ARTICLE

GENOTYPE CATALOGIZATION OF BRUCELLA STRAINS

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ABSTRACT

Background: Brucellosis is an infectious disease caused by bacteria of the Brucella genus, which infects almost all types of agricultural and many species of wild animals, as well as humans. Recently, molecular genetic methods for the diagnosis of brucellosis have been introduced into veterinary practice, which allow detecting bacterial DNA in biological material. Methods: A commercial kit of reagents Sileks MagNA-G (Sileks, Russia) based on the method of fixing nucleic acid on magnetic particles, was used for DNA isolation. Results and conclusion: The PCR analysis with primers from the AMOS system confirmed the identification of the currently existing B. melitensis biovars, three biovars of B. abortus, and B. ovis and B. suis species by classical methods. BrA, BAbor, WboA, and Eri primers allowed differentiation of B. abortus at the generic and species levels, as well as identification of B. abortus strain 19 from material taken from experimentally infected animals. Thus, the data obtained on the molecular genetic identification of the DNA of different species of Brucella will largely allow solving the problem of diagnosing brucellosis in animals.

KEY WORDS
Brucellosis, infection process, immunity, strain, genotype, vaccine, biovar

INTRODUCTION

Brucellosis is an infectious disease caused by bacteria of the *Brucella* genus, which infects almost all types of agricultural and many species of wild animals, as well as humans. The infectious agent, which easily migrates from animals to humans, causes acute febrile illness (*B. abortus* - undulating fever, or *B. melitensis* - Maltese fever) and can progress to a chronic form leading to serious complications affecting the musculoskeletal and other body systems. In the system of anti-Brucellar measures, diagnosis is of great importance. Over the years, a large number of diagnostic agents and methods have been tested in experimental and production conditions. The duration of development and decrement of immunobiological responses and their diagnostic value in various animal species have been studied. Recently, molecular genetic diagnostic methods for the diagnosis of brucellosis have been introduced into veterinary practice. These methods detect the DNA of bacteria in biological material and are highly sensitive, specific, fairly simple, and fast (in terms of obtaining the result). One of them is PCR (polymerase chain reaction). Due to its high sensitivity, specificity and universality, the PCR method is increasingly used for the diagnosis of infectious diseases. PCR detects DNA from pathological material containing less than 100 *Brucella* cells. Molecular genetic identification of DNA of different *Brucella* strains allows solving the problem of diagnosis of brucellosis in a substantial way [1-7].

The study aimed to determine the pathogens of brucellosis and identify them in the organs and tissues of experimentally infected animals.

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MATERIALS AND METHODS

The study was conducted based on the laboratory of chronic infections and the laboratory of molecular biology and biotechnology of the Vyshnevolotsky branch of the All-Russian Institute of Experimental Veterinary on the Lisy island. Clinical, microbiological, serological, and molecular genetic methods proposed by the Joint FAO/WHO Expert Committee on Brucellosis were used. The following equipment was used: laminar-flow cabinets, regular and carbon dioxide thermostats, refrigeration cabinets, deep-freeze cabinets, centrifuges, autoclaves, light microscopes, equipment for freeze-drying, sterilizing filtration, and ultrafiltration.

Oligonucleotide primers for genus-specific and species-specific differentiation of *B. abortus*

DNA purification was conducted using a commercial reagent kit Sileks MagNA-G (Sileks, Russia), which is based on the method for purification of nucleic acids using magnetic particles. BrA primers were used for genus-specific identification of *B. abortus*. *B. abortus* was differentiated from other *Brucella* strains using BAbor oligonucleotides. Further identification of *B. abortus* strain 19 was carried out using primers Eri and WboA [Table 1].

The 25 µl reaction mixture consisted of 10 µl PCR buffer, 3 mmol MgCl, 0.2 mmol dNTPs, 0.4 µmol of each primer, 2.5 units/100 µl of Taq-polymerase, and 10 µl of DNA.

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PCR was carried out using the programmable thermal cycler Tertsik (DNA-technology, Russia) according to the following program: 95°C for 10 sec, 61/60/63/65°C (BrA/BAbor/Eri/WboA) for 10 sec, and 72°C for 10 sec; 40 cycles, with two-minute preliminary denaturation at 95°C and subsequent three-minute elongation at 72°C.

Table 1: Characteristics of oligonucleotide primers for the genus-specific and species-level differentiation of *B. abortus*

Primer	5'-3' sequence	Length, n	Amplicon size, bp	Annealing point, °C
BrA-F	AGTCAGACGTTGCCTATT	18	260	61
BrA-R	GTGTTCAGCCTTGATATG	19		61
BAbor-F	GTTCTTGCTGGTCTTGCGGTG	21	1054	60
BAbor-R	AGCGCAGGAGATGCAGGCAC	20		60
Eri-F	GCGCCGCGAAGAACTTATCAA	21	178	63
Eri-R	CGCCATGTTAGCGGCGGTGA	20		63
WboA-F	GCCAACCAACCCAAATGCTCACAA	26	400	65
WboA-R	TTAAGCGCTGATGCCATTTCCTTCAC	24		65

Oligonucleotide primers in the AMOS system

AMOS (*abortus*, *melitensis*, *ovis*, and *suis*) method proposed by Bricker et al. was used to identify *B. melitensis* (all biovars), *B. ovis*, first, second, and fourth biovars of *B. abortus* and *B. suis*. The method is based on species-specific or biovar-specific characteristics of the localization of the genetic element IS711 in the chromosomal DNA of *Brucella*. For identification, five primers were used. One of them is specific to the sequence of genetic element IS711. Other four are specific to adjacent DNA sequences, which are characteristic of each type of *Brucella* [Table 2].

The 25 µl reaction mixture consisted of 10 µl PCR buffer, 2.2 mmol MgCl, 0.2 mmol dNTPs, 0.4 µmol of each primer, 2.5 units/100 µl of Taq-polymerase, and 10 µl of DNA.

PCR was carried out using the programmable thermal cycler Tertsik (DNA-technology, Russia) according to the following program: 94°C for 20 sec, the primer annealing temperature ranged from 56.5°C to 60°C, and the primers annealed on the matrix for 20 sec, elongation at 72°C for 40 sec; 35 cycles.

Table 2: Characteristics of the oligonucleotide primers of the AMOS system

Brucella	5'-3' sequence	Direction	Length, n	Amplicon size, bp	Annealing point, °C
B. abortus	GACGAACGGA ATTTTTCCAATCCC	forward	24	498	55.5
	TGCCGATCACT TAAGGGCCTTCAT	reverse	24		56.5
B. ovis	CGGGTTCTG GCACCATCGTCG	forward	21	976	56.0
	TGCCGATCACT TAAGGGCCTTCAT	reverse	24		56.5
B. melitensis	AAATCGCGTC CTTGCTGGTCTGA	forward	23	731	56.0
	TGCCGATCACT TAAGGGCCTTCAT	reverse	24		56.5
B. suis	GCGCGGTTTTC TGAAGGTTCAGG	forward	23	285	55.0
	TGCCGATCACT TAAGGGCCTTCAT	reverse	24		56.5

The amplification results were analyzed using horizontal electrophoresis in 2% agarose gel. 100 bp Ladder DNA Marker (100bp-3000bp) (Axygene, USA) was used as a molecular mass marker.

Cataloging of genotypes of *Brucella* strains from the collection of pathogenic and vaccine strains

Brucella strains in the collection of pathogenic and vaccine strains were studied based on biochemical, serological, and virulent properties. The study considered the ability of *Brucella* to release H2S, the need for CO2 during cultivation, sensitivity to erythritol and aniline dyes (fuchsin, thionin), the dissociation degree in samples with trypaflavine, acriflavin and miostagmic reaction, the color of the grown White Wilson colonies, and the ability to grow in the presence of penicillin antibiotics. “A” and “M” antigenic structures of *Brucella* were determined using monoreceptor serum. Sensitivity to a specific bacteriophage was also studied. Virulent properties were studied in laboratory animals; the infection index and antigenic and allergenic properties were determined. As a result, the current species affiliation existing strains was

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confirmed. Thirty-six strains belong to strain *B. melitensis*, 77 strains belong to *B. abortus*, 25 belong to *B.ovis*, 53 strains belong to *B. suis*, one strain belongs to *B. neotomae*, and two strains belong to *B. canis*.

For the genus-specific and species-level differentiation of *B. abortus* and for the identification of *B. abortus* 19 strain, we used primers that can be applied in the molecular genetic diagnosis of brucellosis in infected animals.

RESULTS AND DISCUSSION

The BrA primers [Table 1] were synthesized based on the BCSP31 gene sequence. This sequence is identical for all types of *Brucella* and therefore, these primers are genus-specific. As a result of PCR, a specific fragment of 260 bp was amplified [Fig. 1]. This sequence is identical for all types of *Brucella* and therefore, these primers are genus-specific. As a result of PCR, a specific fragment of 260 bp was amplified [Fig. 1].

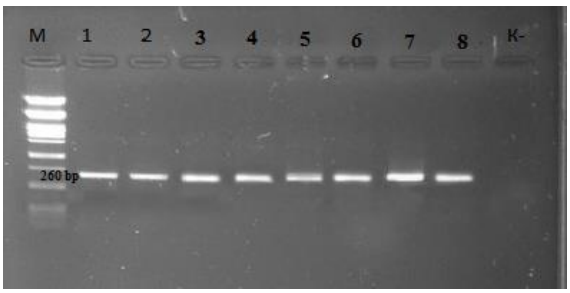

Fig. 1: Results of testing of the effectiveness of BrA primers. Designations: M – DNA marker; 1 – retropharyngeal right lymphnode; 2 – inguinal left lymphnode; 3 – mediastinal lymphnode; 4 – liver; 5 – spleen; 6 – *B. abortus* 19; 7 – *B. abortus* 54; 8 – *B. melitensis* H-102; K- – negative test control

Differentiation of *B. abortus* from other *Brucella* strains is possible due to the absence of a 25 kb region in the chromosomal DNA of all *B. abortus* biovars. PCR was carried out using BAbor oligonucleotides [Table 1], which hybridize with DNA sequences that flank the 25 kp region during annealing. This region is present in all species of *Brucella* except *B. abortus*. A fragment of 1054 bp was amplified for *B. abortus*, and for the remaining species of *Brucella*, amplification did not take place, because the required fragment length was very large (26 kp). [Fig. 2] shows the results of PCR with BrA primers. The amplicon size in the presence of *B. abortus* DNA in the sample was 1054 bp.

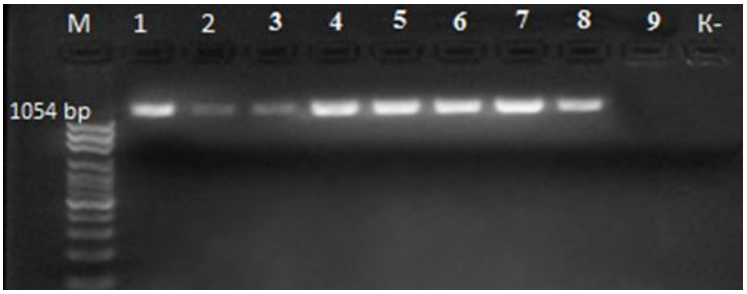

Fig. 2: Results of testing of the effectiveness of BAbor primers. Designations: M – DNA marker; 1 – retropharyngeal right lymphnode; 2 – inguinal left lymphnode; 3 – mediastinal lymphnode; 4 – liver; 5 – spleen; 6 – *B. abortus* 19; 7 – *B. abortus* 54; 8 – popliteal right lymphnode; 9 – *B. melitensis* H-102; K- – negative test control

The identification of *B. abortus* 19 was based on the absence of a 702 bp fragment in the Eri locus. Oligonucleotides were selected in such a way that one of them annealed at the Eri locus common for strain 19, *B. abortus* strains, and other species, while the WboA annealed at the gene that is absent in strain 19. When performing PCR with WboA primers and DNA of all *Brucella* strains (including strain 19), a specific 400 bp fragment was amplified. When performing PCR with the same material and Eri primers, a 178 bp fragment was amplified for all *Brucella* strains, with the exception of strain 19. Using these primers in parallel allowed us to identify *B. abortus* strain 19 [Fig. 3].

From the PCR results presented in [Table 3], it can be seen that the primers BrA, BAbor, WboA, and Eri allowed differentiating *B. abortus* at the generic and species levels and identifying *B. abortus* strain 19 in the material taken from experimentally infected animals.

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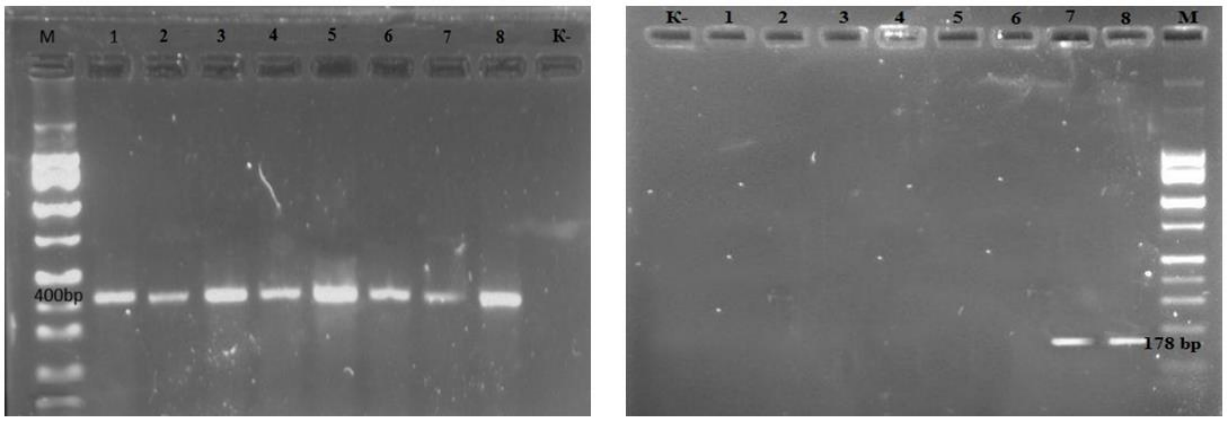

Fig. 3: Results of testing of the effectiveness of WboA (left) and Eri (right) primers. Designations: M – DNA marker; 1 – retropharyngeal right lymphnode; 2 – inguinal left lymphnode; 3 – mediastinal lymphnode; 4 – liver; 5 – spleen; 6 – *B. abortus* 19; 7 – *B. abortus* 54; 8 – *B. melitensis* H-102; K- – negative test control.

Table 3: Results of PCR with primers BrA, BAbor, WboA, and Eri (number of positive samples /total number of samples)

Material	Primers			
	BrA	BAbor	WboA	Eri
Retropharyngeal right lymphnode	4/4	4/4	4/4	0/4
Retropharyngeal left lymphnode	4/4	4/4	4/4	0/4
Inguinal right lymphnode	4/4	3/4	4/4	0/4
Inguinal left lymphnode	4/4	4/4	4/4	0/4
Popliteal right lymphnode	4/4	4/4	4/4	0/4
Popliteal left lymphnode	4/4	3/4	4/4	0/4
Mediastinal lymphnode	4/4	4/4	4/4	0/4
Paraaortal right lymphnode	3/3	3/3	3/3	0/3
Paraaortal left lymphnode	3/3	3/3	3/3	0/3
Antescapular left lymphnode	4/4	4/4	4/4	0/4
Spleen	4/4	4/4	4/4	0/4
Kidneys	4/4	4/4	4/4	0/4
Liver	4/4	4/4	4/4	0/4
Suspension of *B. abortus* 19	+	+	+	-
Suspension of *B. abortus* 54	+	+	+	+
Suspension of *melitensis* H-102	+	-	+	+

PCR was performed using five primers of the AMOS system, one of which is specific to the IS711 sequence, and the other four are specific to adjacent DNA regions specific for each *Brucella* species. When testing strains of *B. melitensis*, *B. ovis*, and *B. suis*, specific fragments of 731 bp, 976 bp, and 285 bp were amplified, respectively. The specific product of PCR analysis of *B. abortus* was 498 bp [Fig. 4].

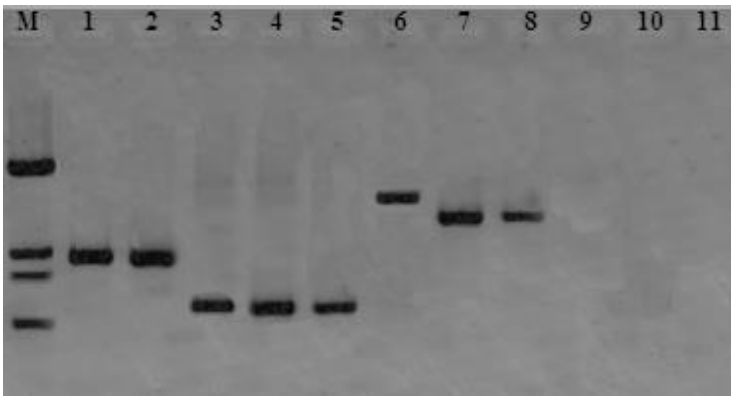

Fig. 4: Results of testing of the effectiveness of AMOS primers. Designations: M – DNA marker; 1 – *B. abortus* 19; 2 – *B. abortus* 54; 3 – *B. suis* HH-1; 4 – *B. suis* 002; 5 – *B. suis* 245; 6 – *B. ovis* 63/91; 7 – *B. melitensis* H-102; 8 – *B. melitensis*; 9 – *B. neotomae* 66/2; 10 – *B. canis* 6/66; 11 – negative test control.

Thus, the obtained PCR data with primers BrA, BAbor, WboA, Eri in each *Brucella* species with corresponding fragment sizes are similar to data of other authors [1, 7].

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CONCLUSION

We performed the catalogization of *Brucella* strain genotypes from a collection of pathogenic and vaccine strains of causative agents of animal infectious diseases of the All-Russian Institute of Experimental Veterinary.

In the study of *Brucella* cultures from the collection of pathogenic and vaccine strains of causative agents of animal infectious diseases of the All-Russian Institute of Experimental Veterinary the cultures were attributed to species using cultural-biochemical, tinctorial, and serological methods. Thirty-six strains belong to *B. melitensis*, 77 strains belong to *B. abortus*, 25 strains belong to *B. ovis*, 53 strain belong to *B. suis*, one strain belongs to *B. neotomae*, and two strains belong to *B. canis*. PCR analysis with the use of primers from the AMOS system confirmed the identification of currently existing biovars of *B. melitensis*, three biovars of *B. abortus*, species *B. ovis* and *B. suis* using classical methods. Primers BrA, BAbor, WboA, and Eri allowed differentiating *B. abortus* at the generic and species levels, as well as identifying *B. abortus* strain 19 in the material taken from experimentally infected animals. Consequently, the data of the molecular genetic identification of DNA of different *Brucella* species will significantly simplify the solution to the problem of diagnosing animal brucellosis.

CONFLICT OF INTEREST

The authors declare no competing interests in relation to the work.

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FINANCIAL DISCLOSURE

None.

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