Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2025. – № 2 (010). – P. 44-52. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2025.2(010).1878
UDC 619:616.98:579.852.11 **Research article**

Bacteriological monitoring of infectious epididymitis of rams

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Received: 21-02-2025 **Accepted:** 17-06-2025 **Published:** 27-06-2025

Abstract

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

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

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

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

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

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

Introduction

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

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

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

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

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

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

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

Results and Discussion

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

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

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

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

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

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

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

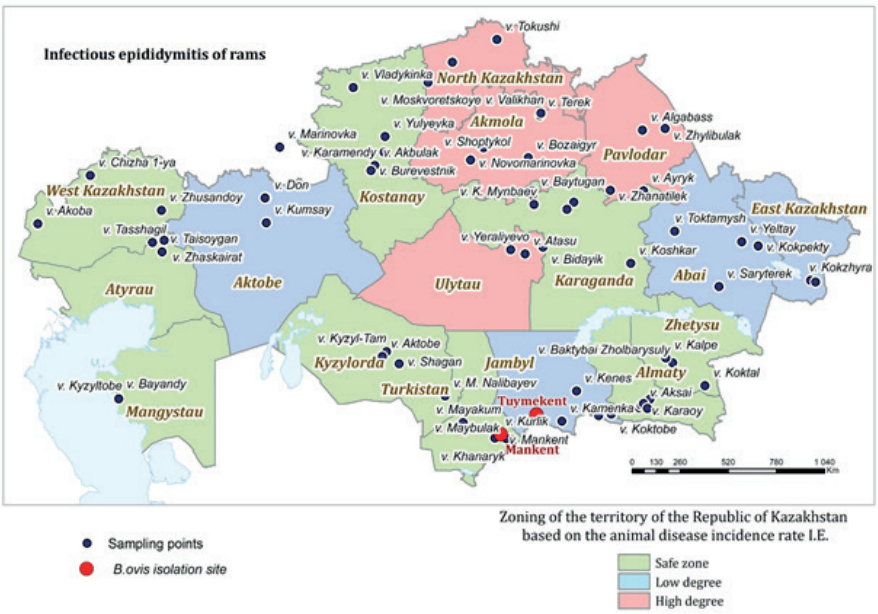

Figure 1 – Geographical sites of sample collection for IER testing and *B. ovis* isolation

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

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

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

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

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

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

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

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

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

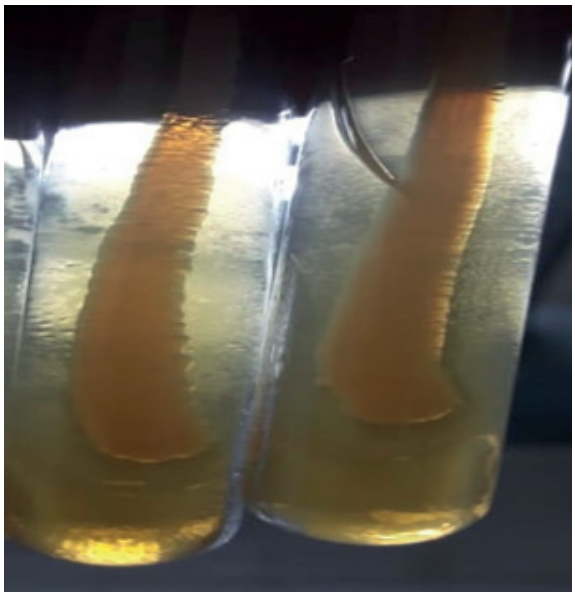

Figure 3 – Active growth of *B. ovis* isolated from the testicles of an infected ram (the Turkestan region) in MPLGGA tubes in the Laboratory of Brucellosis of Kazakh Scientific Research Veterinary Institute

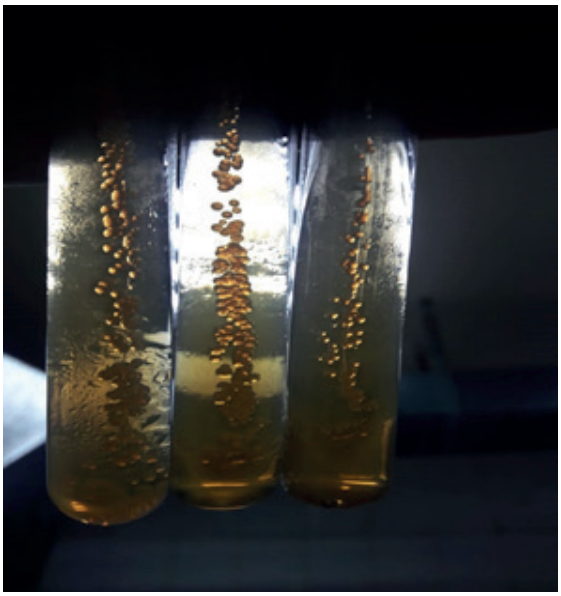

Figure 4 – Growth of *B. ovis* isolated from the whole blood of an infected ram (the Zhambyl region) in the Laboratory of Brucellosis of Kazakh Scientific Research Veterinary Institute

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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

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

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

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

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

Authors' Contributions

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

Acknowledgement

The work was carried out within the framework of NTP BR218004/0223 "Improvement of measures to ensure biological safety in Kazakhstan: counteraction to dangerous and especially dangerous infections" for 2023-2025. Source of funding Committee of Science of the Ministry of Science and Higher Education of the Republic of Kazakhstan.

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