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MODERN MOLECULAR BIOLOGICAL METHODS OF DIAGNOSTICS OF BOVINE BRUCELLOSIS USING POLYMERASE CHAIN REACTION (PCR)

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The article presents the results of a comprehensive molecular diagnostic study aimed at detecting infection with the brucellosis pathogen Brucella abortus in cattle from Aisary village, Kostanay District, Kostanay Region. At the first stage, all 94 blood samples were tested using the complement fixation test (CFT), which yielded 10 positive results. To clarify the diagnosis, the same samples were further examined by the agglutination reaction (AR) method, which revealed 3 positive and 7 inconclusive results.

To obtain more reliable data and confirm the presence of the pathogen, PCR analysis was performed using specific primers for Brucella abortus DNA.

As a result of the molecular study, the presence of pathogenic DNA was confirmed in only 2 out of 10 samples; both had previously tested positive in the AR. The remaining 8 samples, including 7 inconclusive and one suspected false-positive AR result, showed no evidence of Brucella abortus DNA.

Thus, PCR confirmed infection in only two animals and excluded the disease in the rest, highlighting the high accuracy and diagnostic value of molecular genetic methods when combined with conventional serological approaches for brucellosis detection.

Key words: *PCR, DNA, AR, complement fixation test, brucellosis, molecular genetic, diagnostics.*

ПОЛИМЕРАЗДЫ ТІЗБЕКТІ РЕАКЦИЯНЫ (ПТР) ПАЙДАЛАНА ОТЫРЫП, ЗАМАНАУИ МОЛЕКУЛЯРЛЫҚ-БИОЛОГИЯЛЫҚ ӘДІСТЕРІМЕН ІРІ ҚАРА МАЛ БРУЦЕЛЛЕЗІН ДИАГНОСТИКАЛАУ

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Мақалада Қостанай облысы Қостанай ауданы Айсара ауылында ірі қара малдың бруцеллез қоздырғышы Brucella abortus ауруын анықтау мақсатында жүргізілген кешенді молекулярлық-диагностикалық зерттеудің нәтижелері баяндалған.

Бірінші кезеңде барлық 94 қан сынамасы комплементті байланыстыру реакциясы (КБР) әдісімен талданды, нәтижесінде 10 үлгі оң нәтиже берді. Диагнозды нақтылау үшін осы үлгілер қосымша агглютинация реакциясы (АР) әдісімен зерттелді, оның нәтижелері бойынша 3 сынама оң, ал 7 сынама күмәнді болып шықты.

Анағұрлым сенімді мәліметтер алу және қоздырғыштың бар-жоғын растау мақсатында Brucella abortus ДНК-сына арнайы праймерлерді пайдалана отырып, полимеразды тізбекті реакция-сында талдау жүргізілді.

Молекулалық зерттеу нәтижесінде патогенді ДНҚ-ның болуы 10 үлгінің 2-сінде ғана расталды: екеуі де бұрын агглютинация реакциясы бойынша оң болған. Қалған 8 сынама, 7 күмәнді және бір болжамды жалған оң агглютинация реакциясын қоса алғанда, ДНК B. abortus болған жоқ.

Осылайша, полимеразды тізбекті реакциясы (ПТР))тек екі жануарда ғана жұқтырғандығын растады және қалғандарында ауруды болдырмауға мүмкіндік берді, бұл бруцеллезді анықтауда молекулалық-генетикалық әдістердің жоғары дәлдігі мен диагностикалық құндылығын көрсетеді.

Түйінді сөздер: *ПТР, ДНК, АР, КБР, бруцеллез, молекулярлық-генетикалық, диагностика.*

СОВРЕМЕННЫЕ МОЛЕКУЛЯРНО-БИОЛОГИЧЕСКИЕ МЕТОДЫ ДИАГНОСТИКИ БРУЦЕЛЛЁЗА КРУПНОГО РОГАТОГО СКОТА С ИСПОЛЬЗОВАНИЕМ ПОЛИМЕРАЗНОЙ ЦЕПНОЙ РЕАКЦИИ (ПЦР)

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В статье изложены результаты комплексного молекулярно-диагностического исследования, проведённого с целью выявления инфицирования крупного рогатого скота возбудителем бруцеллёза – Brucella abortus – в селе Айсары Костанайского района Костанайской области.

На первом этапе все 94 пробы крови были проанализированы методом реакции связывания комплемента (РСК), в результате чего 10 образцов дали положительные результаты. Для уточнения диагноза эти же образцы были дополнительно исследованы методом реакции агглютинации (РА), по результатам которой 3 пробы оказались положительными, а 7 – сомнительными.

С целью получения более достоверных данных и подтверждения наличия возбудителя был проведён ПЦР-анализ с использованием специфических праймеров к ДНК Brucella abortus.

В результате молекулярного исследования наличие патогенной ДНК было подтверждено лишь в 2 из 10 образцов: оба ранее были положительными по РА. Остальные 8 проб, включая 7 сомнительных и одну предположительно ложноположительную по РА, не показали присутствия ДНК B. abortus.

Таким образом, ПЦР подтвердила инфицирование лишь у двух животных и позволила исключить заболевание у остальных, что подчёркивает высокую точность и диагностическую ценность молекулярно-генетических методов в сочетании с традиционными серологическими подходами при выявлении бруцеллёза.

Ключевые слова: *ПЦР, ДНК, РА, РСК, бруцеллез, молекулярно-генетическая, диагностика.*

Introduction. Brucellosis is a zoonotic disease caused by bacteria of the *Brucella* genus, which are capable of penetrating host cells, thereby complicating pathogen detection by classical diagnostic methods.

Despite the active use of serological diagnostics, such methods often fail to detect seronegative cases, particularly at the early stages of infection [1, p. 47]. Serological tests (RBT, CFT, ELISA) facilitate large-scale screening but are ineffective in chronic and seronegative forms of infection [2, p. 67].

Molecular biological approaches, primarily PCR and its modifications, have significantly expanded diagnostic capabilities. For example, real-time PCR targeting the IS711 gene enables the identification of *Brucella* spp. DNA in tissues, blood and milk with high sensitivity and specificity. Daugalieva et al. (2021) identified *B. melitensis* and *B. abortus* by PCR and MLVA-16 in samples from cattle and small ruminants in Kazakhstan [3, p.5-7].

The development of digital PCR (ddPCR) has further improved diagnostic accuracy and enabled quantification of the bacterial load in blood samples. In the Wengniute Banner study (2021–2022), ddPCR demonstrated a sensitivity of up to several ng/μL and high reproducibility of results (see *Sensitivity and Repeatability* section) [4, pp. 5–9].

Thus, modern molecular biological technologies make it possible to:

1. Overcome the limitations of serological testing in seronegative infections.
2. Increase the sensitivity and speed of diagnosis (within a single working day).
3. Perform quantitative assessment of bacterial load.
4. Apply rapid diagnostic methods under field conditions [5, pp. 5–7].

It should be emphasized that the integration of molecular diagnostics (PCR, ddPCR, LAMP, CRISPR) with traditional methods can significantly enhance the efficiency of brucellosis surveillance and control in both veterinary medicine and clinical practice [6, p. 5].

In recent years, monitoring of the epizootic situation and the diagnosis of brucellosis have been actively modernized in accordance with the recommendations of the WOAH and regional studies. For instance, Mikailov et al. (2024) emphasized that classical diagnostic methods, such as the in vitro agglutination reaction (AR) and the complement fixation test (CFT), are inferior in sensitivity and specificity to modern molecular techniques [7, p. 817].

A comparative study of various serological tests for the diagnosis of bovine brucellosis, including AR, CFT, ELISA, and the indirect hemagglutination assay (IHA), showed that ELISA (96%) and AR (100%) had the highest sensitivity, whereas IHA demonstrated significantly lower performance [8, p. 3].

Results of Epizootic Monitoring in Kazakhstan for 2023–2024

Brucellosis diagnosis was carried out using RBT and CFT, with 155 out of 2,981 samples testing positive (5.2%). The authors emphasized the importance of implementing the WOAH (2022) international recommendations and noted an increase in diagnostic sensitivity when transitioning to ELISA [9, p. 3].

The sensitivity and specificity of tests such as the Rose Bengal test (RBT), CFT, and the lateral flow test (LFT) were also assessed. LFT and RBT were found to be suitable for screening, while CFT was primarily used to confirm the diagnosis due to its high specificity [10, p. 4].

The aim of the present study was to confirm the presence of *Brucella abortus* DNA in bovine serum samples that yielded questionable or positive serological results using PCR.

Objectives

1. To analyze bovine blood samples using the complement fixation test (CFT) and the agglutination reaction (AR).

2. To perform a one-step PCR assay followed by electrophoretic detection of the amplicons in agarose gel.

Materials and methods. Within the framework of epizootic monitoring, 94 blood samples were collected from cattle in Aysary village, Kostanay District, Kostanay Region. All samples were collected in sterile vacuum tubes (vacutainers) and transported to the laboratory of the Kostanay National Institute of Veterinary Science (NIVS) in thermocontainers within 6 hours of collection. Serological studies were performed in accordance with GOST 34105-2017 [11]. Of the 94 samples, 10 tested positive by the complement fixation test (CFT). These 10 samples were further examined using the agglutination reaction (AR), of which 3 samples were positive and 7 were inconclusive.

Molecular diagnostic analysis was also conducted. DNA was extracted using the BRU-COM kit (FGUN TSNIIE, Rospotrebnadzor). PCR amplification was performed on a Mastercycler Gradient thermocycler using specific primers for *Brucella abortus* DNA.

The amplification conditions were as follows: denaturation at 95 °C, annealing at 60 °C, and elongation at 72 °C for 35 cycles. Amplification products were analyzed on a 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination.

Results

Polymerase chain reaction (PCR) is currently one of the most sensitive and specific methods for the molecular diagnosis of infectious diseases, including brucellosis. However, the efficiency of PCR depends on several critical factors: proper preparation of clinical material to prevent DNA loss or degradation, optimal selection of primers specific to the target sequences of the pathogen, and appropriate choice of the method for visualizing amplification products.

In this study, a one-step PCR assay followed by electrophoretic detection of amplicons in an agarose gel was employed. This approach is commonly used in both laboratory practice and scientific research because it allows for extremely high sensitivity, detecting as few as 5–10 DNA molecules per sample. However, such sensitivity is typically achieved only with highly purified DNA samples, which necessitates careful attention to sample preparation.

At the first stage of the study, all 94 samples (n = 94) were analyzed using the complement fixation test (CFT) in accordance with established veterinary standards. Ten samples (10.64%) tested positive, which warranted additional serological testing. The same samples were subsequently examined using the agglutination reaction (AR), a simpler and widely employed serological technique. Based on the AR results, 3 samples (3.19%) were classified as positive, and 7 samples (7.45%) were inconclusive, complicating the establishment of a definitive diagnosis.

To obtain more reliable data and clarify the infection status of the animals, PCR analysis was performed on these 10 samples using specific primers for *Brucella abortus* DNA (Table 1).

Table 1 – Comparative results of AR, CFT, and PCR analyses

№	AR	CFT	PCR
1	Inconclusive	+	-
2	Inconclusive	+	-
3	+	+	+
4	Inconclusive	+	-
5	Inconclusive	+	-
6	+	+	+
7	Inconclusive	+	-
8	Inconclusive	+	-
9	Inconclusive	+	-
10	+	+	-

Notes:

- AR – Agglutination Reaction
- CFT – Complement Fixation Test
- PCR – Polymerase Chain Reaction

According to the data in Table 1, the analysis revealed the following:

- All 10 samples (10.64%) previously testing positive by CFT out of 94 were included in the PCR analysis.
- Of these, 3 samples (3.19%) that were positive by AR were further analyzed using PCR. As a result, 2 samples (2.13%) were confirmed to contain *Brucella abortus* DNA, while one sample tested negative.
- The 7 samples (7.45%) with inconclusive AR results were negative by PCR, indicating the absence of pathogenic DNA in these samples.

Thus, PCR confirmed the presence of *Brucella abortus* in only 2 animals initially positive by serological methods and allowed the exclusion of infection in the remaining 8 animals, including 7 with inconclusive serological results and 1 with a possible false-positive AR result.

The results obtained clearly demonstrate the high sensitivity and specificity of the PCR method, particularly in cases where serological methods yield ambiguous or inconclusive results. The use of PCR in combination with CFT and AR significantly enhances diagnostic accuracy, minimizes the likelihood of diagnostic errors, and enables timely detection and isolation of infected animals, which is especially important within the framework of epizootic control.

The PCR amplification results are presented in Figure 1, showing characteristic amplicons in the two positive samples corresponding to specific regions of the *Brucella abortus* genome.

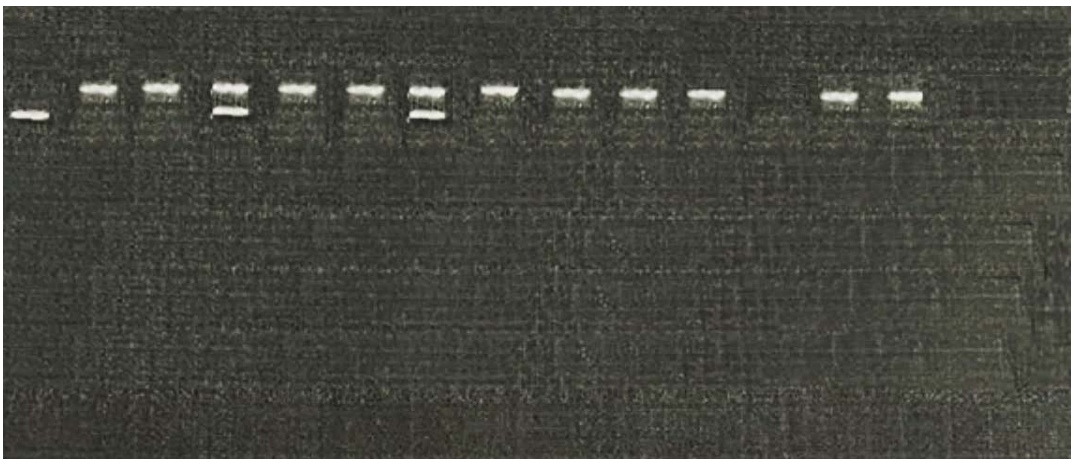

Figure 1 – Analysis of PCR results of 10 serum samples by agarose gel electrophoresis

Figure 1 presents the results of a PCR assay analyzed by agarose gel electrophoresis. Ten blood serum samples collected from cattle suspected of brucellosis based on serological methods (CFT and AR) were tested.

Each lane on the gel corresponds to an individual sample, numbered from 1 to 10. Control samples include a positive control (*Brucella abortus* DNA) and a negative control (reaction mixture without DNA).

As shown in the figure, characteristic amplicon bands corresponding to a specific fragment of *Brucella abortus* DNA are clearly visible in the lanes corresponding to samples 3 and 6. The presence of these bands indicates a positive PCR result, confirming the presence of the pathogen DNA in these samples. These results verify that the animals from which these samples were collected were indeed infected with *Brucella abortus*.

At the same time, the remaining samples, including sample 10, did not display amplified products of the expected size. The absence of characteristic bands indicates a negative PCR result and, therefore, the absence of detectable pathogen DNA in the sample. This is particularly important for sample 10, as the animal had previously yielded inconclusive results based on serological tests; PCR analysis confirmed that it was not infected. This result helped prevent the erroneous culling of the animal, which has both economic and epizootic significance.

Thus, the electrophoregram shown in Figure 1 demonstrates the diagnostic accuracy of the PCR method: only the two truly infected animals (samples 3 and 6) were identified, while all other animals were correctly excluded from the group of infected animals. These findings confirm the high sensitivity and specificity of PCR and highlight its importance in clarifying diagnoses, particularly when serological results are doubtful or contradictory.

Discussion. The results of this study clearly demonstrate the diagnostic advantages of the polymerase chain reaction (PCR) method over traditional serological tests, such as the agglutination reaction (AR) and the complement fixation test (CFT). PCR provides higher sensitivity and specificity, which is particularly important when analyzing samples with ambiguous or inconclusive serological results.

Of the 10 samples tested, 7 were classified as inconclusive based on AR. If only serological methods had been used, it would have been difficult to make an accurate diagnosis, potentially leading to premature or erroneous management decisions, such as the culling of animals. However, PCR analysis confirmed the absence of infection in all seven of these animals with high accuracy, indicating that *Brucella abortus* DNA was not present in the tested samples.

Particularly noteworthy is sample 10, which tested positive by serological methods but did not display a specific amplicon by PCR. This discrepancy may reflect a false-positive result in the serological assay, possibly due to cross-reactivity with other antigens, or an extremely low concentration of bacterial DNA in the sample, below the sensitivity threshold of the PCR method. In any case, the negative PCR result in this instance prevented the unnecessary culling of a clinically healthy animal, which is significant from both economic and ethical perspectives.

The particular value of PCR lies in its ability to directly detect the genetic material of the pathogen, in contrast to serological methods that rely on antibody detection. Since antibody production may depend on the individual animal's immune response, the stage of infection, or prior vaccination, PCR enables diagnosis independent of these factors.

Thus, PCR has proven to be a highly effective method, especially in borderline or controversial cases. The use of this approach enhances the objectivity of diagnosis, minimizes the risk of erroneous culling, facilitates more accurate identification of infected individuals, and reduces the likelihood of further spread of infection within the herd. Moreover, the high reliability of PCR results fosters confidence among farms and animal owners in veterinary interventions.

Taken together, the results of this study underscore the necessity of integrating PCR diagnostics into standard epizootic surveillance and brucellosis control schemes, particularly in cases where serological test results are doubtful or contradictory.

Conclusion

This study confirmed the high diagnostic value of the polymerase chain reaction (PCR) method for detection of *Brucella abortus* in cattle. The use of PCR enabled the acquisition of more accurate information regarding the actual infection status, particularly in cases where serological methods yielded doubtful or contradictory results.

The analysis revealed instances of discrepancies between serological and molecular data, highlighting the importance of a comprehensive approach in the diagnosis of brucellosis. The application of PCR helped prevent false-positive results and unjustified culling of animals, thereby reducing potential economic losses for agricultural enterprises.

The method demonstrated the potential for early detection of infection through the direct identification of pathogen DNA, independent of the stage of the immune response. This renders PCR particularly valuable for livestock monitoring, planning preventive measures, and enabling prompt management decisions in infection foci.

From a practical perspective, the integration of PCR diagnostics into veterinary surveillance systems contributes not only to more effective control of brucellosis but also to the strengthening of regional epizootic health. In the context of the increasing relevance of zoonotic infections, this method offers prospects for further enhancement of prevention programs, early detection, and the prevention of the spread of hazardous diseases.

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