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Phenotypic and Genotypic Characteristics of *Brucella* Strains Isolated from Animals on the Territory of the Republic of Kazakhstan

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ABSTRACT

Brucellosis is one of the most dangerous infectious diseases of humans and animals. Therefore, monitoring the epidemiological situation in Kazakhstan and identifying sources of population infection make such studies a priority. The study aims to investigate brucellosis's phenotypic and genotypic diversity, which caused the disease in the population and animals on the country's territory. Serological methods were used in the study – immunoenzyme analysis using commercial test kit Ingezim Brucella Compac 2.0, microbiological methods – cultivation and microscopy of microorganisms isolated from pathological material, and polymerase chain reaction with multiplex procedure according to Bruce-Ladder method using commercial kit INgene Bruce-ladder V. As a result of the study, 48 animals with high antibody titers were selected from 990 animals, of which there were 413 cows and heifers, 552 goats and sheep and 25 animals of other farm animal species for sampling for microbiological culturing. As a result of culturing, microbiological culture growth was obtained in 28 samples. Microscopic examination of colonies on nutrient media and microorganisms did not reveal significant differences between pathogens from different disease foci. Polymerase chain reaction (PCR) typing revealed that in 70% of cases of the population, the source of the disease was *Brucella melitensis*, which was detected by PCR. In the other 30%, the causative agent was identified as type *B. abortus*, which was isolated mainly from the country's northern regions. The studies that were conducted allowed us to clarify the circulation regions of different types of pathological brucellosis in Kazakhstan and to improve some methodological approaches in an epizootic survey of infection foci.

Key words: Immunoassay, Bacteriological tests, Polymerase chain reaction, Antibody titer, Test system.

INTRODUCTION

Brucellosis is one of the most dangerous infectious diseases that can simultaneously affect many species of animals and humans. This disease is widespread throughout the world, and according to the World Health Organisation (WHO) (2020), in the last decade, it has been observed in more than 170 countries, with up to 500 thousand cases of confirmed brucellosis in humans per year. New outbreaks of human brucellosis are most often observed in Central Asia, including the Republic of Kazakhstan. An important factor is the constant growth of disease foci and the number of sick people in the country. This makes research into the sources of infection and ways of spreading this disease an urgent and priority area for medicine and veterinary medicine.

According to the official information of the Ministry of Agriculture of the Republic of Kazakhstan, published on the Forbes Kazakhstan portal (2023), only in 2023, 17.5 thousand heads of sick animals were identified, which was less than 1% of the total number of farm animals in the country. Such indicators showed a 57% decrease in disease incidence compared to the same period in 2022. At the same time, the number of diseases among the population of Kazakhstan is only increasing. Following Urazayeva et al. (2023), a high incidence of brucellosis in the population is also noted in the neighboring countries of Kazakhstan – Iraq, Tajikistan, Saudi Arabia, Iran, and Kyrgyzstan. Infection of people occurs through alimentary and airborne methods of spreading the infection. However, the main route of human infection in most countries of the world is through the consumption of animal products that have not been thermally decontaminated. In Central and East Asia, most cases of brucellosis are caused by consuming milk from infected animals and their products (Ragatova et al. 2024).

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This phenomenon is promoted by extensive development of pasture-based livestock breeding, as well as inadequate sanitary and hygienic approaches in the production of food products in individual livestock farms and their control when sold in markets. One of the factors of persistent infection on the territory of Kazakhstan is chronic manifestation of the disease in people. Similar conclusions were reached by Zaharov et al. (2019), who indicated a significant chronic carriage of brucellae in people in the West Kazakhstan region. According to WHO, this may be why brucellosis is endemic in Central Asia and the South Caucasus, where the highest incidence of brucellosis in the world is observed (Aslan et al. 2023).

All this causes brucellosis to continue to pose a threat to the health of the population of Kazakhstan. In the last year alone, cases of the disease were registered in North Kazakhstan, East Kazakhstan, West Kazakhstan, Kostanay, Zhambyl, Pavlodar, Kyzylorda regions and Zhetysu region (2023). The reason for this increase in the disease among the population and animals, according to Aikimbayev et al. (2021), was the change in the ratio of the number of farm animals in large, collective, and small, individual farms, where the level of veterinary care differs significantly. The decrease in anti-epidemic measures for brucellosis among the population also contributed to this. According to the tendency of spread and the level of morbidity among people and farm animals, Kazakhstan belongs to the countries unfavorable for brucellosis with constant detection of infection (Zholdasbekova et al. 2018).

Based on the above data, in most cases, the cause of brucellosis spread among the population of Kazakhstan is transmission from animals. Establishing the sources of the disease will reduce the intensity of the epizootic and epidemiological process in the country and contribute to its elimination in the Republic of Kazakhstan. Therefore, the study aimed to analyse the genetic and phenotypic diversity of brucellosis in the aetiology of this disease among farm animals in different regions of Kazakhstan.

MATERIALS AND METHODS

Studies were conducted in brucellosis-unfavourable settlements in different regions of Kazakhstan. For the period from 2019 to 2022, 18 outbreaks of brucellosis in humans and animals in West Kazakhstan, East Kazakhstan, Pavlodar, Kostanay, Zhambyl and Almaty regions were investigated. At confirmation of medical diagnosis of brucellosis in inhabitants, pathological material was sampled from animals to carry out typing of the pathogen and to determine the ways of infection and spread of infection in the controlled territory. All types of susceptible farm animals, which were at the centre of the disease, were examined. A study was approved by the Ethics Commission of Kazakh Scientific Research Veterinary Institute, No. 78339.

The first study stage was to examine animals for the presence of specific antibodies in blood serum by enzyme-linked immunosorbent assay (ELISA) using the Ingezim Brucella Compac 2.0 test system from Inmunología Y Genetica Aplicada, S.A., Spain (registration certificate number RK-BP-2-3805-19 dated 28.01.2019). Blood was collected in sterile vacuum tubes followed by sedimentation to obtain serum. The test system allowed the determination of antibody titres in the blood of cattle, sheep, goats, and pigs. For immunoenzyme analysis, a set of equipment from Thermo Fisher Scientific (Finland), in particular, Wellwash Versa vosher and Multiskan FC photometer, was used.

Blood was collected from animals with high antibody titres for bacteriological examination. Vacuum tubes with anticoagulants were used for this purpose. Sowing of pathological material and subsequent cultivation were performed on Erythrit agar (analogue of Brucellagar GRM) prepared using the dry commercial nutrient medium. Incubation was continued for 5-7 days at 36-37°C. After that, morphological examination of microorganisms from colonies characteristic of the brucellosis pathogen was performed.

The last stage of the research was to establish the genetic affiliation of Brucellae to a particular type. This stage of the work was carried out by polymerase chain reaction (PCR) using the standard multiplex Bruce-Ladder procedure. For this purpose, a commercial test system INgene Bruce-ladder V from Inmunología Y Genetica Aplicada, S.A., Spain, was used. For amplification, material obtained by bacteriological culturing from colonies characteristic of the brucellosis pathogen was used. The colony was transferred by microbiological loop into a microtube with 200µl of sterile diluent. Amplification was performed according to the instructions for use of the test system in a Qiagen Rotor-Gene Q amplifier (Germany), which also allows multiplex detection (INGENASA 2023). The temperature profile of reaction performance included an initial denaturation at 95°C for 7min, followed by 25 cycles at 95°C for 35 s, 64°C for 45 s. and 72°C for 3min, the final phase was carried out at 72°C for 6min. Typing of the pathogen was carried out by registration of restriction bands as a result of electrophoresis of amplification products in UV transilluminator WD-9403C using GelRed® Prestain Plus 6X DNA Loading Dye from GelRed® Prestain Plus 6X DNA Loading Dye Company as a dye Biotium.

The results of all stages of the study were stored in a database in the Microsoft Excel 365 software product for further analysis and generalisation for the preparation of conclusions and recommendations for subsequent studies. Statistical analysis was performed using genetic-mathematical and biometric methods with the use of descriptive statistics tools in the Tibco Statistica 14 application package.

RESULTS

Regular detection of cases of brucellosis infection among the population in the Republic of Kazakhstan indicates significant persistence of the pathogen among animals. Detection of sick animals on the territory of the Republic remains difficult. This is related to the fact that relatively inexpensive serological tests, such as reactions of agglutination (RA) or complement binding (CB), which have low sensitivity and specificity, are used for mass diagnostics of animals (Resolution of the Government 2013). Therefore, cases of human disease in settlements “favourable” for brucellosis among animals are often registered (Abzhaliyeva et al. 2018). Therefore, the reverse mechanism of determining the source of human

infection was applied in these studies. When a center of the disease among humans was identified, medical species in the area were conducted for clinical and latent forms of brucellosis. As a result of such surveys, 18 foci of infection in 6 regions of the Republic of Kazakhstan were analyzed between 2019 and 2022. The geographical distribution of the surveys is presented in Fig. 1.

Only a part of the country's regions with the highest disease incidence was represented in the study, so cases in the central and south-western regions were not included in the analysis. In addition, one of the reasons for not including these regions in the study was the low number of agricultural enterprises in these regions. As a consequence, the concentration of animals was relatively lower. Studies show that brucellosis is widespread throughout the country, but it is most often manifested by human disease in the southern and south-eastern regions. This is related to the predominance of small ruminants among farm animals in this territory. Predominantly sheep and goats are kept in small farms, where little attention is paid to veterinary care. In the northern regions, livestock breeding is represented by large collective enterprises that are engaged in dairy cattle breeding. In such farms, veterinary measures are carried out regularly, and all livestock are examined for possible diseases and receive specific vaccine prophylaxis. This is a possible reason for the lower incidence of human diseases in these areas.

Serological studies of livestock with a focus on the disease were carried out by ELISA using a commercial test system capable of detecting specific antibodies against brucellosis in the blood serum of farm animals. In each case, cattle and small ruminants that were at the center of the disease, as well as horses and pigs, if there were such on the farm, were subjected to diagnostic testing. As a result, 990 animals were tested, including 413 cattle of different age categories, 128 goats, 424 sheep and 25 other animal species which were represented mainly by pigs. The test results are presented in Table 1.

Table 1: Results of animal blood serum examination by ELISA method in disease foci

Disease cases	Total animals analysed	Amount and percentage of diseases	Including		
			Cattle (total/diseases)	Small cattle (total/diseases)	Others (total/diseases)
1	57	2/3.5	38/2	18/0	1/0
2	61	2/3.3	29/0	28/2	4/0
3	24	3/12.5	16/2	8/1	0/0
4	94	4/4.3	37/0	54/4	3/0
5	41	1/2.4	19/0	22/1	0/0
6	103	6/5.8	39/1	56/5	8/0
7	49	2/4.1	23/0	25/2	1/0
8	36	2/5.6	36/2	0/0	0/0
9	29	4/13.8	3/0	26/4	0/0
10	54	3/5.6	16/0	36/3	2/0
11	32	2/6.3	24/2	8/0	0/0
12	64	7/10.9	12/1	50/6	2/0
13	34	2/5.9	0/0	34/2	0/0
14	83	4/4.8	12/0	71/4	0/0
15	44	2/4.5	39/1	4/1	1/0
16	76	3/3.9	18/0	55/3	3/0
17	38	1/2.6	24/0	14/1	0/0
18	71	2/2.8	28/0	43/2	0/0

Fig. 1: Distribution of analyzed human and animal brucellosis foci by regions of the Republic of Kazakhstan. Source: compiled by the authors.

Animals with an inhibition index exceeding 40% of the difference between the optical density of positive and negative controls were considered positive. Since serological results indicate the level of specific antibodies circulating in the organism, this method was used only to identify animals that had direct contact with the pathogen or were brucellosis carriers at the time of testing. High antibody titers were observed only in individual cattle, sheep, and goats. No signs of disease or characteristic signs of bacterial carriage with changes in the immune status of animals were observed in other types of farm animals. The average infection rate in cattle was 2.7% of the total number of examined animals of this species, while in small ruminants this indicator was much higher – 7.4%.

From all animals with high antibody titers, blood was repeatedly collected for microbiological culturing, which was performed in the bacteriological department of the brucellosis laboratory by thermosetting at 36-37°C on a solid nutrient medium. The results of cultivation were evaluated on day 5. For this purpose, Petri dishes were examined with a microbiological loupe for the formation of colonies characteristic of brucellae. The results of the bacteriological examination are presented in Table 2.

In foci of the disease, where more than 4 animals with high antibody titers were found, blood from only a part of the animals with the highest titers was collected for bacteriological examination. As a consequence of such approaches, 48 blood samples were submitted to the bacteriological laboratory. As a result of microbiological sowing and subsequent culturing, no colony growth characteristic of brucellosis was observed in part of the samples. In a sample from one area, it was not possible to obtain culture growth, which subsequently did not allow specifying the genotype of the pathogen that caused human diseases in one of the foci of the Almaty region. In the process of brucellosis culture growth, the majority of colonies had a smooth, rounded shape, which corresponded to the S-type. A small proportion of colonies had a rough surface and irregular edges. All colonies ranged in size from 1 to 2.5mm regardless of the area or species of animal from which they were isolated. Also, microscopy of bacterial cells obtained from different disease foci showed no particular differences between cultures. All microorganisms had an oval or spherical shape and were located singly or in small groups on prepared smears. In Gram staining, all cells had a reddish color, which corresponded to Gram-negative microorganisms. Therefore, no particular differences in the morphology of the colonies or microorganisms were found. It is planned to further study the biochemical characteristics of microorganism cultures that were isolated in previous studies.

The next study stage aimed to typify the brucellae obtained in the process of cultivation by polymerase chain reaction using a commercial reagent kit INgene Bruce-ladder V and subsequent visualization of amplification products by electrophoretic separation in a gel with the addition of DNA dye (Fig. 2).

Brucellae were typed according to the position of the bands according to the reagent kit instructions (INGENASA 2023). Brucella abortus species is characterized by the location of bands in the position 1682 i 587 nucleotide pairs (bp), whereas Brucella melitensis has three bands corresponding to 1682, 1071 i 587 bp. The results of the analysis of microbiological cultures grown from pathological material from disease foci are shown in Table 3.

Based on the conducted studies, the main type of Brucella that caused human diseases was Brucella melitensis. This subtype was isolated in most regions of the Republic of Kazakhstan and practically in all types of ruminant farm animals, such as sheep and goats, as the main sources of infection characteristic for this type of pathogen, as well as in cows – for which these Brucellae are not characteristic. The prevalence of this type of microorganism in Kazakhstan is related to the large population of sheep in the southern and south-eastern regions, from where it spreads throughout the country. The only region where only Brucella abortus species caused human disease was the Kostanay region. This type of Brucella abortus is the main causative agent of brucellosis in cattle, and due to the significant population of dairy cows in this area, it has become the dominant type in this region. The circulation of this type of brucellosis in the northern regions of Kazakhstan is related to this.

Table 2: Results of bacteriological and morphological examination of brucellae from animal blood samples from disease foci

Disease cause	Number of analyzed samples	Positive result	% of typical colonies (S-type)	Colony size, mm	Brucella form	Gram stain
1	2	2	90	1.5-2	Oval	Negative
2	2	1	100	1-2	Oval	Negative
3	3	2	85	1.5-2	Spherical	Negative
4	4	2	90	1.5-2.5	Oval	Negative
5	1	1	95	2-2.5	Oval	Negative
6	4	2	95	2-2.5	Oval	Negative
7	2	1	100	1.5-2	Oval	Negative
8	2	1	100	1-2	Oval	Negative
9	4	3	85	1.5-2.5	Spherical	Negative
10	3	2	80	1.5-2	Spherical	Negative
11	3	1	95	2-2.5	Oval	Negative
12	4	2	100	1.5-2	Oval	Negative
13	2	2	85	2-2.5	Spherical	Negative
14	4	2	95	1-2	Oval	Negative
15	2	2	80	1-1.5	Spherical	Negative
16	3	1	100	1.5-2	Oval	Negative
17	1	0	0	-	-	-
18	2	1	95	2-2.5	Oval	Negative

Table 3: Results of PCR typification of brucellae from bacteriological culture

Disease cause	Pathogen type	The type of animal from which the pathogen was isolated	Pathogen region origin
1	*B. melitensis*	Cattle	West Kazakhstan
2	*B. melitensis*	Small cattle	East Kazakhstan
3	*B. abortus*	Cattle	Kostanay
4	*B. melitensis*	Small cattle	Zhambyl
5	*B. melitensis*	Small cattle	Zhambyl
6	*B. melitensis*	Small cattle	Almaty
7	*B. melitensis*	Small cattle	East Kazakhstan
8	*B. abortus*	Cattle	Kostanay
9	*B. melitensis*	Small cattle	Zhambyl
10	*B. abortus*	Small cattle	West Kazakhstan
11	*B. abortus*	Cattle	Kostanay
12	*B. melitensis*	Small cattle	Almaty
13	*B. abortus*	Small cattle	Pavlodar
14	*B. melitensis*	Small cattle	Zhambyl
15	*B. melitensis*	Cattle	Pavlodar
16	*B. melitensis*	Small cattle	Zhambyl
17	-	-	Almaty
18	*B. melitensis*	Small cattle	East Kazakhstan

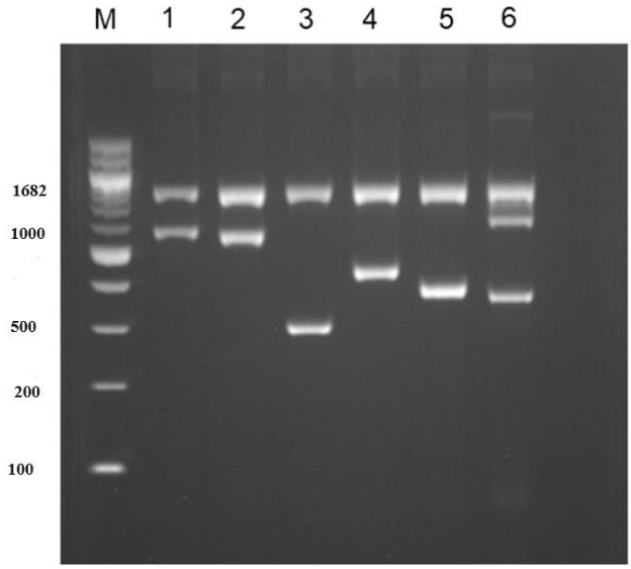

Fig. 2: PCR electropherogram of amplification products of a bacteriological culture grown from a disease focus in Pavlodar region.

DISCUSSION

The challenging epidemiological situation of brucellosis in the population in Kazakhstan makes all research aimed at studying how to spread this disease a priority. Since the only source of human disease in natural conditions is sick animals, namely food products obtained from such animals, the main direction of work was the study of phenotypic and genetic characteristics of pathogens in the foci of infection, where confirmed cases of brucellosis in humans were registered. For this purpose, all types of farm animals located in the territory of the locality were tested where the outbreak of the disease was observed. The use of serological methods for preliminary testing of animals is a fairly common methodology of epizootological surveys, which is used in most countries of the world. In our studies, the use of enzyme-linked immunosorbent assay allowed us to identify in all cases animals with a high titer of antibrucellosis antibodies, which became the main candidates for isolation of the pathogen (Biyashev et al. 2016). This approach does not allow simultaneous typing of Brucella species to determine which of them caused the disease, and the relatively low sensitivity of the test allows its use only for preliminary diagnosis.

Such results are confirmed by numerous studies conducted in Tanzania, which indicate that the accuracy of diagnostic commercial tests was between 55 and 72% (Lukambagire et al. 2021). Vaccine antibody levels or other abnormalities may have induced a significant proportion of false-positive results in serological diagnostics. This may have been the reason for the relatively low percentage of pathogens cultured from pathological material. In one case it was not possible to isolate the pathogen from the pathological material at all. This situation could occur only in the absence of microorganisms in the blood of animals with atypical or chronic forms of the disease manifestation. This is confirmed by the results of the study of Yagupsky et al. (2019), which indicate that the stage of bacterial septicemia in brucellosis is rather short, and all the rest of the time, brucellae as facultative intracellular organisms are practically absent in the blood. Therefore, the sensitivity of the method of using blood to confirm the diagnosis of brucellosis in different periods of the disease had a wide range (from 10 to 90%). This was the reason why, in the conducted studies, the efficiency of obtaining a microbiological culture of the pathogen was only 58% of all samples of selected pathological material from animals with high antibody titers.

This conclusion is supported by the study of Avijgan et al. (2019), in which it is indicated that, unlike most infectious diseases, antibodies against brucellosis (immunoglobulins of class G) often disappear in the blood after a relative improvement in the general condition of the sick animal. In this case, a high concentration of antibodies in the blood may be an indicator only of recent contact of the animal organism with the infectious agent but is not an indicator of the presence of brucellae in the blood, which is also confirmed by the results of studies in Dal et al. (2019), conducted in medical institutions. Therefore, a problem arises in finding lifetime pathological material from animals with asymptomatic infection, which could guarantee the detection of brucellae in microbiological

cultivation. This direction is planned to be continued in further work on brucellosis research.

The results of the bacteriological study, conducted by sowing and culturing microbiological culture obtained from pathological material from animals with high antibody titers, indicated that no significant differences were found between brucellae isolated from different regions of the Republic of Kazakhstan and from different animal species. The majority of colonies typical for brucellosis had sizes from 1 to 2.5mm, they were small, whitish colonies with smooth edges (Umitzhanov et al. 2014). Only a small number of colonies had rough edges, which corresponded to the R-form of colonies. Accordingly, the microscopic characteristics of the grown *Brucella* cells from different cultures also did not differ practically. Regardless of the type of S- or R-colonies, they were spherical or oval-shaped Gram-negative microorganisms that were located singly or in small clusters. Focusing only on the morphological characteristics of colonies and pathogens, it was impossible to perform interspecific differentiation of brucellae and distinguish practically between cultures from pathological material from different regions. Similar results were obtained in the work of Kurmanov et al. (2022) in the cultivation of brucellae of *B. abortus*, *B. melitensis* and *B. suis* species, and no interspecific morphological differences between the pathogens were found. Studies by Di Bonaventura et al. (2021) showed that different species of Brucellae are very closely related to each other. The percentage of similarity between the main species is close to 100%. They can be considered as different subspecies belonging to the same species, and they are distinguished only at the biochemical level. Therefore, further study of the ways of spreading the disease in Kazakhstan should include the cultivation of brucellae on selective media, which allows typing them by their biochemical features.

Since culture and morphological and bacteriological methods were ineffective in *Brucella* typing, the next step in the research was the use of PCR to determine the source of human infection in each of the disease foci. For this purpose, a commercial kit for the identification and differentiation of Brucellae by multilocus PCR was used. As a result of studies of pathological material from human foci of disease in Kazakhstan, two types of brucellae *B. abortus* and *B. melitensis* were isolated, while the prevailing type was *B. melitensis*, which was isolated in 70% of human cases. Kurmanov et al. (2022) also indicate that in other Asian countries, the most dangerous species of brucellae, which are highly likely to cause disease in humans, are the same species. Thus, according to Dadar et al. (2019), *Brucella melitensis* species predominate in Iran, while the incidence of disease in humans in the same country in the studies of Ahmed et al. (2020) was caused in 86% of cases by *B. melitensis* and only 6% by *B. abortus*.

The *Brucella melitensis* microorganism type was isolated almost throughout the country, indicating its widespread occurrence in Kazakhstan. This may be due to rather intensive trade and movement of sheep within the country since these are small ruminants that are the main source of this type of Brucella infection. Only in the northern regions of the Republic of Kazakhstan were biovars of *B. abortus* identified. Such a result is attributed to the predominant development of dairy cattle breeding in this region, which was the isolation of a limited region of bovine-type brucellosis distribution among animal and human populations. Similar results of the distribution of this type of microorganisms were obtained by Yespembetov et al. (2019), where it is indicated that more than 90% of the total number of *B. abortus* samples were isolated from the northern regions of Eastern and Western Kazakhstan. While *B. melitensis* strains were registered predominantly in the south-east of Kazakhstan. This is not entirely consistent with the results obtained in this article, as the distribution of this species had a wider geographical scope. This may be a consequence of the significant time interval between the surveys, or it may be due to an increase in the pathogenicity of *B. melitensis* on the territory of the Republic of Kazakhstan. Therefore, continuing monitoring studies to study the prevalence and intensity of the infection process in the country is considered relevant research in the future.

Conclusion

Summarizing the results of the conducted research on the study of prevalence and sources of human brucellosis morbidity in the Republic of Kazakhstan, it is possible to make the following conclusions and suggestions for future scientific works. The main causative agent of brucellosis in Kazakhstan is the species *B. melitensis*, which was isolated in 70% of the surveyed foci of the disease, and only in 30% of cases, the causative agent was attributed to the species *B. abortus*. Other species of pathological brucellae were not isolated in Kazakhstan. At that, while *B. melitensis* species were registered throughout the country, and *B. abortus* was registered only in the northern regions. Phenotypic differences between different species of brucellae were not detected during microbiological examination or when culture-morphological or bacteriological methods were applied.

The incidence of brucellosis in the population is observed in all regions of the country. Still, the intensity of the disease was higher in the southern and south-eastern regions, where sheep and goats are predominantly kept in individual farms of the population, where veterinary measures are limited. In the northern regions of Kazakhstan, livestock production is concentrated in large farms with appropriate veterinary services. Due to this, the incidence of disease in these areas was lower. The use of whole blood as pathological material for brucellosis isolation from animals with high titers of specific antibodies allowed to the isolation of the infectious agent only in 58% of cases, which prompts to search for other sources of pathological material for lifetime diagnostics of brucellosis in animals with the chronic and latent form of the disease course.

In future studies, it is planned to continue work on monitoring the incidence of brucellosis in Kazakhstan and determining the type of pathogen that causes it. It is also planned to continue efforts to typify brucellosis species circulating in the country, both by biochemical markers and molecular genotyping methods.

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Authors Contribution

Conceptualization, YO, AB; methodology, AA, AM, MB; software, AM, AB; investigation, YO, MB; resources, AM, AA, MB; data curation, AA, YO; writing—original draft preparation, AA, YO, AM, MB, AB; writing—review and editing, AA, AM; visualization, YO, MB, AB.

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