depending on the regions of the country, although these figures are clearly underestimated [8].

The difficulty of combating brucellosis is, first of all, explained by the lack of a reliable test for the timely detection of an infected animals. Currently widely used serological tests, such as the agglutination test (AT), complement fixation test (CFT), rose Bengal test (RBT) and enzyme-linked immunosorbent assay (ELISA), diagnose brucellosis based on the detection of antibodies against lipopolysaccharides (LPS) - surface immunogenic antigen of the pathogen. *Brucella* and related bacteria have a very similar LPS structure, which often causes false-positive results [9-11].

Kazakhstan has an unsuccessful experience in implementing the “Test-and-Slaughter” policy (2008-2011), when vaccination and classical serological reactions were canceled, and culling of animals was carried out only according to the ELISA kit indications. The innovation has led to a sharp increase in the number of animals reacting positively to brucellosis [12]. This situation forced the Committee for Veterinary Surveillance and Control of the RK to make a decision to return to traditional serological tests and immunoprophylaxis using vaccines registered in the country, as well as in the member states of the Eurasian Economic Union. As would be expected, since 2012 the number of reflectors began to decrease markedly. Practice has shown that the use of ELISA kits can be used in the serodiagnosis of brucellosis only in the availability of *Brucella* specific antigen [13].

Both in the RF and in the RK, live attenuated Brucella abortus 19 (S) and Brucella abortus 82 (SR) vaccines have been most used to create immunity in animals against brucellosis. These vaccines, as immunogenic preparations, have given rise to the problem of differentiating infected from vaccinated animals, which remains an important issue of veterinary science to this day. Thus, for intravital brucellosis diagnosis simple methods are needed that are superior to traditional serological tests in specificity and allow distinguishing post-infectious antibodies from post-vaccination ones.

Among the non-traditional serological tests currently used to diagnose brucellosis, the indirect hemagglutination assay (IHA) has the greatest potential for widespread introduction into veterinary practice, which can diagnose brucellosis at an early stage, when conventional tests give questionable or negative results [14, 15]. In previous study, we developed a method for preparing Brucella erythrocyte antigen (EA) for IHA [16], which was used for the serodiagnosis of cattle and sheep brucellosis. IHA based on the new EA, which is an extract of B. abortus 19, obtained by autoclaving cells at 0.5-0.7 atm. within 45 min. (pH 8.0-9.0), significantly exceeded known serological tests in sensitivity and specificity [17,18].

The advantage of IHA is that the analyte for it can be milk, an easily accessible, non-invasive biological material [19]. It has been established that in lactating animals with brucellosis, antibodies can be detected not only in the blood of animals, but also in milk. Moreover, with local damage to the mammary gland, antibodies may be absent in the blood, but detected in milk due to the production of antibodies by B-cells of the udder lymph nodes [20-23]. However, so far the possibility of using milk as a test sample in IHA for screening vaccinated livestock for brucellosis remains unexplored. The purpose of the work was to study the serological potential of IHA with milk (IHA/m) in differentiating post-infectious from post-vaccination antibodies in cows immunized with B. abortus 82.

Materials and methods

Animals. The study used 40 lactating cows, of which 10 were immunized with the live attenuated *B. abortus* 82(SR) vaccine (Shchelkovo Bioplant, Moscow, RF) in accordance with the manufacturer's instructions, 20 were unvaccinated reactors isolated for slaughter according to AT and/or CFT indications, and 10 were healthy unvaccinated animals kept on a brucellosis-free farm.

Biological analytes. Blood serum samples for serological studies were taken from cows on the 30th, 60th and 90th days post-vaccination (p.v.) in vacutainers in a volume of 7-8 ml, milk samples - in sterile tubes in the same days from four udder lobes in a total volume of 20 ml. At the same time, cows kept in a brucellosis-free farm were subjected to serological testing. Similar analytes from seropositive cows were taken once before culling.

Serological tests, namely AT, CFT, radial immunodiffusion with O-polysaccharide antigen (RID/O-PS), milk ring test (MRT), IHA with serum (IHA/s) were carried out in accordance with the Interstate Standard GOST 34105-2017 [24].

The milk and blood serum samples were tested by IHA starting from the dilutions of 1:25 and 1:50, respectively, according to our previously described methods [25]. The IHA results were considered positive in the titer of anti-*Brucella* antibodies 1:100 and 1:200 with a score of no less than four crosses ("#") or three crosses ("+++") when examining milk and/or blood serum of unvaccinated and vaccinated cows, respectively. The results with a score of two crosses ("++"), one cross ("+") and minus ("-") were considered negative.

Control sera. As a control for serological tests, negative brucellosis serum and/or positive brucellosis serum (Scientific-Production Company "Biocenter", Omsk, RF) were used. The latter had titers of 1:200, 1:20#, 1:400# and 1:200# for AT, CFT, IHA/s and IHA/m, respectively.

Control milk samples. Milk from a known healthy cow with negative results by classical serological tests and/or a mixture of milk from a healthy cow with positive brucellosis serum (0.1 ml of serum per 2 ml of milk) were used as negative and/or positive control milk samples.

Testing cows for mastitis. Subclinical mastitis was excluded using the "Al-Test" (Caspian Zonal Research Veterinary Institute, Makhachkala, Russia) before testing milk by IHA and MRT for anti-Brucella antibodies.

Statistical processing of serological tests results was carried out according to the method described by T.S. Sayduldin (1992) [26].

Results

Examination of blood sera from healthy cows (n=10) from a brucellosis-free farm by AT, CFT and IHA/s, as well as milk by MRT and IHA/m showed negative results, which indicates the specificity of the serological tests used.

Blood serum and milk samples from unvaccinated cows (n=20), recognized as *Brucella*-infected based on the results of a planned examination of the livestock by the farm's veterinary service, were additionally subjected to serological tests (Table 1).

Table 1 – Titers of agglutinating and complement-fixing antibodies in the blood serum and milk of cows culled due to brucellosis

Animal numbers	Samples examined in serological tests				
	blood serum			milk	
	AT	CFT	IHA/s	IHA/m	MRT
1	1:400#	1:20+++	1:400#	1:400#	+ve
2	1:100+++	1:40#	1:800#	1:400+++	+ve
3	-ve	1:10+++	1:200++	1:200#	+ve
4	1:200#	1:20#	1:400#	1:200+++	-ve
5	1:400#	1:40#	1:800+++	1:800+++	+ve
6	1:200+++	-ve	1:400+++	1:200#	-ve

Continuation of Table 1

7	1:50#	1:10+++	1:200+++	1:200+++	+ve
8	1:100#	1:40#	1:200#	1:400+++	+ve
9	1:200+++	1:20#	1:400+++	1:400#	+ve
10	1:200+++	1:40+++	1:800+++	1:800+++	+ve
11	1:200#	1:10#	1:400+++	1:200#	-ve
12	1:200#	1:20#	1:400#	1:400+++	+ve
13	1:50+++	1:10+++	1:200+++	1:200+++	+ve
14	1;400+++	1:40#	1:400#	1:400#	+ve
15	1:200+++	1:20+++	1:400#	1:400#	+ve
16	1:400#	1:40#	1:800#	1:800+++	+ve
17	1:200#	1:40+++	1:400#	1:400+++	+ve
18	-ve	1:20#	1:400+++	1:400#	+ve
19	1:100+++	-ve	1:200#	1:200+++	-ve
20	-ve	1:10+++	1:100#	1:100#	+ve
Average antibody titer	1:260,0 (+8,7%;-8,0%)	1:17,5 (+8,7%;-8,0%)	1:760,0 (+13,3%;-1,7%)	1:700,0 (+13,3%;-11,7%)	
Notes: (+ve) - positive result; (-ve) - negative result					

Table 1 shows that antibodies in diagnostic titers were detected in all cows by IHA/s, while AT results were negative in 3 and questionable in 5 cows (1:50-1:100). A significant difference was established between the average values of agglutinin titers detected by IHA/s (1:760 (+13.3%; -11.7%) and AT (1:260 (+8.7%; -8.0%) (P ≤0.01). Moreover, the average antibody titer by IHA/s in AT-negative cows - 1:235 (+29.2%; -22.6%) was significantly lower than that of AT-positives - 1:475 (+6.4%; -22.6%). -6.0%) (P≤0.05). CFT was more sensitive than AT, detecting complement-fixing antibodies in 7 sera with negative or equivocal results for anti-*Brucella* agglutinins. It should be noted that among the culled livestock, a cow №19 showed a questionable result by AT (1:100), negative results by CFT and MRT, however, hemagglutinins were detected both in serum and in milk in a titer of 1:200 with an assessment four and three crosses, respectively. In general, both variants of IHA detected anti-*Brucella* antibodies in 45% of cases where AT and/or CFT had equivocal or negative results.

Testing of cows for subclinical mastitis showed the absence of this pathology in culled cows. *Brucella*-specific antibodies by IHA/m were detected in all cows with an average titer of 1:700 (+13.3%; -11.7%), while MRT did not reveal the presence of antibodies in the milk of four cows, two of which were also negative by CFT. The difference between the average titers of IHA/m-antibodies detected in AT-negative and/or positive cows was not significant - 1:235 (+29.2%; -22.6%) and/or 1:400 (+13.3%; -11.7%), respectively. A high direct correlation was established between IHA antibody titers in blood serum and milk (r=0.793), as well as a noticeable direct relationship was noted between the indicators of CFT and IHA/s (r=0.543) and CFT and IHA/m (r=0.564).

The study of the dynamics of post-vaccination antibodies in blood sera by AT, CFT, RID/O-PS and IHA/s, as well as in milk samples by MRT and IHA/m, was carried out during the first three months post vaccination (p.v.) with live attenuated vaccine *B. abortus* 82. Table 2 shows the state of the humoral immune response of cows at the 30th day p.v.

Table 2 – The results of serological study of milk and blood serum samples of cows at 30th day p.v. with *B. abortus* 82

№	IHA/m (1:50-1:400)	MRT	IHA/s				CFT				AT			RID/O-PS
			1:50	1:100	1:200	1:400	1:5	1:10	1:20	1:40	1:50	1:100	1:200	
1	-ve	-ve	#	#	#	#	#	#	#	+++	#	#	+++	+ve
2	-ve	-ve	#	#	#	#	#	#	+++	+++	#	#	#	+ve
3	-ve	-ve	#	#	#	#	#	#	#	#	#	#	+++	+ve
4	-ve	-ve	#	#	#	#	#	#	#	#	#	#	#	+ve
5	-ve	-ve	#	#	#	#	#	#	#	#	#	#	#	+ve
6	-ve	-ve	#	#	#	#	#	#	#	#	#	#	#	+ve
7	-ve	-ve	#	#	#	#	#	#	#	#	#	#	+++	+ve
8	-ve	-ve	#	#	#	+++	#	#	+++	+++	#	#	#	+ve
9	-ve	-ve	#	#	#	#	#	#	#	+++	#	#	++	+ve
10	-ve	-ve	#	#	#	#	#	#	+++	+++	#	#	#	+ve
Notes: (+ve) - positive result; (-ve) - negative result														

From Table 2 it follows that the live attenuated *B. abortus* 82 vaccine has good immunogenicity. Agglutinins were detected up to the maximum dilutions of blood sera by AT (1:200) and IHA (1:400) in 60% and 90% of cases with a score of four crosses, respectively. With a similar assessment, complement-fixing antibodies were detected up to a dilution of 1:40 in half of the livestock. Moreover, precipitating antibodies were also detected by RID/O-PS in all experimental animals. Despite the intense antibody immune response to the vaccination, anti-*Brucella* agglutinins were absent in the milk of all cows, both by MRT and IHA/m.

Table 3 shows the results of testing the analytes at the 60th day p.v.

Table 3 – The results of serological assay of milk and blood serum samples of cows at 60th day p.v. with *B. abortus* 82

№	IHA/m (1:50-1:400)	MRT	IHA/s				CFT				AT			RID/O-PS
			1:50	1:100	1:200	1:400	1:5	1:10	1:20	1:40	1:50	1:100	1:200	
1	-ve	-ve	#	#	+++	-ve	#	#	#	++	#	#	++	+ve
2	-ve	-ve	#	#	++	-ve	#	#	+++	++	#	#	-ve	+ve
3	-ve	-ve	#	#	+++	++	#	#	#	#	#	#	++	+ve
4	-ve	-ve	#	#	++	-ve	#	#	#	+++	#	+++	-ve	-ve
5	-ve	-ve	#	#	+++	+++	#	#	+++	++	#	#	#	+ve
6	-ve	-ve	#	+++	-ve	-ve	#	#	#	++	#	#	-ve	+ve
7	-ve	-ve	#	#	#	+++	#	#	#	-ve	#	#	+++	-ve
8	-ve	-ve	#	#	+++	++	#	#	+++	+++	#	#	-ve	+ve
9	-ve	-ve	#	#	#	++	#	#	#	++	#	#	-ve	+ve
10	-ve	-ve	#	#	+++	+++	#	#	+++	++	#	#	#	+ve
Notes: (+ve) - positive result; (-ve) - negative result														

As can be seen from Table 3, at the 60th day of immunization of cows, a weakening of antibody formation occurred. For example, precipitating antibodies were not detected in two animals, and the results of IHA/s and AT were negative at maximum dilutions in four and five animals, respectively.

Complement-fixing antibodies were characterized by relative stability. Both analyzes designed to test milk for brucellosis (MRT and IHA/m) gave clear negative results.

The immune status of vaccinated cows at the end of the experiment is shown in Table 4.

Table 4 – The results of serological examination of milk and blood serum samples of cows at 90th day p.v. with *B. abortus* 82

№	IHA/m (1:50-1:400)	MRT	IHA/s				CFT				AT			RID/ O-PS
			1:50	1:100	1:200	1:400	1:5	1:10	1:20	1:40	1:50	1:100	1:200	
1	-ve	-ve	#	#	+++	-ve	#	#	+++	-ve	#	#	-ve	-ve
2	-ve	-ve	#	#	-ve	-ve	#	#	++	-ve	#	#	-ve	-ve
3	-ve	-ve	#	#	++	-ve	#	#	+++	-ve	#	#	-ve	-ve
4	-ve	-ve	#	#	++	-ve	#	#	+++	-ve	#	-ve	-ve	-ve
5	-ve	-ve	#	#	++	-ve	#	#	++	-ve	#	#	-ve	-ve
6	-ve	-ve	#	++	-ve	-ve	#	#	++	-ve	#	+++	-ve	-ve
7	-ve	-ve	#	#	++	-ve	#	#	++	-ve	#	#	-ve	+ve
8	-ve	-ve	#	#	+++	-ve	#	#	-	-ve	#	+++	-ve	-ve
9	-ve	-ve	#	#	++	-ve	#	#	++	-ve	#	#	-ve	-ve
10	-ve	-ve	#	#	++	-ve	#	#	++	-ve	#	#	-ve	-ve
Notes: (+ve) - positive result; (-ve) - negative result														

At the 90th day p.v. questionable AT results for brucellosis were detected in 9 immunized animals in a titer of 1:100 with a score of one to four crosses, and in one cow (№4) agglutinins were not detected in the diagnostic titer. The precipitating and hemagglutinating antibodies in diagnostic values were found in the blood sera of one (№7) and two cows (№1 and №8; 1:200+++), respectively. It should be noted that complement-fixing antibodies remained in diagnostic titers (1:5-1:10) with a score of four crosses in all animals until the end of the experiment. MRT and IHA/m, as in the previous periods of the observation, did not detect post-vaccination antibodies in immunized cows.

Discussion and conclusion

Milk, as an alternative biological analyte, is of great interest in the intravital diagnosis of brucellosis, since, firstly, in addition to serum antibodies, it may also contain antibodies produced by B - lymphocytes of the mammary lymph nodes due to local brucellosis; secondly, it can be easily collected without the use of special equipment [27, 28]. As a non-invasive biological material milk is preferable to blood serum for many infectious diseases of lactating cattle since they allow to determine the health status of not only an individual animal but also the entire herd with minimal material costs. Today, MRT is used to determine anti-brucellosis antibodies in milk in countries (Russia, Kazakhstan, Armenia, Belarus and Kyrgyzstan) that have adopted the interstate standard GOST 34105-2017 [24]. The test is characterized by sufficient sensitivity, however, sometimes it gives false positive results, but much more often it produces a negative reaction in cows infected with brucellosis, which is associated with the influence of factors such as fat content and/or acidity of milk, size of fat globules, clinical and subclinical forms of mastitis, as well as the state of pregnancy [29-31]. MRT was no more successful in diagnosing sheep and goats brucellosis than in cows. It has been suggested that the small fat globules in sheep and goat milk are less active in absorbing agglutinated stained *Brucella* cells, do not rise to form a typical colored ring, and settle at the bottom of the tube [32]. Antigens absorbed on the surface of the solid phase (erythrocytes) and the IHA protocol neutralize the indicated negative effects of milk factors on the assay results. For example, it has been established that IHA/m is characterized by specificity, and its results do not depend on inflammation of the mammary gland or pregnancy. In cows with milk hemagglutinins, the results of culture isolation and polymerase chain reaction (PCR) were positive in 67 and 83% of cases, respectively [33].

Our results showed that among the serological tests used, the most sensitive were IHA/s and IHA/m, which confirmed the diagnosis of brucellosis when AT and/or CFT gave questionable and negative readings. Moreover, the degree of correlation between the results of the two variants of IHA was very high (r = 793), and hemagglutinin titers in the blood were significantly higher (1:760) than that of agglutinins detected by AT (1:260; P <0.01). In the blood serum of 10% of animals culled due to brucellosis, antibodies were not detected by CFT, while IHA/m in these cows was positive in a titer of 1:200 with a score of three or four crosses.

Vaccination of cows caused increased production of complement-fixing, agglutinating and precipitating antibodies, which tended to weaken during observation of the animals: 30th, 60th and 90th days p.v. By the end of the experiment, all vaccinated animals showed a questionable and/or negative reaction to brucellosis by AT; positive RID/O-PS and IHA/s were noted in 5% and 10% of cases, respectively. However, complement-fixing antibodies remained at diagnostic values in all animals until the end of the experiment. Despite the intense antibody immune response to the vaccine injection, there were no *Brucella*-specific agglutinins in the milk already on the 30th day p.v., both by IHA/m and MRT.

Thus, the data obtained indicate the need for further studies on a large cow population to determine the diagnostic value of IHA/m for differentiating animals immunized against brucellosis with live attenuated vaccines from infected ones in comparison with culture isolation and/or PCR.

Information on funding

The study was carried out within the project IRN AP19677302 with the grant-targeted funding of the Ministry of Science and Higher Education of the Republic of Kazakhstan for 2023–2025.

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doi.org/ 10.51452/kazatuvc.2024.1(005).1630
UDC 636.09: 619: 616.98

EPIZOOTOLOGICAL MONITORING OF THE INCIDENCE OF PARVOVIRUS ENTERITIS IN DOGS IN KOSTANAY, REPUBLIC OF KAZAKHSTAN

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Abstract

Parvovirus enteritis of dogs is the cause of a highly contagious acute disease of many carnivorous animals, which leads to severe gastroenteritis and myocarditis. Parvovirus infection is characterized by severe enteritis and vomiting, as well as dehydration, fever, leukopenia and diarrhea. The article presents data on the frequency of occurrence of the disease in dogs with parvovirus enteritis in Kostanay, the Republic of Kazakhstan. In order to assess the epizootological situation of parvovirus enteritis, the logs of registration of sick animals, the results of rapid tests for infectious diseases of dogs of private veterinary clinics of the city for the period from 2020 to 2023 were analyzed. The object of the study was dogs of different breeds and age groups. Data analysis has shown that parvovirus enteritis is the most common infectious diseases of dogs. It was diagnosed in 20.3% of veterinary clinic patients, in two forms of intestinal and myocarditis. The disease is seasonal. Infection of dogs with parvovirus occurs mainly in spring (35,6%) and autumn (37,8%). The analysis of the sex and age structure of patients with parvovirus enteritis indicates a greater susceptibility to the disease of puppies under the age of one year. 86% of patients belonged to this age group. Dogs get sick regardless of gender.

Key words: CPV; dog; epizootological monitoring; infectious diseases; intestinal form; myocarditis form; parvovirus enteritis.

Introduction

In addition to the main pathology of animals, which is mainly determined by diseases of a diverse nature, epizootic infections remain relevant [1]. In modern veterinary medicine, from the scientific and practical side, little attention is paid to the problem of infectious diseases of domestic animals (dogs, cats, etc.) of viral etiology, which do not belong to particularly dangerous diseases for humans and animals [2]. The improvement of the epizootic state for a number of infectious diseases is largely the key to the epidemic well-being of the population in the regions and the country as a whole. Viral infections represent one of the numerous groups of infectious diseases among dogs, as a rule, have a different form

of clinical course, including the development of complications [3]. Taking into account the position of dogs in society, these animals, especially high-bred ones, are susceptible to many types of infectious diseases. When studying infectious diseases, special attention should be paid to viral infections, among which the most contagious are distinguished, one of such common diseases is parvovirus enteritis.

Parvavirus enteritis of dogs is the cause of a highly contagious acute disease of many carnivorous animals, which leads to severe gastroenteritis and myocarditis [4]. Parvovirus infection is characterized by severe enteritis and vomiting, as well as dehydration, fever, leukopenia and diarrhea. Treatment of this infection is mainly symptomatic, antimicrobial and antiemetic drugs are also used. The disease has very low survival rates in dogs that have not been treated [5]. The pathogen causes severe clinical disease in puppies under the age of 5 months and adult dogs with insufficient immunity [6]. The susceptibility and mortality rate of this disease in various animal species varies widely. In populations of non–immune dogs and fur-bearing animals, mortality from parvovirus enteritis among adult animals can reach 10-15%, among young animals it is significantly higher - 30-50%, in acute cases it reaches 100% [7].

Enteritis is caused by canine parvovirus (CPV), which has maintained pandemic circulation among dogs for more than 40 years [8]. The pathogen is currently enzootically circulating in the dog population around the world. This virus has a huge ability to mutate. Over 40 years of existence, the original strain has undergone several mutations that gave rise to 3 subtypes: CPV-2a, b, c. The latter are characterized by increased virulence and the ability to recover quickly [9, 10]. In addition to resistance in the external environment, canine parvovirus is characterized by the speed and ease of spread, which is the reason for its spread around the world [11].

Undoubtedly, parvovirus enteritis is one of the most common infectious diseases of dogs, which is registered all over the world and has a different form of clinical course. Currently, domestic dog breeding is developed and at the same time pedigreed animals are most susceptible to viral infections with a complicated form of the course [12].

In this regard, special attention should be paid to the study of the prevalence and frequency of parvavirus enteritis. This will undoubtedly make it possible to periodically monitor frequently reported infectious diseases in dogs, which is of particular relevance for timely prevention and correct diagnostic algorithms using effective therapies [13, 14].

The aim of the study was to conduct epizootological monitoring of parvovirus enteritis of dogs in Kostanay.

Materials and methods

The research was carried out on the basis of five private veterinary clinics in Kostanay, the Department of Veterinary Medicine and the Research Institute of Applied Biotechnology of the Akhmet Baitursynuly Kostanay Regional University.

The object of research was dogs of different breeds and age groups. When assessing the epizootological situation, were used the materials of the logs of registration of sick animals, the results of rapid tests for infectious diseases of dogs of private veterinary clinics of the city for the period from January 2020 to December 2023.

The diagnosis of parvovirus enteritis in dogs was made comprehensively, taking into account epizootological data, anamnestic survey of the owners, clinical examination according to generally accepted methods, studies of general and biochemical blood analysis, if necessary, ultrasound of the abdominal cavity and X-ray.

The final diagnosis was made based on the results of immunochromatographic analysis for the detection of specific antigens of canine parvovirus strains CPV2, CPV2a, CPV2b and CPV2c in dog faeces (QBQVET, Moscow, Russia).

At diagnosis, gastroenteritis of alimentary origin, gastroenteritis of parasitic origin, colibacteriosis, coronavirus enteritis, infectious hepatitis, rotavirus infection were differentiated.

Results

During the period from January 2020 to December 2023, in five private veterinary clinics of the city of Kostanay, 2234 dogs with various infectious and non-infectious pathologies were accepted and clinically examined, of which 24.7% were animals with infectious diseases. Analyzing the data from the

logs of registration of sick animals of veterinary clinics, as well as the results of immunochromatographic analysis for infectious diseases of dogs, it was found that a significant percentage (20.3) accounted for parvovirus enteritis of dogs, was registered in 455 animals. Of these, 103 cases of the disease were observed in 2020, 128 cases in 2021, 115 cases in 2022, and 109 cases of canine parvovirus enteritis in 2023 (Figure 3).

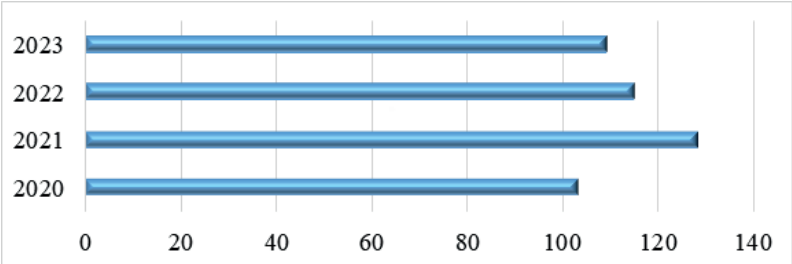

Figure 3 – The number of dogs with parvovirus enteritis in Kostanay, in the period 2020-2023.

An analysis of dog cases of parvovirus enteritis by year showed that in 2021, compared with 2020, the incidence of dogs increased by 24.3% (an absolute increase of 25), and in 2022 and 2023 there was a noticeable decrease in incidence by 10.2% (abs.13) and 5.2% (abs. 6), respectively. On average, 113.75±6.17 cases of canine parvovirus enteritis are detected annually (Table 1).

Table 1 – Dynamics of the increase in the incidence of parvovirus enteritis in dogs in Kostanay, in the period 2020-2023

Year	Number of patients	Absolute		The indicator of visibility, %	Growth rate, %	Pace, %	
		increase	decrease			growth	decrease
2020	103	no		100.0	no	no	
2021	128	25.0		124.3	124.3	24.3	
2022	115		13.0	111.7	89.8		10.2
2023	109		6.0	105.8	94.8		5.2

An analysis of the seasonal dynamics of the incidence of parvovirus in dogs showed that the disease is registered year-round in the form of local enzooties with spring and autumn rises. Most often, parvovirus diseases in dogs were observed in October (19.8%) and April (16.7%). A slightly smaller number of dogs with parvovirus were registered in March, May, July, August, September, December. The frequency of disease detection in these months of the year ranged from 5.5% to 12.5%. January, February, and June can be considered relatively prosperous months of the year for parvovirus enteritis. The frequency of diagnosis of the disease in these months did not exceed 3.9% (Figure 4).

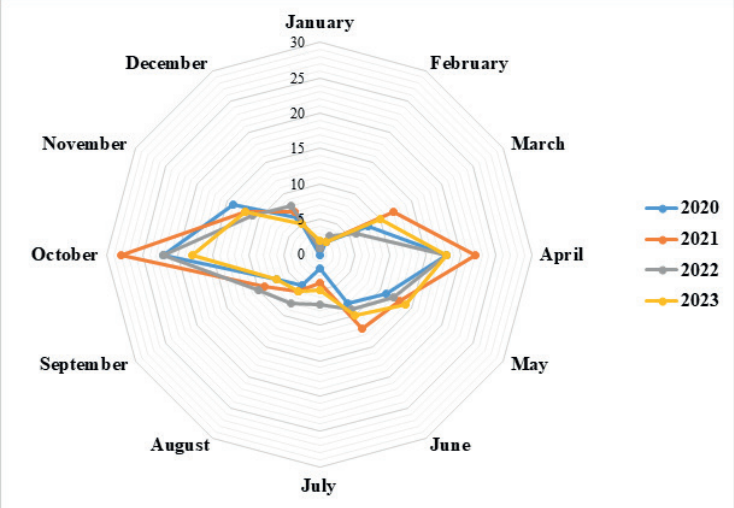

Figure 4 – Seasonal dynamics of the incidence of parvovirus in dogs

Parvovirus enteritis was mainly detected in young, unvaccinated dogs aged two months to a year, the frequency of diagnosis of the disease in dogs at this age was 86% (391 animals), of the total number of cases of the disease, of which 69% were dogs under six months old, and in animals older than 1 year – 14% (64 animals), which turned out to be statistically significant ($\chi 2 = 350.2$; $p < 0.001$) (Figure 5).

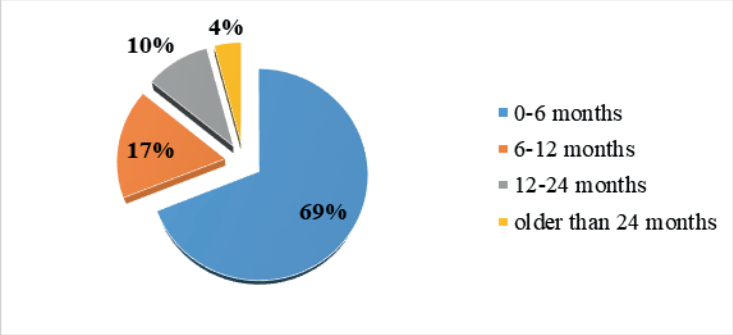

Figure 5 – Distribution of parvovirus cases depending on age

The disease was registered in two forms – intestinal and myocarditis, while the myocarditis form was detected mainly in puppies up to two months of age (Table 2).

Table 2 – Symptoms and forms of parvovirus enteritis

Form	Age	Symptoms
Intestinal	Dogs of all ages	Vomiting, and vomiting movements are repeated at intervals of 30...40 minutes, an increase in body temperature to 41 °C, diarrhea, rapidly increasing dehydration from 8% and above, abdominal pain, pallor of the mucous membranes, tachycardia.
Myocarditis	In 98% of cases, puppies under 2 months old	Signs of heart failure. Cyanosis of the mucous membranes, tachycardia or bradycardia, pulse of weak filling. Shallow and saccaded breathing, collapse

The analysis of the breed susceptibility of dogs to parvovirus enteritis showed that the frequency of diagnosis of the disease in mongrel dogs was 24.8%, and in pedigreed dogs -75.2%, which turned out to be statistically significant ($\chi 2 = 54{,}8$; $p < 0{,}001$). Rottweiler dogs were susceptible to parvovirus enteritis – 32 cases (9.3%), German Shepherd – 42 cases (12.3%), collie – 12 cases (3.5%), pit Bull Terrier - 19 cases (5.5%), Caucasian Shepherd - 34 cases (9.9%), Dachshund- 26 cases (7.6%), spaniel- 23 cases (6.7%), pygmy pinscher- 18 cases (5.2%), Doberman- 16 cases (4.6%), poodle- 12 cases (3.5%), Labrador- 42 cases (12.2%), Toy Terrier- 24 cases (7%), Pomeranian- 28 cases (8.2%), corgi – 14 cases (4%).

The sexual predisposition of dogs to parvovirus has not been established. Thus,
the incidence of females was 49%, and males - 51% ($\chi 2 = 0.55$; $p > 0.5$).

Discussion

Parvovirus enteritis of dogs is widespread all over the world and causes the death of young dogs, despite the availability of effective vaccines [15-17]. The analysis of the epizooological situation in the city of Kostanay, Republic of Kazakhstan for the period from January 2020 to December 2023 also showed that the first place among infectious diseases is occupied by parvavirus enteritis, which is registered in 20.3% (n=455) of cases, in two forms: intestinal and myocarditis. On average, within one year, according to five veterinary clinics, about 108 cases of the disease are registered in the city. On average, during one year, according to five veterinary clinics, about 113.75±6.17 cases of dog disease are registered in the city. In 2021, compared with 2020, there was an increase in the incidence of parvovirus in dogs, which amounted to 24.3%, and in 2022 and 2023, a decrease in the incidence of 10.2% and 5.2%, respectively, was observed.

The disease is fixed year-round, but the frequency of reported cases depends on the season. Infection of dogs with parvovirus occurs more often in autumn (37.8%) and in spring (35.6%). This may be due primarily to the fact that it is during this period that there are sharp temperature fluctuations during the

day, high humidity and, as a result, a decrease in the overall resistance of the body, which increases the risk of the disease.

We found that out of the total number of registered animals with parvovirus, the disease is most often observed in puppies under the age of 1 year, the disease was registered in 86% of cases, these results correlate with studies conducted in other countries where the incidence of parvovirus among animals of the first year of life is more than 80% [18, 19]. In adult dogs older than one year, the incidence was less frequent, mainly in unvaccinated animals or in animals with reduced immunity, i.e. who have been ill with other infectious (colibacteriosis) and invasive (babesiosis) diseases.

The analysis of the breed susceptibility of dogs to parvovirus enteritis showed that the frequency of diagnosis of the disease in mongrel dogs was 24.8%, and in pedigreed dogs -75.2%, which turned out to be statistically significant ($\chi 2 = 54,8$; $p < 0,001$). Dogs of the Rottweiler breed, German Shepherd, collie, Pit Bull terrier, Caucasian Shepherd, Dachshund, spaniel, dwarf pinscher, Doberman, poodle, Labrador, Toy terrier, Pomeranian, Corgi were susceptible to parvovirus enteritis.

The sexual predisposition of dogs to parvovirus has not been established.

Despite extensive vaccination, the main reason for the widespread spread of the virus is still represented either by the intervention of maternal antibodies in vaccinated puppies (the so-called susceptibility window), or by the low effectiveness of immune protection in adult dogs [18, 19].

Conclusion

The analysis of the epizootological situation of parvovirus enteritis of dogs in the city of Kostanay, for 2020-2023, showed that the disease is widespread, occupies 20.3% in infectious pathology in dogs. The disease is registered all year round, but the frequency of reported cases depends on the season. During the year, there are two rises in the disease – spring and autumn, when dogs are infected with parvovirus. Puppies and young dogs are more likely to get sick, especially with a lack of immunization.

Scientific research was carried out within the framework of fulfilling the tasks of dissertation research and will subsequently be submitted for public defense of a doctoral dissertation.

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doi.org/ 10.51452/kazatuvc.2024.1(005).1627
UDC 571.27

MONOCLONAL ANTIBODIES AGAINST CTLA-4 AND PD-L1 RECEPTORS OF THE CATTLE IMMUNE SYSTEM

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Abstract

With the progression of bovine leukemia virus (BLV), the concentration of T-cells, as well as CTLA-4 and PD-1 receptors on their cytoplasmic membrane increases. Elevated concentrations of regulatory T-cells lead to increased production of transforming growth factor-β (TGF-β), suppression of interferon-γ (IFN-γ) expression, tumor necrosis factor-α (TNF-α), and inhibition of natural killer (NK) cells. Effector and cytotoxic T-lymphocytes, as well as the production of cytokines IFN-γ and TNF-α, play a crucial role in immune response against viral infections. However, at late subclinical stages, T-lymphocyte activity decreases due to the activity of regulatory T-lymphocytes, contributing to infection growth and progression to clinical disease. Blockade of CTLA-4 and PD-1/PD-L1 receptors with specific antibodies restores the immune response against BLV.

Monoclonal antibodies (mAbs) against recombinant bovine CTLA-4 and PD-L1 were obtained using hybridoma technology methods. The obtained mAbs were analyzed using enzyme-linked immunosorbent assay (ELISA) and western blotting methods.

As a result of the study, hybridoma cell lines producing mAbs to recombinant bovine CTLA-4 and PD-L1 receptors were obtained. The hybridomas produced IgG1 class mAbs that specifically reacted with standard proteins and had a binding constant: 3b3 - 2.9×108 M-1, 4h10 - 1.9×108 M-1. The obtained mAbs effectively blocked the reaction of commercial bovine CTLA-4 and PD-L1 proteins with specific polyclonal antibodies in an indirect fluorescent antibody assay (IFA).

Key words: bovine leukemia virus; CTLA-4 and PD-1 receptors; ELISA; immune system; monoclonal antibodies.

Introduction

Immunotherapy of chronic infections in cattle represents one of the alternative approaches to prevent disease spread within this population. The efficacy of checkpoint blockade-based immunotherapy has been demonstrated in studies related to bovine leukemia virus (BLV) [1]. Interest in this disease stems from the fact that BLV is a delta retrovirus belonging to the Retroviridae family. It is closely related to human T-cell leukemia viruses' types 1 and 2 (HTLV-1 and HTLV-2) and simian T-cell leukemia viruses (STLV) [2, 3]. BLV is widespread among dairy and beef cattle and manifests as a non-neoplastic disease [4, 5]. In 70% of cases, BLV infection is asymptomatic, significantly complicating control over infection spread in the animal population due to the virus's high replication rate. Approximately 30% of infected cattle develop persistent lymphocytosis, and 1-5% of the population develops malignant B-cell lymphosarcoma [6].

Disruption of the immune system function in BLV-infected cattle occurs at all stages of infection, leading to decreased productivity. BLV infection can affect both innate and adaptive immune system cells and alter the proper functioning of uninfected cells. It has been found that the PD-1/PD-L1 signaling pathway is activated during BLV infection, exerting immunoinhibitory effects [7,8]. Another immune system inhibitor is the CTLA-4 receptor, a co-stimulatory receptor of the B7 receptor family. By competing with the CD28 receptor, the CTLA-4 receptor blocks the CD28:B7 signaling pathway, thereby reducing immune activation. Additionally, the CTLA-4 receptor can disrupt the TCR and MHC signaling pathways and, by blocking the B7.1 receptor on dendritic cells, prevent the development of anti-tumor immunity [9,10,11]. On the other hand, blocking the binding of CTLA-4 and CD80 or CD86 with antibodies restores the immune response against these diseases. In several studies using recombinant bovine CTLA-4-Ig, the inhibitory immune function of bovine CTLA-4 has been demonstrated. Immunization of mice with recombinant bovine CTLA4-Ig induced the formation of anti-CTLA-4 antibodies. Administration of anti-CTLA-4 antibodies significantly increased IFN-γ production by immune system cells in healthy and leukemia-infected cows. According to the authors, antibodies against CTLA-4 may be useful for developing new therapies against BLV infection [12,1]. A similar effect was observed with PD-1/PD-L1 pathway blockade, which enhanced T-cell function and led to BLV proliferation inhibition. It has been demonstrated that reducing the concentration of PD-1+ T-cells by binding them with PD-L1 on B-cells contributes to viral infection progression. According to the authors, PD-1/PD-L1 pathway blockade has potential clinical application for enhancing host antiviral immunity in the treatment of chronic infections [13].

Thus, PD-1, PD-L1, and CTLA-4 receptors are potential targets for restoring the function of exhausted T-cells in chronic viral infections. Studies on checkpoint blockade in cattle with specific antibodies have shown increased IFN-γ production in BLV-infected cattle [1,12,13]. Watari K. et al., (2019) used recombinant bovine CTLA4-Ig to produce antibodies that blocked only the region of the CTLA-4 receptor that reacts with the B7 receptor of T lymphocytes. The aim of this study was to obtain mAbs against recombinant bovine PD-L1 and CTLA-4 receptors of the immune system and study their biochemical properties. To obtain mAbs, recombinant extracellular fragments of PD-L1 and CTLA-4 receptors carrying several epitopes, including the region reacting with the B7 receptor, were used.

Materials and methods

Ethical approval

The article was prepared within the framework of project AP14870156. To implement the project, a positive decision was received from the local ethical commission at the National Center for Biotechnology LLP (IRB 00013497).

Balb/c mice (n=10) 6-8 weeks old (Institute of Cytology and Genetics, Novosibirsk, Russian Federation) were immunized with recombinant bovine CTLA-4 and PD-L1 proteins using a two-week schedule. Recombinant proteins were administered intraperitoneally at a concentration of 25 μg/mL for CTLA-4 (NCB, Astana, Kazakhstan) and 125 μg/mL for PD-L1 (NCB, Astana, Kazakhstan) in 0.1 mL of incomplete Freund's adjuvant. Three days after the final immunization, the serum of immunized mice was examined for the presence of antibodies to recombinant CTLA-4 and PD-L1 proteins. Splenocytes from mice with high antibody titers were used for hybridization. Hybridization of myeloma cells X63-Ag8.653 and immune splenocytes was carried out according to the method described by Oi and Herzenberg [14]. Hybridoma clones producing antibodies were transferred into the wells of a 24-well plate (Corning Incorporated, Kennebunk, USA) as they grew. Cells were cultured until an average monolayer density or concentration of 5x105 cells/ml was achieved. Cloning of hybrid cultured cells was carried out using the limiting dilution method [15].

To obtain preparative quantities of mAbs, hybridoma cells were cultured in 25-50 mL tissue culture flasks containing complete growth medium RPMI-1640 (Sartorius, Beit HaEmek, Israel) for 3-4 days in a CO2 incubator at 37°C. The cells were harvested from the flasks surface by pipetting and centrifuged at 1000 rpm for 7 minutes. The cell pellet was resuspended in incomplete growth medium and 2×106 cells were injected intraperitoneally into BALB/c mice previously injected with pristane (Sigma, St. Louis, USA) at a dose of 0.5 mL per head 7-10 days before hybridoma injection.

Purification of mAbs from as cites fluid was carried out by salting out with ammonium sulfate to 50% saturation with stirring for 12 hours at 4 °C. The resulting precipitate was centrifuged at 5000 rpm for 30 minutes at 4 °C. The antibody pellet was resuspended in PBS (pH 7.2) and dialyzed for 24 hours. The concentration of mAbs was determined using Bradford method [16]. For additional purification, the MAbTrap™ Kit (GE Healthcare, Uppsala, Sweden) was used. Ascitic fluid was diluted with prepared binding buffer at a 1:1 ratio. Subsequently, the sample was applied to a prepared column using a syringe.

The immunochemical properties were determined as follows: the binding constant of mAbs was determined using the method described by J. Beatty [17]. The class and subclass of immunoglobulins were determined using the Rapid ELISA Mouse mAb Isotyping Kit Pierce™ (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. The specificity of mAbswas determined using an indirect ELISA. For this, wells of a 96-well plate (Corning Incorporated, Kennebunk, USA) were sensitized with commercial bovine PD-L1 and CTLA-4 proteins (Kingfisher Biotech, Inc., Saint Paul, USA) at a concentration of 5 μg/mL in 0.05 M carbonate buffer with a pH of 9.6 and incubated for 12 hours at 4 °C. After washing the plate and blocking with a 1 % BSA solution (PAA Laboratories GmbH, Pasching, Austria) the wells were filled with serial dilutions of mAbs, starting from a concentration of 10 μg/mL, and incubated at 37 °C for 1 hour. After washing, an anti-species conjugate was added at a dilution of 1:1000 and incubated at 37 °C for 1 hour. The reaction was developed using a substrate, and the results were recorded on a spectrophotometer iMark (BioRad, Tokyo, Japan) at a wavelength of 492 nm.

Results

One of the key conditions for successful hybridoma generation producing mAbs is the increase in the concentration of positive B-lymphocytes. Therefore, in the optimization of mouse immunization parameters, two different protein administration regimens were employed. In the first case, antigens were injected twice into the hind paw pads with an interval of two weeks. The principle of the second variant of immunization is described in the materials and methods section. It was found that the highest antibody titers were obtained using 5-fold intraperitoneal injections of protein (Table 1).

Table 1 – Optical density of serum from mice immunized with recombinant PD-L1 and CTLA-4 proteins in ELISA

Scheme Number	Route of protein administration	Protein injection dose,μg/mL	OD at 492 nm, M/m				
			day1	day7	day11	day12	day13
Recombinant bovine PD-L1							
Scheme1	Hind foot pad	100	0,155 0,042	-	-	-	0,500 0,047
		200	0,262 0,020	-	-	-	1,107 0,062
Scheme2	Intraperitoneally	100	0,103 0,006	0,252 0,024	0,361 0,031	0,456 0,028	0,541 0,040
		125	0,163 0,023	0,271 0,031	0,431 0,034	0,833 0,046	1,646 0,039
		300	0,322 0,031	0,725 0,041	lethal outcome	-	-
Recombinant bovine CTLA-4							
Scheme1	Hind foot pad	50	0,348 0,028	-	-	-	1,341 0,040
		100	0,665 0,040	-	-	-	lethal outcome

Continuation of Table 1

Scheme2	Intraperitoneally	25	0,213 0,047	0,576 0,029	0,937 0,036	1,345 0,029	1,652 0,057
		100	0,322 0,018	0,742 0,023	lethal outcome	-	-
		200	0,537 0,027	lethal outcome	-	-	-

From Table 1 it can be seen that during immunization according to scheme 2, the optical density in the ELISA were higher and amounted to 1.646 for PD-L1 and 1.652 for CTLA-4. The optimal immunization dose for recombinant PD-L1 and CTLA-4 proteins was 125 and 25 µg/mL, respectively. The obtained results demonstrate that scheme 2 is preferable, since when using lower concentrations of proteins, it made it possible to obtain more intense immunity than scheme 1.

To obtain cell lines producing mAbs against recombinant bovine PD-L1 and CTLA-4 proteins, B-lymphocytes from immunized mice were subjected to hybridization with X-63 myeloma cell line. Several experiments were conducted, and the results of the hybridizations are presented in Table 2.

Table 2 – Indicators of hybridization of B-Lymphocytes from immunized mice and X-63 cell line

Number of hybridizations	Number of myeloma cells	Number of lymphocytes	Number of wells	Number of formed clones/%	Number of clones producing mAbs/%
Recombinant bovine PD-L1					
3	$5x10^6$	$40x10^6$	576	126/22	12/10
Recombinant bovine CTLA-4					
3	$5x10^6$	$40x10^6$	576	100/14	8/8

To obtain stable hybrid cell lines producing mAbs, hybridomas were cloned using the limiting dilution method in 3 replicates. The binding affinity constant (Kaff) of the mAbs was determined using OD-50 sigmoidal curves in ELISA. The method is based on the law of mass action and utilizes the total concentration of antibodies added to the antibody well, rather than the ratio of bound to free antibodies. The ELISA results of the antibodies, 3b3 against rCTLA-4 and 4h10 against rPD-L1, are presented in Figure 1.

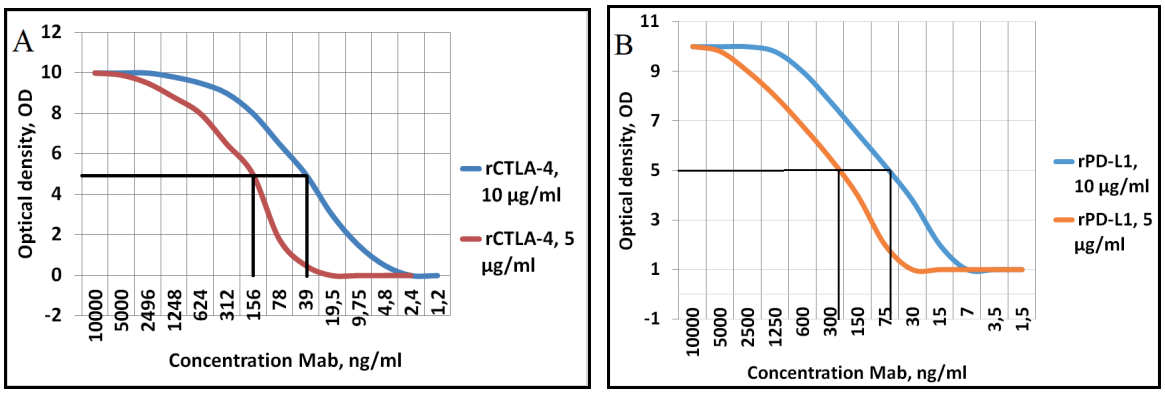

Figure 1 – Experimental ELISA curve for mAbs against two concentrations of bovine rCTLA-4 and rPD-L1 proteins. (A) 3b3 mAb; (B) 4h10 mAb

From the figure 1, it can be observed that the OD-50 sigmoidal curves of the 3b3 mAb strain at two concentrations of rCTLA-4 protein were 156 and 39 ng/ml, while for 4h10 it was 225 and 58 ng/ml. After calculations using the formula, the binding affinity constant (Kaff (M-1)) was determined to be 2.9×108M-1 for 3b3 and 1.9×108M-1 for 4h10.

The subclass of mAbs was determined using the Rapid ELISA Mouse mAb Isotyping Kit Pierce™. The results showed that the mAbs to bovine rCTLA-4 and rPD-L1 proteins belong to the immunoglobulin class IgG1.

To determine the specificity of the obtained mAbs, commercialbovineCTLA-4 and PD-L1 proteins from Bovine CTLA-4 ELISA Kit and Bovine PD-L1 ELISA Kit (Kingfisher Biotech, Inc., USA), were used. The results of specificity testing are presented in Figure 2.

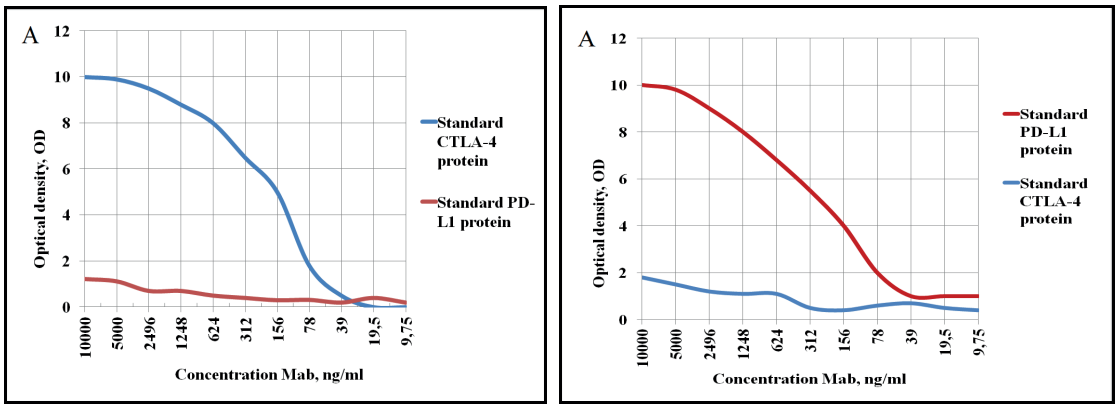

Figure 2 – Experimental ELISA curve for mAbs against two commercial bovine CTLA-4 and PD-L1 proteins. (A) 3b3 monoclonal antibody; (B) 4h10 monoclonal antibody

From Figure 2, it is evident that the mAbs 3b3 and 4h10 specifically bind to commercial bovine CTLA-4 and PD-L1 proteins at concentrations ranging from 5 μg/ml to 78 μg/ml. No reactions of the mAbs with heterogeneous proteins were observed.

Discussion

Despite the veterinary measures being implemented, the significant prevalence of BLV has intensified the interest in studying immune checkpoints for treating chronic infections in cattle. It has been determined that the progression of bovine leukemia virus-related disease (BLV-RS) increases the concentration of regulatory T lymphocytes, leading to elevated production of TGF-β, which, in turn, suppresses the expression of IFN-γ and TNF-α, as well as NK cell suppression [18]. A correlation with the PD-1/PD-L1 signaling pathway and lymphocyte activation gene 3 (LAG-3) has been noted in BLV-RS infection [8]. Additionally, it has been reported that increased expression of CTLA-4+ T cells leads to the progression of BLV infection [19].

Information is available on obtaining mAbs by immunizing mice with CTLA-4-Ig protein. Purified mAbs were found to bind to bovine cells expressing CTLA-4 and did not bind to CD28-expressing cattle Cos-7 cells. The application of anti-CTLA-4 antibodies significantly increased IFN-γ production by the immune cells of healthy and BLV-infected cattle. According to the authors, anti-CTLA-4 antibodies could be useful for developing new therapies against BLV infection [1]. A similar effect was observed with PD-L1 blockade antibodies, which enhanced T-cell function and inhibited BLV proliferation [20].

As a result of the research, an effective mouse immunization regimen and protein injection dose were selected, which allowed for a high level of antibody concentration in immunized mice. Thus, when mice were immunized with rPD-L1 protein, the OD value in the ELISA assay was 1.651, while immunization with rCTLA-4 protein yielded an OD of 1.731. Hybridization of immune mouse B-lymphocytes with the myeloma cell line X-63 resulted in 12 hybrid clones producing antibodies against bovine rPD-L1 and 8 against bovine rCTLA-4 proteins. For further work, clones 3b3 (anti-CTLA-4) and 4H10 (anti-PD-L1), demonstrating the highest antibody activity in ELISA, were selected. The obtained clones produced IgG1 class antibodies, specifically bound to commercial bovine CTLA-4 and PD-L1 proteins, and had a binding constant: 3b3 - 2.9×10^{8} M^{-1}, 4H10 - 1.9×10^{8} M^{-1}.

Conclusion

Blocking CTLA-4 and PD-L1 receptors of cattle with antibodies contributes to increasing the concentration of T lymphocytes and the expression of IFN-γ, thereby effectively inhibiting BLV. As a result of the study, hybridoma cell lines producing mAbs to bovine CTLA-4 and PD-L1 receptors

were obtained. The obtained mAbs belong to the IgG1 class, specifically reacted with commercial bovine proteins, and had a binding constant: 3b3 - 2.9×10^{8} M^{-1}, 4H10 - 1.9×10^{8} M^{-1}. The obtained mAbs efficiently blocked the reaction of commercial bovine CTLA-4 and rPD-L1 proteins with specific polyclonal antibodies in ELISA.

Information on funding

This research was performed within the framework of the budget program 217 of the Ministry of Education and Science, the Republic of Kazakhstan on the project AP14870156 "Obtaining producers of monoclonal antibodies to bovine PD-L1 and CTLA-4 receptors for treating chronic viral and bacterial infections", for the period 2022–2024.

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2024. – N1(005). – P. 30-36. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2024.1(005).1639
UDC 619:616

PREVALENCE OF THEILERIA ANNULATA AMONG CATTLE IN THE TURKESTAN REGION

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Abstract

Theileriosis is a severe blood-parasitic disease, an important problem in veterinary protozoology, since the damage it causes remains significant. The recently widespread practice of importing breeding stock to improve local breeds leads to the fact that in the first summer season, upon contact with infested ticks, the imported animals become very seriously ill, with a mortality rate of up to 90-100%. In this work, 738 samples of cattle were examined using the polymerase chain reaction method, which showed the presence of infection in 598 DNA samples isolated from cattle from 10 districts, 19 settlements of the Turkestan region. Whole blood samples were taken from cattle aged 3 years and older. Upon examination, the animals seemed healthy. The overall positivity rate for the entire region was 81%, highlighting the prevalence of infection among livestock. There are different levels of infection in the regions; in 6 settlements the infection rate of cattle was 100%. In addition, 7 villages showed high PCR positivity rates, ranging from 70% to 97%. While the lowest prevalence of infection showed from 18% to 69% in 6 villages, respectively.

Key words: cattle; polymerase chain reaction; theileriosis; *Theileria annulata.*

Introduction

Theileriosis is an acute or subacute vector-borne disease of domestic and some wild animals caused by pigmented protozoa of the genus *Theileria.* In sick animals, an increase in lymph nodes, high fever, parasitemia of lymph node cells and parenchymal organs, and then red blood cells, impaired function of the cardiovascular and digestive systems are noted [1].

Theileria spp. pathogens are specific to different species and affect both wild and domestic animals. Several species of Theileria spp. have been identified that are parasitic in cattle: *Theileria parva, T. annulata, T. mutans, T. velifera, T. sergenti, T. taurotragi* and *T. orientalis*. The most pathogenic are *T. parva* and *T. annulata*, which are highly lethal and induce the transformation of infected lymphocyte cells or macrophages. Other types of theileria do not cause uncontrolled proliferation of infected white blood cells, but instead multiply predominantly in infected red blood cells. T.parva is found in southern

Africa, while T. annulata has been recorded in southern Europe, North Africa and Asia [2]. Infection of *Theileria orientalis (T. orientalis)* occurs in the Far East, Central Asia and in cattle usually occurs subclinically [3].

Bovine theileriosis caused by *Theileria annulata (T. annulata)* is an economically important infection causing serious damage to livestock. Theileriosis causes pathological changes in the organs and systems of the animal body, which lead to the fact that they do not recover to the physiological norm for a long time. Milk yields in sick cows do not return to normal during this lactation. Among the livestock of the meat sector, this disease leads to a sharp emaciation of animals, loss of up to 30% of body weight and deterioration in the quality of meat products from slaughtered animals [4,5]. In Kazakhstan, theileriosis caused by *T. annulata* is common in Turkestan, Kyzylorda, Almaty, Zhambyl regions [6]. Every year in the South Kazakhstan region, there is a case among cattle from theileriosis. Over-sick cattle restore productivity for a long time (1-2 months) only up to 80%. A high percentage of infection leads to the fact that imported highly productive cattle are very difficult to tolerate the disease with a high level of lethality. This, of course, hinders the intensification of animal husbandry [7]. Since ticks of the genus *Hyalomma*, which are carriers of *T. annulata*, are registered in almost all regions of Kazakhstan, there is a danger of spreading the invasion to new regions [8]. Most of the works describing the spread of theileriosis in Kazakhstan are based on microscopic research methods and affect different ages of animals [9]. The purpose of our work was a monitoring study the carriage of *T. annulata* in cattle older than three years in the Turkestan region, which will allow to assess the infection rate of animals that have had contact with ticks for at least two pasture seasons.

Materials and methods

Ethical approval

This study was approved by the local ethics committee in the Kazakh National Center for Biotechnology (Protocol #1 dated April, 2022). The respective cattle owners gave their approval for sampling. No animal was harmed during the sampling.

Study area and objects

The territory of the Turkestan region is in the south of Kazakhstan, within the eastern part of the Turan lowland and the western spurs of the Tien Shan and has a warm and mild climate, which is favorable for the development and reproduction of many pathogens and vectors of invasive diseases of farm animals, which cause great economic losses to livestock. The number of cattle in Turkestan at the end of 2023 was 1 082 702, respectively, which is 12.6% of the total number of cattle in Kazakhstan according to the Bureau of National Statistics of the Republic of Kazakhstan [10].

Sampling

The sampling period is from mid-June to the end of July 2023. Whole blood samples were taken from cattle aged 3 years and older. Upon examination, the animals seemed healthy, special clinical examination or investigation for current tick infestation was not carried out. The blood was collected in vacuum tubes with ethylenediaminetetraacetic acid (EDTA) and delivered at a temperature of 4 °C to the laboratory within 48 hours. 738 DNA samples isolated from cattle from 10 districts and 19 settlements of the Turkestan region were examined.

DNA isolation

DNA was isolated from 300 µl of whole blood using a modified method of Boom using silica powder with preliminary lysis of erythrocytes [11]. Erythrocytes were lysed RBL (Red Blood Cell Lysis Buffer, NH4Cl (150 mM), NaHCO3 (10 mM), EDTA (1 mM, pH 8.0), H2O) in a ratio of 1:3. Lysis of leukocyte mass was carried out in a lysing buffer (30mM Tris-HCl, 30mM EDTA, 5% Tween20, 0,5% Triton X-100, 3,2 M GuaSCN), followed by sorption of DNA onto silica. The silica with bound DNA was washed twice with a washing buffer (75 % Ethanol, 10mM Tris), dried and eluted in 100 µl of 1 x TE (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) buffer.

Polymerase chain reaction

For the screening of samples, a standard polymerase chain reaction was used using primers that were previously described in the article by Kuibagarov M. et al. 2023 [9]. Matched to the enolase gene: Eno_T.anul_F 5'-ttgcgagatggagacaaaagc-3' and Eno_T.anul_R 5'-tcagggtgtgataaacttctgcc-3'. Distilled sterile water was used as a negative control. DNA samples in which the corresponding pathogen was confirmed by PCR and direct Sanger sequencing were used as a positive control. The PCR reaction

was performed in a total volume of a reaction mixture of 25 µl: 10 pmol of each primer, 5 µl of matrix DNA, 12.5 µl of UDG HS-qPCR BioMaster (2x) and water up to 25 µl. The PCR amplification program included: anticontamination treatment of the PCR mixture for 2 minutes at 50 °C, prolonged denaturation of 95 °C for 5 minutes, 40 cycles of 95 °C – 30 seconds, 60 °C – 40 seconds, 72 °C – 50 seconds, final elongation of 5 minutes at 72 °C, the PCR program was performed using an amplifier Mastercycler ProS (Eppendorf, Germany).

The analysis of DNA fragments amplified by PCR was performed by electrophoresis in 1.5% agarose gel containing ethidium bromide. Electrophoresis was performed in a horizontal Powerpack electrophoresis chamber using a current source for Bio Rad electrophoretic bath. A 1X TAE buffer was used as a buffer for electrophoresis. The results were documented using the Gel Doc (Bio-Rad) gel documentation system and Quantity One (Bio-Rad) software. The sizes (bp) of PCR amplifications were determined by comparing their electrophoretic mobility in a gel with the mobility of a marker ladder (Biolabmix, a marker with a molecular weight of 100 – 3000 bp, in increments of 100 bp).

Statistics

The exact Clopper-Pearson method, which is quite conventional and tends to produce wider intervals than necessary, was used based on the beta distribution to calculate the 95% confidence interval (CI).

Results

A total of 738 DNA samples isolated from cattle from 10 districts and 19 settlements of the Turkestan region were examined (Figure 1).

1 – Map of Kazakhstan (as of 2024), presenting the location of selected villages in the provinces of Turkestan

A PCR fragment characteristic of the *T. annulata* genome was detected in 598 DNA samples by PCR (Figure 2).

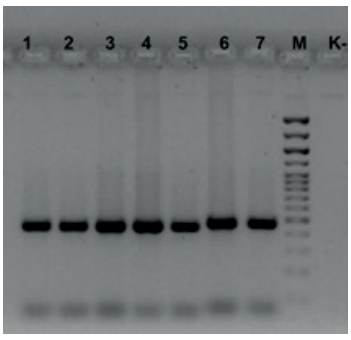

Figure 2 – Electrophoregram of PCR products of 7 positive samples; (M) molecular weight marker (Biolabmix) (100 – 3000 bp, in increments of 100 bp), (K-) negative control sample

Figure 1 shows the result of PCR with species-specific primers of 7 samples, in all samples a specific fragment of 450 bp was amplified. No PCR products were observed in the negative control sample, which indicates the absence of contamination in the reaction.

Thus, in our study, the infection rate was 81%. At the same time, there is a difference in infection rates in different localities (Table 1).

Table 1– Data on the spread of theileriosis in the Turkestan region

District	Village	N cattle examined	N cattle positive by PCR	% cattle positive (CI) 95% confidence interval
Sayram	Karabulak	41	36	87.8 (73.8-95.9)
	Karamurt	20	7	35.0 (15.4-59.2)
	Mankent	20	12	60.0 (36.0-80.9)
Ordabasy	Badam	39	38	97.4 (86.5-100)
	Burdjar	40	40	100 (91.1-100)
Otyrar	Aktobe	30	25	83.3 (65.3-94.4)
	Talapty	39	30	77 (60.7-88.9)
Tulkibas	Abai	88	83	94.3 (84.2-98.1)
Kazygurt	Karzhan	50	43	86.0 (73.3-94.2)
	Zhanabazar	70	35	50.0 (37.8-62.2)
Tole Bi	Kogaly	23	8	34.8 (16.4-57.3)
Sauran	Karashik	17	17	100 (80.5-100)
	Shornak	49	49	100 (92.7-100)
Saryagash	Saryagash	45	38	84.4 (70.5-93.5)
	Zhilga	49	49	100 (92.7-100)
Baydibek	Shayan	49	33	67.5 (52.5-80.0)
Sozak	Syzgan	27	27	100 (87.2-100)
	Sozak	25	25	100 (86.3-100)
	Sholakkorgan	17	3	17.7 (0.38-43.4)
Total		738	598	81.0 (78.0-83.9)

In 6 settlements (Kaushik, Shornak, Syzgan, Sozak, Zhilga and Burjar), the infection rate of cattle over 3 years of age was 100%. While the lowest prevalence of infection (18%) was observed in animals from Sholakkorgan in the Sozak region. In addition, in 7 villages (Karabulak, Aktobe, Talenty, Abay, Karazhan, Badam) high PCR positivity rates were detected, ranging from 70% to 97%. The percentage of positive PCR results varied from 30% to 69% in 5 villages, respectively (Karamurt, Mankent, Zanabazar, Shayan, Kogaly).

Discussion

The territory of the Turkestan region is highly endemic for bovine theileriosis. A study of 738 samples of cattle using the polymerase chain reaction (PCR) method showed the presence of infection in 598 DNA samples isolated from cattle from 10 districts and 19 settlements of the Turkestan region. The results are divided into different villages and districts, allowing a more detailed assessment of the spread of infection in different parts of the region. The overall positivity rate for the entire region was 81%, highlighting the significance of the infection in livestock. Our results coincide with previously obtained data from Kuibagarov et al, [9] who determined 84.4% of the prevalence of infections caused by *T. annulata i*n the Turkestan region using the molecular genetic method. Also, one of the works on the prevalence of theileriosis in cattle in Kazakhstan is a microscopic study of blood smears of cattle in the Kyzylorda region; the authors described 23.7% of cases of infection of animals [12].

Differences in positivity rates between different villages and districts show the heterogeneity of infection spread across the region. This may indicate different factors influencing the spread of infection related to relief and microclimatic conditions in different areas. For example, in mountainous areas such as Tole bi and some Sairam villages, positive results were lower, while in steppe areas such as Ordabasy, Sauran, Sozak, Saryagash, positive results were higher.

Attention must be paid to marked geographic differences in pathogen distribution. However, it is worth noting that in addition to the general climate, local microclimatic conditions play a significant role in the spread and maintenance of ixodid tick infestations [13].

The significant range of absolute altitudes of the territory and the peculiarities of the water regime determine the presence in the Turkestan region and have a significant impact on the species composition and number of ixodid ticks parasitizing farm animals. Farm animals in Southern Kazakhstan are parasitized by 12 species of ixodid ticks. The richest fauna is of ixodid ticks of the genus *Hyalomma, species H. anatolicum, H. detritus, H. scupense, H. aziatic, H. plumbeum,* which are potential carriers of the causative agent of theileriosis in cattle grazing in low-mountain steppe and tugai agricultural landscapes - 10 species. The smallest number of tick species is recorded in the desert landscape - 5 species; in the semi-desert landscape, 9 species parasitize [14]. A recent study of 2809 ticks collected from cattle and livestock buildings in the Turkestan region showed a high abundance of *H. anatolicum* (47.3%), *H. scupense* (26.3%) and *H.asiaticum ticks* (7.9%) with infection rates of 0.5%, 0.1% and 0.9%, respectively [15].

In studies by Sang C. et al, it was indicated that *D. marginatus* and *H. asiaticum* are the most common tick species in the five border regions of Kazakhstan. *D. marginatus* and *H. asiaticum* are also dominant in the neighboring Xinjiang Uyghur Autonomous Region, as South-East Kazakhstan and North-West China share a 1,783 km border [16].

Conclusion

In conclusion, our results show that theileriosis in cattle is widespread in the Turkestan region. A study of 738 samples of cattle using the polymerase chain reaction (PCR) method showed the presence of infection in 598 DNA samples isolated from cattle from 10 districts and 19 settlements of the Turkestan region. The overall positivity rate for the entire region was 81%, highlighting the significance of the infection in livestock. The difficult situation regarding blood-parasitic diseases in the Turkestan region must be taken into account when planning to regulate the movement of animals to other regions. Since the species of ticks *H. anatolicum, H. asiaticum, H. scupense, H. aziatic, H. plumbeum a*re distributed over 90% of the territory of Kazakhstan, there remains a potential threat of shifting the area of theilariasis to the northern regions of Kazakhstan.

Information on funding

The study was funded by the Ministry of Education and Science of the Republic of Kazakhstan within the framework of project, grant no. AP14869969 for 2022–2024.

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2024. – N1(005). – P. 37-43. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2024.1(005).1631
UDC 619:616.24:616.9:636.2:005.5(045)

EPIZOOTOLOGICAL MONITORING OF EQUINE HERPESVIRUS IN KAZAKHSTAN

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Abstract

Equine herpesvirus is one of the most common infectious diseases of horses, causing huge economic losses associated with fetal abortion and subsequent chronic course. At the same time, a difficult epizootological situation regarding herpesvirus infections remains in the republic. Therefore, epizootological surveillance and planned monitoring studies of the epidemiological process in a certain territory are one of the effective tools for preventing the spread of equine herpesvirus in horse breeding farms. This article presents the results of an analysis of epizootological monitoring and ongoing diagnostic measures regarding equine herpesvirus on the territory of the Republic of Kazakhstan. According to epizootological data, over the past 10 years, 35 outbreaks of equine herpesvirus have been registered in Kazakhstan. Serological monitoring confirmed the circulation of the causative agent of equine herpesvirus in the country's horse breeding farms. In a study of 1391 blood serum samples, antibodies to equine herpesvirus were detected in 93 cases (6.7%). The data obtained give reason to believe that in disadvantaged areas it is necessary to take a full range of measures for timely diagnosis, prevention and elimination of foci of infection.

Key words: diagnostics; equine herpesvirus; epizootological monitoring; herpesvirus; horses; prevention.

Introduction

In the Republic of Kazakhstan, horse breeding is a traditional and profita-ble branch of animal husbandry: according to the State Statistics Service of the Agency for Strategic Planning and Reform of the Republic of Kazakhstan, in 2023 there were more than 4 million horses in the country [1]. Along with meat and dairy horse breeding, the breeding of stallions and mares of the purebred horse breed is promising. Currently, in all regions of the republic, various types of personal, subsidiary, farm and state horse breeding farms are organized, the number of local and imported horses purchased in Russia, the CIS countries and many other foreign countries is gradually increasing [2].

At the same time, despite the intensive development of horse breeding in Kazakhstan, one of the hindering factors for its development in the country is diseases of contagious etiology. In this regard, equine herpesvirus (equine her-pesvirus infection, equine viral abortion) poses a constant threat to horse breed-ing.

Equine herpesvirus (EHV) is a highly contagious viral infection that causes upper respiratory tract disease, neurological impairment, abortion and neonatal death. EHV is the historical common name of a disease caused by two DNA viruses of the herpesviridae family – equine herpesvirus-1 and equine herpesvirus-4 (EHV-1 and EHV-4) [3,4]. Both viruses belong to the alpha herpesvirus genus; their genomes are 55-84% identical in nucleotide composition and up to 96% similar in amino acid composition. Today, EHV-1 and EHV-4 belong to the sub-family of alphaherpesviruses of the Varicellovirus genus [5,6,7].

EHV causes significant economic damage to horse breeding, which consists of the loss of reproductive ability of mares, lack of offspring, culling of horses that are valuable for breeding, and the implementation of veterinary and sanitary measures [8, 9]. In addition to direct damage, this infection creates barriers to in-ternational trade and transportation of both horses and a large arsenal of goods under the control of the veterinary service [10].

The epizootic situation regarding EHV in many countries of the world re-mains tense and ambiguous. The disease is registered on almost all continents and annually results in large economic losses for horse breeding farms. In recent years, a significant increase in the incidence of equine herpesvirus infection has been observed in countries with developed horse breeding (USA, Canada and the EU) [11,12]. EHV outbreaks, in addition to causing direct economic losses to horse farms, can also disrupt important economic sectors such as horse racing. The occurrence of EHV-1 infection in different countries varies from isolated sporadic cases to infection of 90% of the herd. Mortality also varies during out-breaks and can reach 40–50% [13,14].

At the same time, in Kazakhstan there is very little research in the field of epizootology of EHV, there is no new data on the dynamics of the epizootic pro-cess of the disease, depending on the impact of natural, climatic and economic factors.

According to epizootological data, equine herpesvirus is often recorded on farms where special preventive measures are not taken. According to literature data and official veterinary statistics, equine herpesvirus began to occur in Kazakhstan in 2011. The first time equine herpesvirus was recorded in the Almaty region. Further, in 2012, serotypes EHV-1 and EHV-4 were detected in two dis-tricts of Zhambyl region [15]. In subsequent years, foci of equine herpesvirus were registered in other regions of the country.

Based on the above, the purpose of this research is to assess the current epizootological situation of EHV in Kazakhstan, by conducting epizootological monitoring and diagnostic studies in various regions of the country.

Materials and methods

The research was carried out at the Department of Veterinary Sanitation of the S. Seifullin Kazakh Agrotechnical Research University and on the basis of the RSE on the REM «National Reference Center for Veterinary Medicine» (NRCVM), Committee for Veterinary Control and Supervision, Ministry of Agri-culture, Republic of Kazakhstan.

For epizootological analysis, veterinary reporting forms, data of own stud-ies were used, as well as analysis of statistical data and epizootological surveys of a number of affected farm horse farms in the Republic of Kazakhstan, based on data on the geography of the regions included in the zones of well-being from equine herpesvirus of horses with vaccination, and other necessary information. Also, in epizootological studies, the materials of periodic bulletins of the International Epizootic Bureau on cases of registration of horse EHV in various countries of the world were used.

«Diagnostics of Infectious Diseases Laboratory, NRCVM, conducts moni-toring studies for equine herpesvirus of horses every year. Serological supervision of equine herpesvirus is based on the detection of antibodies by enzyme-linked immunoassay (ELISA) (Indezim Rhinopneumonitis test system, Spain) in monitoring studies. Molecular genetic studies were performed by real-time polymerase chain reaction (PCR) using a Equid herpesvirus 1 qPCR Kit is designed for the in vitro detection of EHV-1 genomes (NZYTech, Lisboa, Portugal).

The 2023 plan included laboratory diagnostic studies for serological moni-toring of rhinopneumonia-susceptible animals (horses), regardless of age, sex and breed. The sampling locations were determined by using data from the Farm Animal Identification Program used in the Republic of Kazakhstan. The method includes a randomized method of sampling blood sera in each settlement where horse breeding is available. The flocks themselves for sampling are determined by local veterinary services under the supervision and supervision of the veterinary inspection.

Materials for serological and molecular genetic studies were 1391 samples of equine blood sera collected in all 14 regions of the country.

Results

A retrospective analysis of epizootological data on EHV confirms the country's poor situation with regard to this infection. Information on the registration of EHV in Kazakhstan over the past 10 years is presented in Table 1.

Table 1 – Cases of registration of EHV in the Republic of Kazakhstan (2014-2023)

Name of the regions	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023
North Kazakhstan Region	-	1	2	-	-	2	-	2	8	1
Akmola	1	-	-	-	-	-	-	-	1	-
Atyrau	-	-	-	-	1	-	-	-	1	-
Aktobe	-	-	-	-	-	-	-	1	-	-
West Kazakstan Region	-	-	1	-	-	-	-	1	-	-
Karaganda	-	1	-	1	1	-	2	1	-	-
Pavlodar	-	2	1	-	1	-	-	-	2	-
Total	1	4	4	1	3	2	2	5	12	1

As can be seen from the table, in the period 2014-2023, EHV was regis-tered in 7 regions out of 14. Moreover, in contrast to the early periods (2011-2012), foci of infection were detected in all regions except the southern regions. A total of 35 outbreaks of equine herpesvirus were recorded during the observation period in Kazakhstan. And if in certain regions (Akmola, Atyrau, Aktobe, West Kazakhstan) only isolated cases of horse disease with EHV were recorded for 10 years, then in the North Kazakhstan region 16 outbreaks or 45.7% of all were recorded during this period cases of disease. In addition, in the Akkayin and Kyzyzhar districts in the North Kazakhstan region and the Karkaraly district of the Karaganda region, a certain stationarity of the disease is observed.

Taking into account the area of registration of EHV in the country, we collected blood serum samples for monitoring diagnostic studies. Sampling in areas for serological studies was carried out taking into account the current epidemio-logical situation and the number of susceptible livestock in a particular area (Figure).

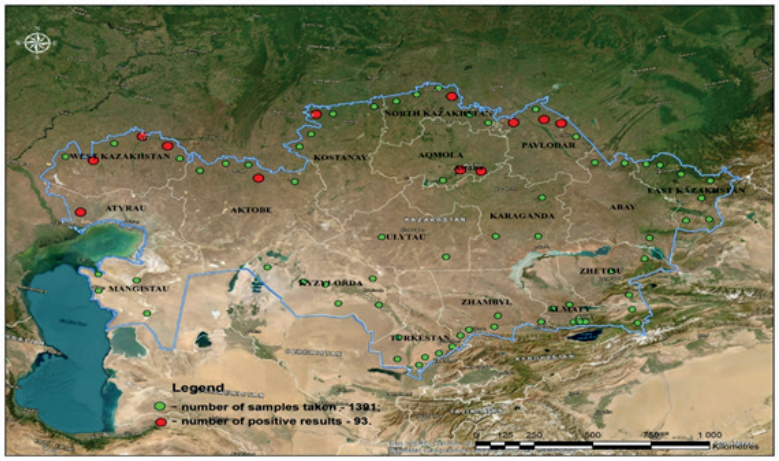

Figure 1– Visualization of sampling sites for monitoring studies of equine herpesvirus in 2023

As a result, 1391 blood serum samples were taken from farms of various forms of ownership in 14 regions of the republic and Almaty. Taking into account the presence of various risk factors (the number and density of susceptible livestock, retrospective analysis of the epizootic situation, economic ties, the risk of introducing the pathogen from outside, etc.), the largest number of samples were taken in West Kazakhstan (198), Almaty (147), Turkestan (136), Kyzylorda (114), East Kazakhstan (105), Kostanay (103) and Karaganda (102) regions.

The obtained horse blood serum samples were examined by ELISA using the Indezim Rhinopneumonitis test system (Spain). Next, in order to detect the DNA of the pathogen, serum samples that reacted positively to equine herpesvi-rus in ELISA were examined by PCR. The research results are presented in Table 2.

Table 2 – Results of diagnostic studies for equine herpesvirus by region in 2023

No	Regions	Number of explored regions	Number of settlements studied	Number of samples studied	Positive results by ELISA (antibodies)	Positive results by PCR
1	Akmola	3	14	35	3	0
2	Aktobe	6	30	94	9	0
3	Atyrau	2	7	31	9	0
4	West Kazakstan Region	10	67	198	29	0
5	Karaganda	6	21	102	0	-
6	Kostanay	5	37	103	15	0
7	Mangystau	5	25	70	6	0
8	Pavlodar	6	28	75	7	0
9	North Kazakhstan Region	5	30	83	15	0
10	East Kazakhstan Region	6	29	105	0	-
11	Almaty	11	58	147	0	-
12	Zhambyl	5	35	89	0	-
13	Kyzylorda	6	41	114	0	-
14	Turkestan	5	51	136	0	-
15	Almaty city	-	-	9	0	-
	TOTAL:	44	277	1391	93	0

Based on the results of serological monitoring of 1391 blood serum samples, antibodies to equine herpesvirus were detected in 93 cases (6.7%). At the same time, out of 15 regions of the country, positive samples were found in 8 regions. In quantitative terms, the largest number of positive samples were found in West Kazakhstan (29), Kostanay (15) and North Kazakhstan (15) regions. The highest proportion of positive samples from the total number of samples examined was also observed in the above regions: West Kazakhstan (14.6%), Kostanay (15.5%) and North Kazakhstan (18.1%).

At the same time, samples that showed a positive result by ELISA were further examined using the PCR method in order to detect the causative agent of EHV. A total of 93 molecular genetic studies were carried out, but in all cases a negative result was obtained.

Discussion

EHV is one of the most common infectious diseases of horses, causing huge economic losses associated with abortion of the fetus and the subsequent chronic course of the disease. Therefore, systematic monitoring and study of the dynamics of the development of the epizootological process in a certain territory is one of the effective tools for monitoring the situation, which allows timely organization and implementation of the necessary preventive and anti-epizootic measures [2].

In order to maintain a favorable epizootic situation in the republic, veterinary measures are planned annually for the prevention and diagnosis of EHV. The policy of the state veterinary service is aimed at

continuous monitoring and timely diagnosis of the disease. Every year, at the expense of the republican budget, more than 1000 heads of horses are subjected to diagnostic tests in the republic, which is no more than 0.025% of the coverage of the entire livestock. Preventive vaccination is carried out on no more than 70.0 thousand animals in regions where there are outbreaks of infection, which is 2.1% of the total horse population in the republic. However, despite the measures taken, against the backdrop of a difficult epizootic situation in the world and neighboring countries, a difficult situation is developing in the republic, which requires not only a practical approach but also a scientific approach in the study of the epizootic process of the disease.

The data obtained confirm that strains of the pathogen of EHV circulate in the republic and their distribution area covers almost all regions of the country. At the moment, EHV is registered both in organized horse breeding farms and among horse owners in a private farmstead. In addition, taking into account the trend of gradual growth of local and imported horses, the development of various types of equestrian sports, the threat of infection from disadvantaged countries increases.

In this regard, the most important thing for the country's veterinary service is systematic and targeted work on timely diagnosis of infection, the formation of a sufficient number of animals with an immune response and the prevention of the introduction of infection from outside.

Conclusion

Systematic epizootological monitoring measures are the most effective way to prevent the occurrence and spread of EHV in the territory. At the same time, the results obtained confirm the epizootological problems of the country with re-gard to EHV. Analysis of serological studies of blood serum samples collected in various areas of the country showed that the EHVcirculates among various horse populations in at least 8 regions of Kazakhstan. And although molecular genetic studies did not confirm the results of serological studies, due to the specifics of these tests, they do not exclude the presence of pathogenic field strains in the body of the studied animals. Therefore, in affected areas it is necessary to take a full range of measures for timely diagnosis, prevention and elimination of foci of infection.

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Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2024. – N1(005). – P. 44-49. - ISSN 2958-5430, ISSN 2958-5449

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UDC 619:616.995.7:636

SPECIES DIVERSITY AND PREVALENCE OF ZOOPHILIC FLIES IN KOSTANAY REGION (NORTHERN KAZAKHSTAN)

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Abstract

This study delves into the understudied realm of zoophilic flies in the climatically distinct Kostanay region of Northern Kazakhstan. Amidst the region's diverse dipteran fauna, these parasitic flies pose a significant threat to both livestock and human health, acting as vectors for numerous infectious and invasive diseases.

This study aimed to elucidate the spatiotemporal dynamics of zoophilic *Diptera* in the Kostanay region during the summer of 2023.

As a result of the research, it was found that zoophilic flies of 8 families - representatives of 16 species - are registered on feedlots and pastures of farm animals of the Kostanay region of Northern Kazakhstan. Of these, the main species of flies (*Musca domestica, M. stabulans, Calliphora unralensis, C. vicina and Wohlfartia magnifica*) caused the greatest concern to animals. The total summer duration of zoophilic flies per season is 104-162 days or 3.5 - 5.5 months. Of the farm animals examined, cattle were the most intensively attacked.

Key words: distribution index; farm animals; flies; habitat; insects; species.

Introduction

Zoophilic flies pose a serious problem in animal husbandry, as they contribute to the transfer of various pathogens of infectious and invasive etiology, which affects the veterinary well-being of animal husbandry. The species composition of flies attacking animals is diverse and depends on the location of livestock facilities and pastures. There are over 100 species of these insects that come into contact with animals. Of these, 92 species are found in pastures and 57 in cowsheds [1].

Zoophilic flies in the environment of humans and animals are of no small importance. The harm caused by these diptera is due to their close trophic (primarily), topical and trophic relationships with domestic animals. Zoophilic flies cause significant economic damage, which consists of a decrease in the quantity and quality of livestock products (meat, milk, wool, etc.), spoilage and loss of feed, animal diseases with infectious and invasive diseases, and a decrease in the productivity of livestock workers. Young animals, which are more sensitive to these insects, suffer the most from the attack of flies [2].

Veterinary and medical significance the purpose of synanthropic flies, which are mechanical carriers of pathogens of many human and animal diseases, necessitates research to create effective and environmentally safe means of suppressing the number of parasitic insects [3, 4].

In the world's fauna, only the family of true flies (Muscidae) includes more than 100 genera. About 850 species from 52 genera and 5 subfamilies are known in the Palearctic. Houseflies are of great interest because some species contribute to the transfer of various bacteria and viruses [5].

Traditional methods of fly control include preventive and extermination measures. The essence of the first is to exclude potential fly breeding sites based on compliance with sanitary and hygienic requirements. The advantage of preventive measures is their environmental harmlessness, however, they are not able to provide a significant reduction in the number of flies. Only the destruction of flies by physical, chemical, biological methods and their combination gives a good result [6].

The housefly can carry pathogens of respiratory infections in cattle, which causes great damage to many livestock complexes [7].

Houseflies are a distributor of diseases such as dysentery, typhoid fever, tuberculosis, cholera. In addition to the housefly, the Muscidae family includes a large number of not only synanthropic species, but also those living outside settlements. In addition to the diseases listed above, real flies carry the polio virus, paratyphoid bacteria, tularemia, brucellosis, botulism, staphylococcal and micrococcal infections, as well as eggs of parasitic worms: ascariids, pinworms and lentipedes are most severely affected by fly attacks by young animals more sensitive to these insects. In addition, pathogenic and conditionally pathogenic microorganisms - *Salmonella, Escherichia, Pasteurella, spore bacteria*. The coccal group of microorganisms is represented by staphylococci and streptococci. Four species of fungi have been isolated from the outer integuments of flies, among which the genus Aspergillus prevails. Fungi are isolated mainly from adult insects [8].

The Kostanay region's diverse biogeographical landscape harbors a vast and understudied fauna of zoophilic flies, posing a potential public health risk due to zoonotic disease transmission. Zoophilic flies are sources of the spread of pathogens of many infectious and invasive diseases, and are also capable of causing independent diseases in the larval phase. The measures currently being carried out in livestock farms of the Kostanay region do not ensure a steady decrease in their numbers to a virtually harmless level. The epidemiological significance of flies and the real danger of their transferring pathogens of a number of dangerous human and animal diseases are the reasons for constant attention to this problem, the relevance of which is undoubted [9, 10].

The purpose of the study was to investigate the species diversity and prevalence of zoophilic flies in the Kostanay region, as well as to determine the duration of flight of zoophilic flies in places where animals are kept and grazed.

Materials and methods

Extensive ecological and faunal surveys were conducted across diverse livestock breeding landscapes within the Kostanay region, Kazakhstan, during May-October 2023. The expedition encompassed not only agricultural settings in the Denisov, Sarykol, Altynsarin, Auliekol, and Naurzum districts but also ecologically distinct habitats like the Karatamar and Verkhnetobol reservoirs and the Tobol and Ayat river floodplains.

Complementary laboratory analyses were conducted at the Department of Veterinary Medicine and the Museum of Entomology of the Faculty of Agricultural Sciences, Non-Profit Limited Company «Akhmet Baitursynuly Kostanay Regional University», Kostanay.

This comprehensive investigation aimed to gather robust insect material, quantify fly populations, and elucidate key aspects of zoophilic fly biology, including seasonal and diel activity patterns, reproductive strategies, and economic significance.

Observation sites for zoophilic flies were strategically established across livestock farms in the Kostanay region. These sites encompassed not only animal housing facilities (feedlots, summer camps) but also adjoining vegetated areas and designated grazing grounds. Species composition of flies was determined through a multi-pronged approach. First, clinical examinations of diverse livestock species (cattle, horses, sheep) within enclosures provided initial insights. Second, strategically placed spinning traps in animal keeping and grazing areas facilitated targeted fly collection. Finally, complementary sampling methods employing entomological nets and sticky paper ensured comprehensive capture, adhering to established protocols [6].

Extensive expeditionary, entomological, and laboratory investigations yielded a comprehensive collection exceeding 2,000 fly specimens. Species identification was meticulously conducted under the magnification of an MBS-10 microscope, utilizing established taxonomic keys and authoritative insect identification guides [7, 8].

Larvae and pupae were systematically collected from diverse substrates associated with animal activity and potential breeding grounds within animal housing and grazing areas.

Population density of parasite species (or groups) was assessed through the abundance index, defined as the average number of individuals encountered per recording unit. The primary method employed calculated the abundance index per host individual, encompassing both parasitized and non-parasitized objects within the study [9].

Data collection for this metric was rigorous, involving daily surveys at all livestock housing and grazing sites throughout the day, with a standardized 2-hour sampling interval.

Results

Analysis of fly assemblages across the study areas revealed remarkable taxonomic homogeneity. Both species composition and abundance demonstrated negligible variation, as detailed in the accompanying table. At the same time, a total of 16 species of zoophilic flies belonging to 8 genera were identified.

Table 1 - Species composition and abundance of flies collected in grazing areas

№	Name of the family	Quantity		Total flies collected	The dominance index, in %
		genus	view		
1	Syrphoidae	1	1	94	3,8
2	Sphaeroceridae	1	1	28	1,1
3	Otitidae	1	1	121	4,9
4	Anthomyiidae	1	1	154	6,2
5	Muscidae	1	5	1236	50,1
6	Fanniidae	1	1	102	4,1
7	Calliphoridae	1	3	572	23,2
8	Sarcophagidae	1	3	158	6,4
	ВСЕГО:	8	16	2465	100

Analysis of Table 1 reveals the dominance of two fly families in terms of species richness: Muscidae (5 species) and Calliphoridae (3 species). Notably, Muscidae exhibits a substantial dominance index (DI) of 50.1%, driven primarily by *Musca domestica* (DI 26.5%). *Muscina stabulans* emerges as the subdominant within Muscidae, contributing a DI of 3.6%. Calliphoridae follows in importance with an average DI of 23.2%.

Within the Calliphoridae, *Calliphora unralensis* emerged as the dominant species, contributing a 7.1% dominance index (DI), followed by the subdominant *C. vomitoria L* with a DI of 5.0%. Notably, across both animal housing and grazing sites, five zoophilic fly species consistently exhibited the highest abundance: *Musca domestica, Muscina stabulans, Calliphora unralensis, C. vicina,* and *Wohlfartia magnifica.* This consistent top five highlights the ecological resilience and adaptability of these specific zoophilic species within the studied livestock environments.

Quantitative assessment of zoophilic fly attacks on livestock revealed a distinct pattern. Cattle experienced the highest burden, with an average of 25.9 fly landings per animal (based on 10 individuals sampled). Horses and sheep followed with average attack rates of 9 and 8.8 flies per animal, respectively. Notably, Table 2 further details the flight initiation times of these harmful fly species under natural outdoor conditions, crucial for understanding their seasonal activity patterns. This distinction is important as dipterans typically exhibit activity indoors about a month earlier.

As such, Table 2 provides valuable insights into the timing of fly emergence and potential attack risk for livestock across different housing and grazing periods (Figure 1).

Table 2 – Duration of flight of zoophilic flies in places of keeping and grazing of animals

Species	Start of fly flight (date)	End of fly flight (date)	Duration of flight per season (days)
Musca domestica	19.04	29.09	162
Muscina stabulans	23.04	20.09	157
Fannia cannicularis	17.04	24.09	157
Lucilia sericata	04.06	14.09	104
Protophormia temenovae	14.04	03.10	158

Table 2 reveals a temporal sequence in the emergence of harmful zoophilic flies from wintering, ordered by their calendar dates of flight initiation. The earliest active flyers, observed on March 14th and 17th respectively, were *Protophormia terraenovae* and *Fannia cannicularis*. This vanguard is closely followed by *Musca domestica, Muscina stabulans,* and a second emergence of *Fannia canicularis*.

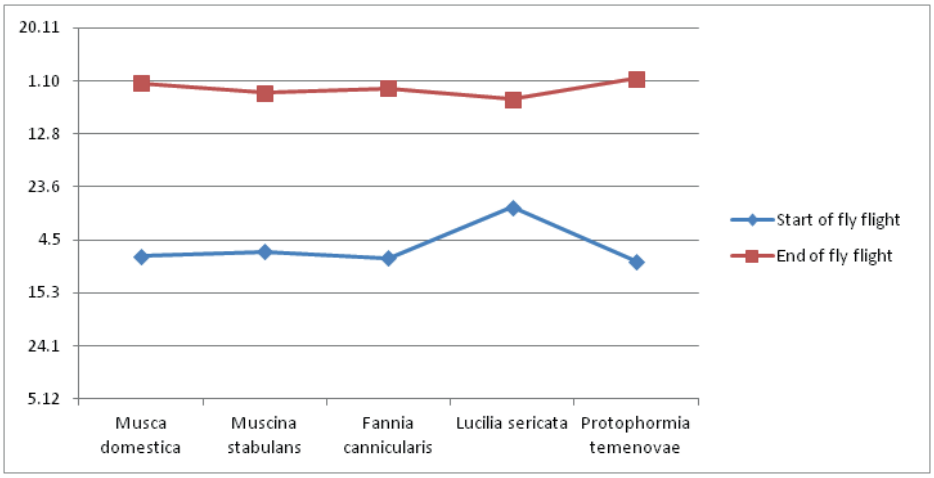

Figure 1 – The beginning and end of the summer of zoophilic flies in places where animals are kept and grazed

Discussion

Intriguingly, our observations reveal an unusually early emergence for the spring blue fly in 2023. Flight initiation commenced on March 14th, significantly preceding the typical second ten days of April. This early activity was followed by a prolonged flight period, extending until June 4th. While the main flight season for pasture flies typically concludes by September 20-30, a notable exception was observed. A small portion of populations, including field flies, *Protophormia,* and others, persisted at summer livestock camps, even overwintering in these locations. This phenomenon, in our view, suggests a potential adaptation for population resilience. By dispersing across different biotopes for wintering, these flies may mitigate the risks associated with unfavorable conditions in any single habitat, thus ensuring their overall survival.

Fly activity extended into early October, with the latest confirmed sightings of *M. domestica* (housefly), *Protophormia spp.*, and *C. vicina* occurring on October 3rd, 2023. This late persistence coincided with air temperatures ranging from 3-5 °C in the shade to 12-14 °C in direct sunlight, demonstrating remarkable adaptability in these species. Notably, cattle experienced the highest burden of fly attacks, with an average of 25.9 landings per individual (n=10). Horse and sheep attack rates followed at 9.4 and 8.8 landings per animal, respectively, suggesting potential species-specific preferences or varying susceptibility among livestock.

Conclusion

Extensive ecological and faunal surveys across diverse livestock facilities in Northern Kazakhstan's Kostanay region, encompassing districts (Denisov, Sarykol, Altynsarin, Auliekol, Naurzum), water reservoirs (Karatamar, Verkhnetobol), and river floodplains (Tobol, Ayat), revealed a rich assemblage of zoophilic flies. A total of 16 species representing 8 families were documented on animal feedlots and pastures. Notably, five dominant species (*Musca domestica, M. stabulans, Calliphora unralensis, C. vicina,* and *Wohlfartia magnifica*) comprised 87.8% of all collected flies, posing the greatest concern to livestock. These species exhibited extended flight seasons, ranging from 104 to 162 days (3.5-5.5 months), highlighting their ecological resilience and potential pest pressure. Interestingly, cattle emerged as the most heavily attacked livestock species among the three studied (cattle, horses, sheep), suggesting potential variations in fly preferences or host susceptibility.

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doi.org/ 10.51452/kazatuvc.2024.1(005).1604
UDC : 619:616.98:578.832.1:636.52/.58:616-097.3

THE IMMUNITY DURATION AND INTENSITY IN INDUSTRIAL LAYING HENS FOLLOWING VACCINATION WITH INACTIVATED H5N1 AVIAN INFLUENZA VACCINE

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Abstract

After the severe AIV H5 outbreak in Kazakhstan in 2020 the extensive use of AIV H5 vaccines started in the industrial poultry farms to limit the H5N1 influenza spread. Traditional methods, such as stamping out are no longer a viable option in countries where Highly Pathogenic Avian Influenza (HPAI) has become endemic. However, available vaccines and vaccination protocols have been widely researched in laboratory conditions, and limited testing has been conducted on commercial layers for the immunity persistence in field conditions. Immunity persistence after AIV vaccination can be quite different between laboratory and field conditions. Our basic goal was to assess the intensity and duration of immunity in commercial layers following 1, 2 and 3 vaccinations. H5N1 hemagglutination inhibition (HI) antibodies were observed 370 days after vaccination of chickens using three schemes of vaccination. However, at 370 days post vaccination 1 experimental group obtaining one vaccination had comparatively low HI titers (mean titer 6,5±1,2 log2), the other two groups having two and three vaccinations showed 8,8±2,4 mean log2 and 9,4±2,2 mean log2 respectively. The described results showed that inactivated H5N1 vaccine can produce lengthy, intense and homogenous immunity under field conditions following 2 and 3 vaccinations.

Key word: avian influenza immunity; influenza H5N1; vaccination scheme.

Introduction

Avian influenza is an extremely contagious, pantropic infection of poultry causing severe economic loss to the industry. The highly pathogenic subtype H5N1 of Highly Pathogenic Avian Influenza (HAIV) can be quite devastating to a commercial industry. Kazakhstan poultry industry experienced an outbreak of H5N1 AIV beginning in September 2020. The initial infections were detected in a commercial egg-laying flock and a single noncommercial backyard flock [1]. Later on, in October 2020 the outbreak of H5N1 AI spread to other northern regions and till the end of autumn infection appeared in southern commercial egg production flocks. The severe 2020 AIV outbreak brought huge economical losses for the Kazakhstan poultry industry [2]. Some unauthorized resources indicate that the mortality rate amounted to 98 – 100%. Although commonly attested measures for AIV preventing and control are biosafety and biosecurity including adequate and persistent serologic monitoring, vaccination using strong and antigenically homologous vaccine can also be successful. Moreover, vaccination coverage should be sufficient enough and the vaccine application should be easily controlled in the field conditions to prevent recurrence of AIV outbreaks [3]. Despite the fact that no AIV outbreaks have been registered

in vaccinated birds, a new H5N1 virus infecting these birds, e.g. virus introduced by asymptomatically infected birds, may be spread by vaccinated birds that are immunized only against severe desease [4]. After the AIV outbreak in 2020 in Kazakhstan inactivated Russian vaccine called Flu Protect H5 became very popular due to its production of high humoral immunity in poultry. However, for such long –living hens as layers and breeders it is vital to be immune to AIV till the end of their production life. Vaccination of industrial flocks against avian influenza (AI) requires consideration of many different factors, including scheme of vaccination, financial expenses, labor efforts and the availability to implement surveillance and monitoring programs. It is apparent that the effectiveness of a vaccine in laboratory trials conditions may differ from the use of it in industrial flock [5]. Thus, the persistence of AIV immunity in industrial birds have been tested in a very limited research. Many authors have described the prevention of H5N1 disease in vaccinated birds and reduction of virus shedding after vaccination [6, 7]. However, in these studies the immunity was assessed 1-3 weeks post-vaccination and this period is not relevant to field conditions. The immunity response persistence after H5 vaccination under the conditions of big production farm not determined yet. Moreover, dozens of factors may influence the strength and persistence of the immunity after vaccination in field. Prescription and decision for usage of the inactivated vaccine can rely on broader information included in the original registration file, many scientific investigations, controlled trials, and field studies that have been conducted to increase knowledge regarding its characteristics and performances [8, 9]. Field experience from large commercial layers farm use has also enriched the experience and knowledge about the field performance of this vaccine.

The novelty of this work lies in the fact that a large-scale field study of the intensity and duration of AIV immunity in industrial laying hens in the commercial poultry farm was implemented for the first time in Kazakhstan.

The aim of this research was to compare the duration and intensity of H5 immunity in egg producing hens after their vaccination with the FluProtect H5 inactivated vaccine applying three different schemes.

Materials and methods

Flocks. The study was performed on the industrial site in North-Kazakhstan. Layers in three production houses were vaccinated using three different schemes of vaccination. For certain the layers did not have AIV maternally derived antibodies as all of them were received from non-vaccinated parents. For this research we formed three experimental groups from three production houses. During the production period the vaccinated birds were monitored serologically applying HI test. The living standards did not range essentially between the groups. The three experimental groups were kept providing appropriate biosecurity measures.

Vaccination and Sampling. In this study we used a commercially available H5N1 formaline-inactivated vaccine. According to the research plan the three experimental groups were vaccinated using three different schemes of vaccination. The schemes are presented in the table below.

Table 1 – Vaccination schemes of the experimental groups

Experimental group	Vaccination scheme		
#1			at the age of 95-100 days (full dose)
#2	at 1st day of life (half-dose)		at the age of 95 – 100 days (full dose)
#3	at 1st day of life (half-dose)	at the age of 55-60 days (full dose)	at the age of 95-100 days (full dose)

During the whole study period all experimental groups were sampled every 30-day post-vaccination. It is widely attested that 90-95% confidence level is ensured by collecting of 23 to 30 samples per one epizootological unit for accurate monitoring of the flock immunity status.

Serological Analysis. As it is known hemagglutination Inhibition Assay (HI) is an important technique in the research of viruses, especially influenza viruses. This test provides important information on the immune response to viral infections and it is also useful in vaccine development, epizootological studies, and evaluating the efficacy of vaccination programs. Thus, hemagglutination inhibition assay

was used to assess the immunity duration and intensity. The antigen for the HI test was purchased from GD Animal Health, Deventer, Netherlands. The HI test was conducted according to a generally accepted technique. Sera samples were pretreated with chicken erythrocytes to exclude non-specific agglutination.

Geometric Mean Titer (GMT) was calculated for all groups of the experimental birds. GMT was calculated as the antilogarithm of the mean of the logarithms of each value. Negative titres (<16) were regarded as 4 for the calculation of GMT.

Results

Immunity persistence in productive layers after the inactivated H5N1 vaccine was measured using HI test (Fig. 1).

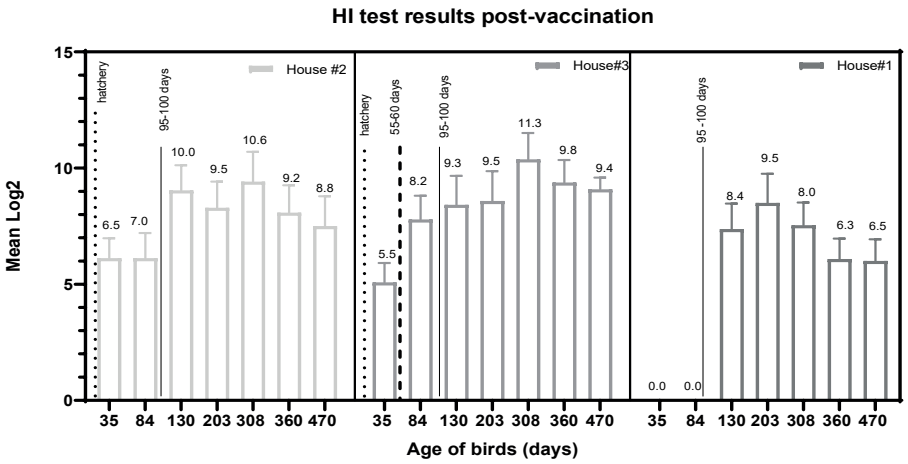

Figure 1 – HI test results showing antibody titters in layers during the whole period of the experiment. Vertical lines mark the period of vaccination

Layers administered a single vaccine dose at the age of 95-100 days showed HI titers (mean titer 8.4 log2) 30 days post-vaccination. Three months later this titer increased non-significantly (mean titer 9.5 log2). Furthermore, HI titers in this experimental group declined throughout the study (mean titer 6,5 log 2) but remained detectable at 470 days of life, till the end of production period. Experimental group number 2 was vaccinated twice: first vaccination was in hatchery using 0,5 of the recommended doses and second vaccination was at 95-100 days at the start of production period with full dose. As it is seen from the Table 2, 35 days post-vaccination chickens had HI titers at the level of 6,5±1,5log2 with 12% of seronegative samples. After 84 days post-vaccination the HI titers increased slightly (mean titer 7,0 ± 1,2 log2) with still some seronegative samples (10%). Furthermore, mean titer in layers rose to 10,0±2,4 log2 post second vaccination and remained nearly unchanged till 360 days of life. Then the titers declined to 8,8±2,4log 2 but remained detectable in 100% of layers till the end of production period. In HI tests conducted in the third experimental group which was vaccinated three times: at hatchery with half a dose of the vaccine, using full dose both at the age of 55-60 days and at the age of 95-100 days, detected extremely minor difference between this group and group number 2 (Table 2).

Table 2 – H5 antibodies in laying hens after inactivated vaccine (1, 2 and 3 vaccinations)

Number of vaccinations	Mean Log2 ± SD/seropositive %				
	dpv	30 dpv	100 dpv	200 dpv	360
Single vaccination	At the age of 95-100 days	8,4± 2,5/100	9,5± 1,8/100	6,3± 2,2/100	6,5± 1,2/100

Continuation of Table 2

Two vaccinations	30 dpv 60 dpv				30 dpb (100dpv)	100 dpb (200 dpv)	200 dpb (300 dpv)	360 dpb (460 dpv)	
	At hatchery (0,5 dose)	6,5 ±1,5/100	7,0 ± 1,2/100	At the age of 95-100 days	10,0± 2,4/100	9,5± 0,8/100	10,6± 2,7/100	8,8± 2,4/100	
Three vaccinations		30 dpv		30 dpb (90 dpv)		30 dpb (100 dpv)	100 dpb (200 dpv)	200 dpb 300 (dpv)	360 dpb (460 dpv)
	At hatchery (0,5 dose)	5,5± 1,1/80	At the age of 55- 60 days	8,2± 2,2/92	At the age of 95-100 days	9,3± 0,7/100	9,5± 1,6/100	11,3± 2,5/100	9,4± 2,2/100

Samples were collected after 30 days post-vaccination as per vaccine manufacturer instruction. dpv – days post primary vaccination, dpb – days post boost vaccination.

The experimental layers had mean titer 5,5±1,1 log2 at the 35 days post-vaccination and some hens were seronegative (20%). After boost vaccination at the age of 55-60 days the HI titer increased to 8,2±2,2, still 8% of samples showed seronegative results. After second boost vaccination at the start of production period the HI titer increased 9,3±0,7 mean log 2 and remained unchanged till the end of production period (mean titer 9,4±2,2 log2) with 100% of seropositive layers.

Fig. 2 shows the changes in HI titers through the whole period of the experiment. HI titers 30 post all boost vaccinations was 8.4, 10.0, 9.3 mean log 2 in 1,2 and 3 experimental groups respectively. All experimental groups had similar HI titters (9,5 log 2) at the age of 130 days (100 days post-vaccination) (Fig. 2).

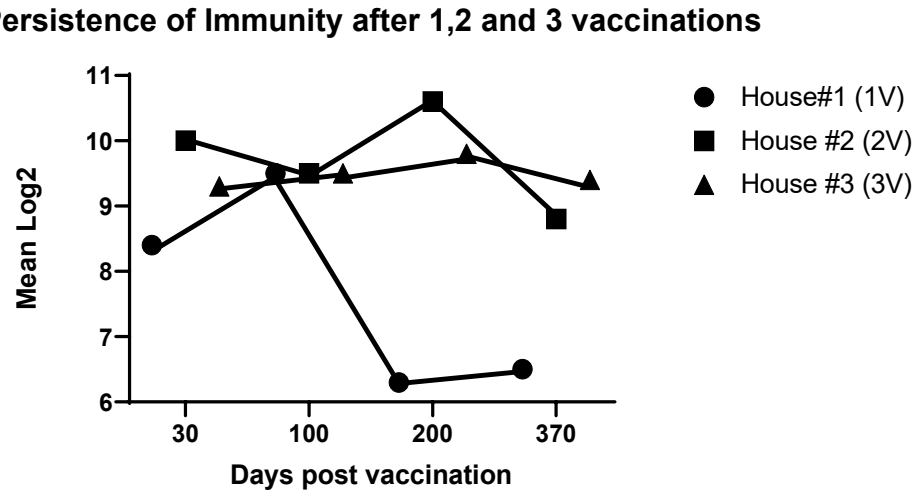

Figure 2 – Immunity persistence in productive layers after 1, 2 and 3 vaccinations. 30 days post vaccination means 30 days post all boost vaccinations in layers having 2 and 3 vaccinations. 370 days post vaccination corresponds to 470 days of life or the end of production period

At 370 days post vaccination 1 experimental group obtaining one vaccination had comparatively low HI titers (mean titer 6,5 log 2), the other two groups having two and three vaccinations showed 8,8 mean log 2 and 9,4 mean log 2 respectively.

Discussion

The antibody responses of the three experimental groups to AI vaccination were different. Experimental group #1 (House #1) achieved 100% seroconversion 30 days post vaccination but by 360 days of age, all of the hens tested had comparatively low titter 6,3 log2. In contrast, experimental groups 2 and 3 were approximately 80% and 92% seropositive, respectively, after first vaccination at 60 days of age by HI. However, 30 days post boost vaccination both groups had remarkably high HI titers. Additionally, both groups had still high HI titers at 360 days of age. The research was implemented on one and the same commercial facility and thus, there were not uncontrolled significant differences between these groups and their environments. There were no substantial vaccine-related differences between the groups. Vaccination of the three groups was carried using the vaccine which was either from the same lot or had very similar viral contents. The adjuvant used was identical. All birds were given the same dose of vaccine at both times and the ages at vaccination were similar among all groups. Based on the clinical signs it can be asserted that there was no challenge with AIV or of any other disease outbreaks in any of the three groups during the experiment. Thus, the differences in HI test results between the groups could be explained only due to the scheme of vaccination. Carol J. Cardona et.al [10] suggest that the immune response measured in experimental group #1 in part resembles a primary antibody response rather than a full anamnestic response. The hemagglutination inhibition titers of all experimental groups began to decline at 360 days of age. However, in the experimental groups 2 and 3 given 1 and 2 boost vaccinations respectively this decrease was slightly different whereas experimental group 1 had significant decline in HI titers to the end of production period. Interestingly, the three groups had the same high HI titers at the age of 130 days (30 days post vaccination or boost vaccination). Perhaps this occurred due to the age, productivity and health condition of layers.

There are scarce studies investigating AIV vaccination of industrial birds under field conditions. Numerous authors describe that H5N1 vaccination has prevented birds [11, 12, 13] from mortality. However, the time limit of the immunity, which is a significant index in the field [14, 15], has not been studied in full volume. Our research results prove that the immunity persistence in layers can differ markedly under field conditions due to the scheme of vaccination. Antibodies were observed in all experimental groups starting the first vaccination. However, the humoral immunity was more persistent, intense and homogenous in layers given two and three vaccinations than that of only one vaccination. Industrial birds grown for egg production, meat, or breeders should be secured from AIV. Kazakhstan poultry industry suggests broilers ready for sale at 39-42 days of age, but layers and breeding stock are maintained for up to 630 days of age. In our study the layers' antibody response was not measured for more than 470 days as birds on this farm are usually kept till 470 days of life. However, AIV immunity having only 6.3 log 2 in HI test lasting to 470 days of life suggests that layers raised for longer period would not be sufficiently secured for life, as intensity of HI antibodies tend to decline with the time elapsed. The antibodies upkeep at the level of 8.8 log2 – 9.4 log2 in the experimental groups #2 and #3 for the whole testing period assumes that application of one or two boost doses in long-living industrial hens such as layers or breeders would be sufficient for longer protection.

Proper vaccination can assist in controlling the highly pathogenic H5N1 viruses' circulation in domestic birds. Certainly, 85% of a flock must be vaccinated having 6.0 log 2 HI titer minimum to ensure flock immunity. Moreover, a lasting antibody response is also required implementing high-quality vaccine. In our study, layers had HI antibodies after inactivated vaccine for 370 days but were not experimentally infected with field virus to confirm protection [16]. Tian et al. [17] described protection from mortality and the virus shedding reduction in birds 52 weeks after primary vaccination with AIV vaccine (9.2 log 2 of HA vs. 6.3 log 2 in our study). Notwithstanding that in our study we did not implement experimental challenge, the results reveal that two and three vaccinations stimulate a 370 days lasting intense immunity in layers. The data from the literature show that under field conditions, most long-living poultry, such as layers and breeders as well as waterfowl and turkeys require a minimum of two or even three vaccinations throughout their life to maintain adequate protection [18, 19]. Single AIV vaccination may be quite sufficient for the protection of short-living birds such as broilers raised for meat.

The immunity duration and intensity in productive laying hens described in this article can assist Kazakhstan authorities in AIV vaccine assessment. In the AIV endemic situation in Kazakhstan the

effective H5N1 vaccine that can induce sufficient immunity in commercial birds will be extremely important for timely AIV outbreaks control, not excluding biosafety and biosecurity measures as well. It is well-known that China and Vietnam realized successful vaccination strategies for excluding the H5N1 transmission from birds to humans. However, after some time AIV outbreaks in poultry repeated. Very likely the two main factors responsible for AIV outbreaks recurring are the practical challenges of implementing the vaccination scheme and the high and rapid AIV antigenic mutability. The ongoing H5N1 circulation in Southeast Asia led to the new strain's formation, demanding that specific vaccine strains be applied in certain countries or regions or renovated every year. In 2003 Hong Kong successfully controlled H5N1 virus transmission by AIV vaccination program [20]. Thus, any industrial flock requires adopted scheme of routine H5N1 vaccination shielding the birds for the whole production period. As it is already known H5N1 has become endemic in Kazakhstan, and as the country is exposed to periodic outbreaks it has become urgent to re-assess the vaccination scheme of AIV vaccination as part of the routine infectious diseases control measures.

Conclusion

The inactivated vaccine application in Kazakhstan was eventually successful in helping to eradicate H5N1 AIV outbreak from the commercial industry, but not as quickly as it might have been. However, there were individual farms that were not successful to eradicate the virus using only one vaccination. The majority of farms that used the inactivated vaccine were multiage egg-production flocks and they used a double vaccination scheme. Our study suggests that using one or two boost AI vaccinations with the inactivated vaccine can provide intense and long – lasting protection to the industrial poultry through its whole production period. However, this vaccination scheme must be applied in association with strict on farm biosecurity to prevent the reintroduction of the virus.

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Correction:

In the article “Trematoda and cestoda species in cyprinid fish from small lakes of the Kostanay region” by Marat Zh. Aubakirov, Evgeniya N. Erenko, Ekaterina A. Laseeva, Akmaral A. Shaimagambetova, published in the previous issue of the Herald of Science: Veterinary Sciences (2023, No. 4), the incorrect interpretation of the data in Fig. 1 was made: «The pathogens Opisthorchis felineus and Methorchis bilis were confirmed in two of the five provided samples through PCR analysis, as shown in Fig.1».

This sentence should read: «As follows from Fig.1 PCR revealed 1 positive sample (band 3) confirming that the pathogen belongs to the Opisthorchis felineus, and Metorchis bilis was not detected».

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Using indirect hemagglutination assay for the diagnosis of cattle brucellosis

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HERALD OF SCIENCE

S. Seifullin Kazakh Agrotechnical Research University

№ 1 (005) 2024
Journal Republic of Kazakhstan
Ministry of Culture, Information and Sports
Registered with the Information and Archives Committee
(№ 5770-Ж certificate)
(№ 13279-Ж certificate)

Compiled by:
Compiled under the editorship of A.K. Bulashev

On the computer page:
S.S. Romanenko

28.03.2024 was signed in the year Format 60 x $84^{1}/_{8}$
Conditional p. p. 4,25 Distribution 300 pieces Order № 2460

Saken Seifullin Kazakh Agrotechnical Research University
published by the research university press.
010011, Astana city, Zhenis avenue, 62 «a»
Phone numbers for inquiries: (7172) 31-02-75
e-mail:office@kazatu.kz
vestniknauki@bk.ru